Serosurvey of pandemic H1N1 influenza A virus in dogs in Andalusia (southern Spain).

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Title: Serosurvey of pandemic H1N1 influenza A virus in dogs in Andalusia (southern Spain).

Running Head: Pandemic H1N1 influenza A virus in dogs in southern Spain.

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Impacts

This is the first report of A(H1N1)pdm09 exposure in dogs in Spain.

Our results indicate a limited A(H1N1)pdm09 circulation in dogs in Spain.

The risk of A(H1N1)pdm09 transmission from dogs to humans can be considered low.

Abstract

In April 2009, a new influenza A virus (IAV) subtype (A(H1N1)pdm09) spread worldwide and triggered the first human influenza pandemic of the 21st century. Since then, exposure to the pandemic H1N1 IAV has been confirmed in different animal species. Serological evidence and clinical infection with A(H1N1)pdm09 have been reported in canines, but the information available about the role of dogs in the epidemiology of this IAV subtype is still very limited in Europe. A cross-sectional study was carried out to determine the seroprevalence of A(H1N1)pdm09 in dogs in **southern** Spain, a **region** with endemic seasonal circulation in human. Sera from 750 companion dogs were collected during the period 2013–2016. Antibodies against pandemic H1N1 IAV were analysed using the haemagglutination inhibition test. Positive samples were also tested by single radial haemolysis assay. Seropositivity was only confirmed by both methods in one (0.13%; 95%CI: 0.00-0.38) adult animal sampled in 2013. To the best of the authors' knowledge, this is the first report of A(H1N1)pdm09 exposure in dogs in Spain. The low seroprevalence obtained indicates a limited exposure history to A(H1N1)pdm09 IAV in dogs in this country and suggests a low risk of transmission of this zoonotic IAV subtype between humans and dogs.

Keywords: Influenza A virus; H1N1; Pandemic; Dogs; Spain; Surveillance

1. INTRODUCTION

Influenza A viruses (IAV; family *Orthomyxoviridae*) are among the most important emerging pathogens worldwide, affecting a wide range of animal species, including human beings. Although wild birds are considered the main reservoir of these viruses, mammalian species can also be implicated in the transmission of IAV (Chen et al., 2018).

In April 2009, a new swine-origin H1N1 IAV subtype labelled A(H1N1)pdm09 was first reported in Mexico and United States. The virus spread rapidly worldwide causing the first influenza pandemic of the 21st century. During the first year, more than 575,000 human deaths were associated with this emergent subtype around the world, and in the United States alone between 43.3 and 89.3 million cases and 12,469 deaths were linked to this virus (CDC, 2019). Nowadays, the A(H1N1)pdm09-like viruses have an endemic seasonal circulation in Europe and represent almost the 40% of the identified IAV in humans (ECDC, 2019).

Influenza A(H1N1)pdm09-like viruses have been confirmed or suggested to be transmitted from humans to different wild and domestic species (Keenliside, 2013; Britton et al., 2019). Furthermore, exposure to this IAV has been detected in different pet animals in several countries (Zhao et al., 2014; Martínez-Orellana et al., 2015; Tangwangvivat et al., 2019). The close contact between companion dogs and humans could be an important interface for the transmission of IAVs with zoonotic potential. Canids have been shown to be susceptible to swine-, equine-, avian- and human-origin IAV (Dubovi, 2010). Moreover, evidence of genetic reassortment between canine H3N2 and human-H1N1 IAV including the pandemic H1N1 subtype has been documented (Song et al., 2012; Chen et al., 2018), which raises a public health concern that dogs may become intermediate hosts for novel emergent IAV.

