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Serological investigation of racehorse vaccination against equine influenza in Morocco

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ABSTRACT

In order to evaluate the vaccination status against equine influenza (EI) in Moroccan racehorses, a serological investigation was carried out on 509 racehorses using three serological tests: an Enzyme-Linked Immunosorbent Assay (ELISA), the Hemagglutination Inhibition (HI) test and the Single Radial Haemolysis (SRH) assay. The serological analysis showed 56% of seropositivity by ELISA, 67% by HI and 89.4% by SRH (with 69.9% above the clinical protection threshold). Using the Kappa test, the SRH and HI assays showed a strong agreement, the SRH and ELISA assays had a moderate agreement and the HI and ELISA assays showed a poor agreement.

Seropositivity was positively correlated with the age of horses and the number of immunisation received. EI vaccines used during the last immunisation before the study had a weak influence on the serological status. This effect was observed when the vaccines Calvenza and Fluvac Innovator® were used, with 94.1% and 100% of seropositivity when measured by HI, and with 100% and 94.7% exceeding the clinical protection threshold when measured by SRH, respectively. No effect was found when other EI vaccines, including Prequenza-Te® (67% coverage (342/509) and Proteqflu-Te® (22% coverage (114/509) were used; with 64% and 67.5% seropositivity (HI) and with 66.4% and 72.8% above the clinical threshold (SRH), respectively. The location and the time since last vaccination have no influence on the serological result. Overall, levels of protective antibody against EI in Moroccan racehorses remain a concern despite mandatory vaccination.

1. Introduction

Equine influenza (EI) is a highly contagious viral respiratory disease common of equids including horses, donkeys, mules and zebras. Two distinct subtypes (H7N7, formerly A-equi-1, and H3N8, formerly A-equi-2) of equine influenza virus (EIV) have been identified as the main causative agents. Viruses of the H7N7 subtype have not been isolated since the late 1970s and the World Organization for Animal Health (OIE) stipulates that there is no longer a requirement for a representative of this subtype in Equine influenza (EI) vaccines (OIE, 2016b). H3N8 EIV were first described in 1963 following a major EI epizooty observed in the United States (Wadell et al., 1963), and then diverged genetically into the Eurasian and American lineages in the 1980s (Borchers et al., 2005; Daly et al., 1996; Martella et al., 2007). The American lineage subsequently evolved into the Kentucky, South American, and Florida sublineages (Lai et al., 2001), with the Florida sublineage dominating in recent years. Recently, the Florida sublineage diverged into two clades (Florida clade 1 and clade 2; FC1 and FC2, respectively), which contain most of EIV isolated worldwide (Bryant et al., 2011; Fougerolle et al., 2016).

Clinically, EI is characterized by high morbidity and low mortality that can be observed in young, older or immuno-depressed animals. Horses infected with EIV develop pyrexia up to 41 °C and typical respiratory disorders, including a serous nasal discharge and persistent cough. Other signs may be observed, such as depression, loss of appetite and muscle pain. Equine influenza is usually treatable with symptomatic treatment. However, horses infected with EIV may develop serious secondary bacterial infection. *Streptococcus equi* subsp. *zooepidemicus* is one of the most common potential pathogens of secondary bacterial pneumonia following EI (Timoney, 1996; Newton et al., 1999;

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Muranaka et al., 2012).

Economically, the losses caused by EI epizootics may be substantial, notably by stopping horse racing, competitions and commercial exchange (Timoney, 1996). Regretfully, this was particularly well illustrated during the 2007 Australian outbreak, when around 75.000 horses were affected and the overall cost reached a 1 billion $ (Cullinane et al., 2010; Paillot and El-Hadge, 2016a,c). Another example, is the EI epizooty confirmed in Algeria in 2011, which has affected more than 900 horses, and resulted in the cancellation of races during 2 months throughout the country (Laabassi and Mamache, 2014). In Morocco, two outbreaks of EI in 1997 and 2004 were previously reported. The antigenic and genetic characterization of the EIV strain isolated during the first outbreak (A/equine/Nador/1/1997) revealed an association to the European lineage. For the second outbreak, the EIV strains A/equine/Essaouira/2/2004 and A/equine/Essaouira/3/2004 were classified as part of the predivergent lineage (Boukharta et al., 2014).

In Morocco, horse racing has experienced a significant growth in recent years by increasing event frequency, which went from 1762 races in 2011 to 2436 in 2016, and the number of participating horses, who rose from 2175 to 3358 during the same period.

In order to participate in these events, racehorses must be vaccinated against EI according to the Moroccan racing code of practice (i.e.: 2 primary doses (V1 and V2) between 21 and 30 days apart followed by a third dose (V3) 5–6 months after V2, and then an annual reminder after V3). The vaccination control is carried out before each race by checking the horse's document and passport.

In order to evaluate the immune status and the associated theoretical protection level against EI, the present study aims to measure EI-specific antibody levels in 509 racehorses in Morocco. Three serological tests were used and compared; namely, the enzyme-linked immunosorbent assay (ELISA), the haemagglutination inhibition test (HI) and the single radial haemolysis (SRH) assay. Single Radial Haemolysis antibody titres are positively correlated with protection against EIV infection.