A(H1N1)pdm09 infections have been reported in dogs in natural and experimental conditions (Lin et al., 2012). Although dog-to-dog transmission of this subtype has been shown to be limited (Lin et al., 2012; Song et al., 2015), infections of dogs with human A(H1N1)pdm09 acquired from their owners were previously documented (Lin et al., 2012; Keenliside, 2013). Serological evidence of A(H1N1)pdm09 has also been reported in dogs worldwide (Sun et al., 2014; Chanvatik et al., 2016; Jang et al., 2017; Su et al., 2019). However, the information available about the role of canines in the epidemiology of this IAV subtype is still very limited in Europe (Dundon, De Benedictis, Viale, & Capua, 2010; Damiani, Kalthoff, Beer, Müller, & Osterrieder, 2012). Hence, we aimed to assess exposure to A(H1N1)pdm09 influenza virus in domestic dogs in Spain, where this subtype was predominately circulating in humans at the time of sampling (ISCIII, 2020).

2. MATERIAL AND METHODS

2.1 Study design and sample colletion

A cross-sectional study was carried out to determine the prevalence of antibodies against A(H1N1)pdm09 IAV in domestic dogs from Andalusia (southern Spain: 36° N-38° 60′ N, 1° 75′ W-7° 25′ W), the Spanish region with the highest census of this species (MAPA, 2019). **The sample size was estimated assuming a prevalence of 50% (the highest sample size for studies with unknown prevalence), with a 95% confidence level and a desired precision of ±3.5% (Thrusfield, 2018)**. **A stratified sampling design was adopted based on the proportion of dogs in the eight provinces of Andalusia. Within each province, animals were selected randomly.** A total of 750 blood samples from dogs admitted into veterinary clinics in 129 municipalities were **finally** collected between 2013 and 2016 (Figure 1). Samples were obtained by cephalic or jugular vein puncture using a sterile collection system. Sera were separated by centrifugation at 400 *g* for 15 minutes and stored at -20ºC until analysis. Epidemiological data, including sex, age (<12, 12–24, 25–36 and >36 months), location, whether pure- or crossbred, activity (pet, hunting or watchdog) and size (height at the withers: small (<40 cm), medium (41–60 cm) or large (>60 cm)) were recorded for each animal (Table 1).

2.2 Serological analysis

Serum samples were tested by an haemagglutination inhibition (HI) test according to standard methods (OIE, 2016). Briefly, sera were pre-treated with receptor-destroying enzyme (RDE; Sigma C8772) (one part of serum and three parts of RDE) for 18 hours at 37ºC, followed by heat inactivation at 56ºC for 30 minutes. Each serum was serially twofold diluted in V-bottomed microtitre plates with 25 µl volume of phosphate-buffered saline (PBS) and incubated with 25 µl of four haemagglutination units (HAU) of A(H1N1)pdm09 IAV (A/California/7/2009 (H1N1) strain) for one hour at room temperature. Afterwards, 50 µl of 0.5% packed cell volume chicken red blood cells was added into each well and incubated at room temperature for 30 minutes. HI antibody titre was defined as the reciprocal of the last dilution that showed complete absence of agglutination. Samples with HI titres ≥ 40 were considered positive.

Screened positive and doubtful samples by HI were further examined for antibodies against A(H1N1)pdm09 influenza subtype using single radial haemolysis assay (SRH) as previously described without modification (OIE, 2016). Samples with a radial zone of lysis around the well were considered positive. The diameter of the zone of haemolysis was measured with digital calipers, and the strain-specific antibody level was expressed as the area (mm^2). Serum from a hyper-immunized experimental pony (Scott et al., 2012) was used as positive control in both diagnostic tests.

2.3 Statistical analyses

The overall prevalence of antibodies was estimated from the ratio of positives to both HI and SRH to the total number of analysed samples, with 95%CI. SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analyses.

2.4 Ethical considerations

Serum samples were opportunistically collected from animals subjected to health programmes, medical check-ups or surgical interventions during the study period, therefore, no ethical approval was necessary for this study.