2. Materials and methods

2.1. Horses

A total of 509 blood samples were taken from 509 racehorses participating in flat race meetings between May and September 2014 in 6 regions in Morocco. The horses were thoroughbred, arabian, arab-barb and anglo-arabian. They ranged in age from 2.17 to 13.35 years old with a mean age of 4.84 ± 015 years. Their EI vaccination was in agreement with the Moroccan racing code of practice and the brand of EI vaccines used in their last immunisation was recorded, the details of previous vaccination history was not available. The majority of horses enrolled in this study were raised in Morocco but a proportion could have initially been imported from Europe (mostly France, Belgium, Spain). Occasionally, the best thoroughbred horses had participated in racehorses organized in France and Spain.

2.2. Samples

Samples were collected by jugular vein puncture on sterile dry tube (without anticoagulant), sera were extracted by centrifugation and stored at −20 °C until serological analysis. All samples were taken with owner consent and respecting the ethical process set by the Royal Society of Horse Encouragement in Morocco (SOREC) regulations.

2.3. Serological analysis

Three serological assays were used to detect EIV antibodies:

a) Enzyme-Linked immunosorbent assay (ELISA):

The ELISA test is a qualitative test, primarily used for the diagnosis of EI. This assay detects antibodies against the viral nucleoprotein (NP) of type A influenza viruses. The analysis was carried out using the ID Screen® Influenza A Antibody Competition Multi-species assay (IDvet). In accordance with the manufacturer's instructions, percent competition (S/N%) was calculated: $S/N\% = (O.D_{sample})/O.D_{negative\ control})*100$. Samples with S/N% ≤ 45% were considered positive. Samples with S/N% ≥ 50% were considered negative and samples with S/N% between 45% and 50% were considered doubtful.

b) *Hemagglutination inhibition (HI) test:*

The HI assay was performed according to the OIE recommendations (OIE, 2016a). The Sera were pretreated to remove non-specific inhibitors of viral haemagglutination using potassium periodate then inactivated at 56 °C for 30 min. This test was carried out using the FC2 A/Equine/Lannilis/1/2012 (H3N8) EIV strain, which was isolated by LABÉO Frank-Duncombe laboratory (Saint-Contest, France) within the framework of the Epidemiological Surveillance Network in Equine Pathology (RESPE, Saint-Contest, France). The HI titres were read as the highest dilution of serum giving complete inhibition of agglutination. A sample was considered positive with an HI titre ≥ 8 (positivity threshold) (Mumford et al., 1983).

c) Single radial haemolysis (SRH) assay:

Antibodies against FC2 EIV strain A/equine/Richmond/1/07 (H3N8) were measured using single radial haemolysis assay (SRH) according to the OIE recommendations (OIE, 2016a). The A/equine/Richmond1/07 strain was isolated in the United Kingdom in 2007 from the surveillance network managed by the Animal Health Trust (a surveillance scheme financed by the UK Horserace Betting Levy Board). The difference in HA amino acid sequences between the two FC2 strains used in SRH and HI assays was limited to one substitution at the 144 position (V144 A substitution, antigenic site A) and 6 substitutions at non-antigenic sites (supplementary Fig. 1). The hemolytic zones resulting from the lysis of the sensitised sheep red blood cells coupled to EIV and guinea pig complement by the antibody in the test sera were measured with a digital calliper. The area of hemolysis was calculated and results were expressed in mm^2. Horses with SRH antibody titres below 85 mm^2 (clinical protection threshold) are considered insufficiently protected and present a real risk of spreading the disease. Sera with SRH antibody titre above 150 mm^2 (virological protection threshold) are considered with sterilising immunity (Paillot et al., 2016c).

As SRH and HI assays require a large amount of viral antigen, both EIV strains were produced on embryonated chicken eggs "Specific Pathogen Free" at the MCI Animal Health's laboratory in Mohammedia (Morocco).

2.4. Statistical analysis

A statistical analysis was performed using the IBM SPSS software (Statistical Package for the Social Sciences). The functions included in the software are Pearson's chi-squared test ($\chi 2$), kappa test, bivariate statistics (Averages, ANOVA1). Tests of significance were carried out at the $\alpha = 5\%$ level. The Microsoft Excel software was also used for data recording.

3. Results

3.1. Serological analysis reveals limited EI vaccine coverage

The serological analysis of the 509 sera by ELISA showed that 56% of samples (285/509) were seropositive, 43.2% (220/509) were seronegative and 0.8% (4/509) were considered borderline/doubtful. Using the HI test, 67.4% of samples (343/509) possessed an HI titre higher than eight (positivity threshold), 15.7% (80/509) with HI titres between two and four and finally 16.9% (86/509) of samples with no measurable HI titre. Using the SRH assay, 69.9% of samples (356/509) were above 85 mm^2 (clinical protection threshold), including 29.1% of