3. RESULTS AND DISCUSSION

Since the introduction of the pandemic A(H1N1)pdm09 IAV in Spain in summer 2009, this subtype has circulated endemically, being responsible for a high number of human cases annually (Larrauri-Cámara, Jiménez-Jorge, Simón-Méndez, & de Mateo-Ontañón, 2010; ISCIII, 2020). Although Spain is one of the European countries with the largest canine population, with more than 7.4 million dogs (MAPA, 2019), serosurveillance of the pandemic H1N1 IAV in this species has not been carried out in this country to date. Our results showed that only one (0.13%; 95%CI: 0.0-0.39) of the 750 examined dogs was positive for anti-H1N1 antibodies with an HI titre of 160. Specific antibodies against A(H1N1)pdm09 were also confirmed in this individual by SRH, obtaining an area of haemolysis of 43.3 mm^2. The seropositive animal was a female, 4.6 years old, sampled in June 2013 in the province of Malaga (Figure 1). **The seropositivity detected in 2013 is consistent with the highest number of A(H1N1)pdm09 cases reported in humans during 2013-2014 (355) compared to 2014-2015 (3), 2015-2016 (192) and 2016-2017 (3) flu seasons in the study region (ISCIII, 2020). Nevertheless, the higher sampling effort in 2013 in our study may explain this finding.**

To the author's knowledge, this is the first surveillance of A(H1N1)pdm09 in dogs in Spain. The low seroprevalence obtained is in accordance with those previously reported in this species in Germany (0.13%; 1/736) (Damiani, Kalthoff, Beer, Müller, & Osterrieder, 2012) and USA (0.14%; 1/731) (Seiler et al., 2010). Slightly higher prevalence of anti-A(H1N1)pdm09 antibodies were found in Thailand (0.38%; 38/9891; 0.64%; 6/932) (Chanvatik et al., 2016; Tangwangvivat et al., 2019), Italy (0.7%; 7/964) (Dundon, De Benedictis, Viale, & Capua, 2010) and northern China (1.5%; 13/882) (Sun et al., 2014). The highest levels of seroprevalence were observed in the USA (4.0%; 43/1082) (Jang et al., 2017), Hong Kong (7.4%; 55/737) (Su et al., 2019) and southern China (20.5%; 393/1920) (Yin, Zhao, Zhou, Wei, & Chang, 2014). Differences between studies include the differences in sample collection periods, diagnostic methods, dog populations studied and number of animals tested. In the present study, sampled were collected throughout the year from veterinary hospitals that did not experience influenza outbreaks.

Dogs have been found to be clinically infected with pandemic H1N1 influenza virus (Lin et al., 2012) and significantly higher seropositivity to A(H1N1)pdm09 was observed in dogs with respiratory illness than in healthy animals (Jang et al., 2017). However, the occurrence of A(H1N1)pdm09 in dogs is likely to be overlooked because most infections in this species are mild or subclinical (Lin et al., 2012; Sun et al., 2014; Yin, Zhao, Zhou, Wei, & Chang, 2014), which may lead in under-diagnosis of cases. Unfortunately, data on the clinical history of respiratory disease could not be gathered in the tested animals in the present study.

In conclusion, the results obtained indicate limited A(H1N1)pdm09 IAV circulation in domestic dogs in Spain during the period 2013–2016. To the best of our knowledge, this is the first report of A(H1N1)pdm09 exposure in dogs in Spain, which may be of public health concern. Despite this being the main subtype of human IAV causing outbreaks in the study country (ISCIII, 2020) and the high census of companion dogs in the study area, the low seroprevalence obtained suggests that the risk of A(H1N1)pdm09 transmission from dogs to other sympatric species, including humans, can be considered low. Nonetheless, due to the presence of anti-A(H1N1)pdm09 antibodies in one of the tested dogs, the continued expansion of IAVs diversity in canines, the close contact between dogs and humans as well as the endemic seasonal circulation of this subtype in humans in Spain, the risk of transmission at the human-animal interface should not be ruled out. Further serological and virological surveys, including dogs with respiratory clinical signs, are warranted to better understand the role of dogs in the epidemiology of A(H1N1)pdm09 and other IAVs with zoonotic potential in this country.

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We are grateful to the clinic vets for provision of serum samples.

CONFLICT OF INTEREST

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Table legend

Table 1. Distribution of the samples by categories.

Figure legend

Figure 1. Map of Andalusia (southern Spain) showing the locations of dogs sampled. Green and red areas indicate seronegative and seropositive municipalities, respectively.

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Serum samples were opportunistically collected from animals subjected to health programmes, medical check-ups or surgical interventions during the study period, therefore, no ethical approval was necessary for this study.

3. RESULTS AND DISCUSSION

Since the introduction of the pandemic A(H1N1)pdm09 IAV in Spain in summer 2009, this subtype has circulated endemically, being responsible for a high number of human cases annually (Larrauri-Cámara, Jiménez-Jorge, Simón-Méndez, & de Mateo-Ontañón, 2010; ISCIII, 2020). Although Spain is one of the European countries with the largest canine population, with more than 7.4 million dogs (MAPA, 2019), serosurveillance of the pandemic H1N1 IAV in this species has not been carried out in this country to date. Our results showed that only one (0.13%; 95%CI: 0.0-0.39) of the 750 examined dogs was positive for anti-H1N1 antibodies with an HI titre of 160. Specific antibodies against A(H1N1)pdm09 were also confirmed in this individual by SRH, obtaining an area of haemolysis of 43.3 mm^2. The seropositive animal was a female, 4.6 years old, sampled in June 2013 in the province of Malaga (Figure 1). The seropositivity detected in 2013 is consistent with the highest number of A(H1N1)pdm09 cases reported in humans during 2013-2014 (355) compared to 2014-2015 (3), 2015-2016 (192) and 2016-2017 (3) flu seasons in the study region (ISCIII, 2020). Nevertheless, the higher sampling effort in 2013 in our study may explain this finding.

To the author's knowledge, this is the first surveillance of A(H1N1)pdm09 in dogs in Spain. The low seroprevalence obtained is in accordance with those previously reported in this species in Germany (0.13%; 1/736) (Damiani, Kalthoff, Beer, Müller, & Osterrieder, 2012) and USA (0.14%; 1/731) (Seiler et al., 2010). Slightly higher prevalence of anti-A(H1N1)pdm09 antibodies were found in Thailand (0.38%; 38/9891; 0.64%; 6/932) (Chanvatik et al., 2016; Tangwangvivat et al., 2019), Italy (0.7%; 7/964) (Dundon, De Benedictis, Viale, & Capua, 2010) and northern China (1.5%; 13/882) (Sun et al., 2014). The highest levels of seroprevalence were observed in the USA (4.0%; 43/1082) (Jang et al., 2017), Hong Kong (7.4%; 55/737) (Su et al., 2019) and southern China (20.5%; 393/1920) (Yin, Zhao, Zhou, Wei, & Chang, 2014). Differences between studies include the differences in sample collection periods, diagnostic methods, dog populations studied and number of animals tested. In the present study, sampled were collected throughout the year from veterinary hospitals that did not experience influenza outbreaks.

Dogs have been found to be clinically infected with pandemic H1N1 influenza virus (Lin et al., 2012) and significantly higher seropositivity to A(H1N1)pdm09 was observed in dogs with respiratory illness than in healthy animals (Jang et al., 2017). However, the occurrence of A(H1N1)pdm09 in dogs is likely to be overlooked because most infections in this species are mild or subclinical (Lin et al., 2012; Sun et al., 2014; Yin, Zhao, Zhou, Wei, & Chang, 2014), which may lead in under-diagnosis of cases. Unfortunately, data on the clinical history of respiratory disease could not be gathered in the tested animals in the present study.

In conclusion, the results obtained indicate limited A(H1N1)pdm09 IAV circulation in domestic dogs in Spain during the period 2013–2016. To the best of our knowledge, this is the first report of A(H1N1)pdm09 exposure in dogs in Spain, which may be of public health concern. Despite this being the main subtype of human IAV causing outbreaks in the study country (ISCIII, 2020) and the high census of companion dogs in the study area, the low seroprevalence obtained suggests that the risk of A(H1N1)pdm09 transmission from dogs to other sympatric species, including humans, can be considered low. Nonetheless, due to the presence of anti-A(H1N1)pdm09 antibodies in one of the tested dogs, the continued expansion of IAVs diversity in canines, the close contact between dogs and humans as well as the endemic seasonal circulation of this subtype in humans in Spain, the risk of transmission at the human-animal interface should not be ruled out. Further serological and virological surveys, including dogs with respiratory clinical signs, are warranted to better understand the role of dogs in the epidemiology of A(H1N1)pdm09 and other IAVs with zoonotic potential in this country.

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CONFLICT OF INTEREST

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Table legend

Table 1. Distribution of the samples by categories.

Figure legend

Figure 1. Map of Andalusia (southern Spain) showing the locations of dogs sampled. Green and red areas indicate seronegative and seropositive municipalities, respectively.

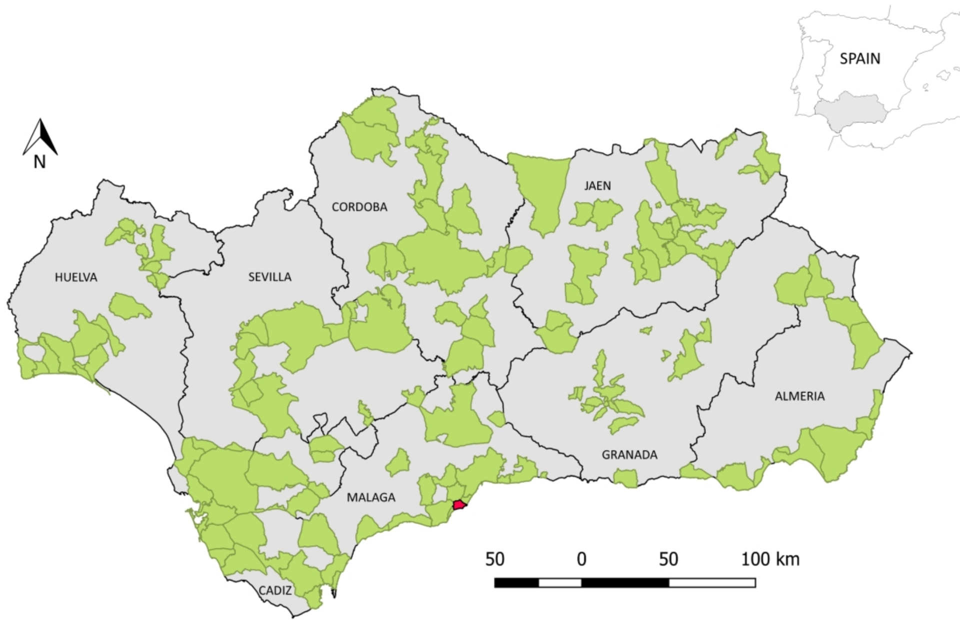

Figure 1. Map of Andalusia (southern Spain) showing the locations of dogs sampled. Green and red areas indicate seronegative and seropositive municipalities, respectively

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Table 1.

Variable	Categories	Nº of samples*	Relative frequency (%)
Location			
	Sevilla	83	10.8
	Cadiz	131	17.0
	Cordoba	100	13.0
	Malaga	105	13.7
	Granada	65	8.4
	Huelva	85	11.1
	Jaen	98	12.7
	Almeria	79	10.3
Year			
	2013	475	61.8
	2014	124	16.1
	2015	121	15.7
	2016	4	0.5
Age			
	<12	32	4.2
	12-24	63	8.4
	25-36	72	9.6
	>36	402	53.6
Sex			
	Male	286	37.2
	Female	309	40.2
Breed			
	Pure	393	51.1
	Crossbred	202	26.3
Activity			
	Pet	605	78.7
	Watchdog	19	2.5
	Hunting	109	14.2
Size			
	Small	105	13.7
	Medium	216	28.1
	Large	104	13.5

*Missing values omitted.