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Streptococcus equi subspecies *equi* in horses in Israel: seroprevalence and strain types

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ABSTRACT

The purpose of this cross-sectional study was to determine the seroprevalence of *Streptococcus equi* in Israel, to monitor seropositive horses over time and to identify archived strains that were recovered from Israeli horses. A serological survey of 200 healthy horses on 20 farms throughout Israel was performed to detect recent exposure to *S equi* antigens A and C via indirect ELISA. Seroprevalence was 9.5 per cent (19/200) and positive horses were found in 30 per cent (6/20) of the farms. Sixteen horses that returned a positive serology result were retested three and six months later. Most (12/16) positive horses remained positive, which suggests the presence of animals with persistent infection. Molecular characterisation of *S equi* strains by sequencing of the SeM gene of 16 archived isolates of *S equi* that were recovered from clinical cases of strangles between 2008 and 2012 identified two strains: SeM-2 and SeM-28.

INTRODUCTION

Strangles, caused by the bacteria *Streptococcus equi* subspecies *equi* (*S equi*), is one of the most prevalent and important equine infectious diseases worldwide (Sweeney and others 2005). Morbidity may be high since *S equi* is very contagious, but mortality is usually low. The severity of clinical disease varies greatly with classical clinical manifestation of an upper respiratory tract infection, and rare potentially fatal complications including acute dyspnoea or dysphagia, internal lymph node abscessation and immune-mediated responses (Sweeney and others 2005; Whelchel and Chaffin 2009).

Most horses recover from strangles over a period of weeks. However, approximately 10 per cent of affected horses become chronic, long-term shedders, most commonly retaining the organism in the guttural pouches (Newton and others 1997). Identification of these chronic carriers is crucial in order to prevent the spread of *S equi*. In recent years, a serological test has been developed to identify horses that have recently been exposed to *S equi* (Robinson and others 2013, Waller 2014). This method has been applied to evaluate the seroprevalence of *S equi* in working horses in Lesotho in Africa and in healthy horses in Ireland, yielding 10.1 per cent and 42 per cent seropositivity, respectively (Ling and others 2011, Walshe and others 2012).

The equine industry in Israel is small and includes 25,000–35,000 horses of which about one-third are sport and show horses. The Israeli equine industry is developing and dozens of horses have been imported from Europe and the USA every year. Horses, mainly Egyptian Arabians, are also exported every year from breeding farms in Israel. There is also a large movement of horses between the Palestinian Authority and Israel for trade, sport and for medical treatment. Israel is located in a unique area in the junction of three continents, Europe, Asia and Africa, neighbouring the Palestinian Authority and Jordan on the east, Lebanon and Syria on the north and Egypt on the south. Data regarding the prevalence of strangles in these countries are very limited with only occasional outbreaks of strangles being reported by the Kimron Veterinary Institute in their annual reports (none in 2010; five in 2011; eight in 2012 and none in 2013) (http://www.vetserv.moag.gov.il/Vet/all_Publications/dochot-shnatiim/default.htm, last accessed January 2016). However, these reports are unlikely to reflect the true prevalence of *S equi* in Israel. This study was constructed to determine the number of seropositive horses present out of a population of 200 horses in Israel located at 20 different farms throughout the country. The serum response of seropositive horses was retested three and six months postinitial sampling to estimate the possibility of persistent infection. The SeM type of 16 archived isolates of *S equi* was also determined to shed

light on the strains circulating within Israeli-resident horses. Since the information regarding the prevalence and incidence of *S equi* in Israel was limited, the authors hypothesised that the prevalence in a healthy horse population will be similar to the findings in other parts of the world (10.1 per cent in South Africa and 42 per cent in Ireland) (Ling and others 2011, Walshe and others 2012). The calculated required sample size for estimated prevalence of 30±7 per cent for a horse population of 35,000 was 223 horses.

MATERIALS AND METHODS

Equine serum samples for detection of antibodies

In total, 200 serum samples were collected from apparently healthy horses at 20 farms between November and December 2014. Blood samples were collected with the owner's consent, and the survey was approved by the Internal Ethics Review Committee of the Koret School of Veterinary Medicine, The Hebrew University (KSVM-VTH/5-2013). Farms sampled were located throughout Israel according to the estimated geographical distribution of horse farms in the country, with higher farm density in the north and in the centre, and lower density in the south of Israel. On each farm, 5–15 of all available horses were randomly selected and sampled to reflect the size of the farm. The history of suspected clinical cases of strangles at each farm was obtained through a telephone survey of the attending veterinarians. Data for each horse were collected from farm managers and included sex, breed, age, housing and recent health condition. Rectal temperature was measured and blood was collected from the jugular vein into a sterile vacuum tube without an anticoagulant agent. Sera were obtained from clotted blood samples by centrifugation (3000 *g* for 8 min) and stored at −80°C until use. Positive horses that were still available to this study were resampled during February to March 2015 and May to June 2015 in a similar fashion.

Indirect ELISA for the detection of antibodies targeting *S equi* proteins

The indirect ELISA (iELISA) for the presence of serum antibodies against *S equi* protein A (SEQ_2190) and protein C (SeM) was performed as described previously (Robinson and others 2013). An OD_{450nm} of ≥0.5 was considered to be a positive result for antigen A or antigen C (Robinson and others 2013).

Bacterial culture and DNA extraction

All available frozen archived *S equi* isolates from 16 clinical samples submitted to the Laboratory for Bacteriology in the Kimron Veterinary Institute in the years 2008–2012 were analysed in this study. Isolates were cultured on blood agar (Tryptose Blood Agar Base; Becton-Dickinson, Sparks, Maryland, USA, enriched with 5 per cent sheep blood). DNA was extracted using QIAGEN DNeasy as per the manufacturer's protocol (QIAGEN, Germany). An antibiogram was performed by the disc diffusion test and interpreted following the Clinical & Laboratory Standards Institute veterinary standards (Clinical & Laboratory Standards Institute, 2013).

SeM gene identification

The forward primer ASW73 (5′-CAG AAAACT AAG TGC CGGTG-3′) and the reverse primer ASW74 (5′-ATT CGG TAA GAGCTT GAC GC-3′) were used to amplify 541 bp of the 5′ region of the SeM gene unique to *S equi*, as described previously (Kelly and others 2006). All PCR products were sequenced and compared with the SeM strains in the MLST database (http://pubmlst.org/szooepidemicus/seM/, last accessed January 2016).

Statistical analysis

Statistical analysis was performed to detect potential risk factors for the presence of *S equi* antibodies. Association with nominal independent variables was assessed by using the χ^2 test, and ORs were calculated. Association with quantitative parameters was assessed using *t*-test. Association between variables was considered statistically significant when the P value was <0.05. All significant parameters in the univariate analysis were included in a multivariable analysis using a forward-stepwise model. When analysing seroprevalence, the data were also analysed using a generalised estimating equation (GEE) with a logit link function, with the farm set as a subject (i.e. random variable) and with an exchangeable working correlation matrix. The analysis was performed using SPSS V.22.0 and Win Pepi V.11.43 statistical software.

RESULTS

Cross-sectional study population

The study population of 200 horses at 20 farms across Israel was comprised of 90 horses (45 per cent) on farms in northern Israel, 33 (16.5 per cent) from central Israel, 43 (21.5 per cent) from southern Israel and 34 (17 per cent) from the Golan Heights. Horses' age ranged between 8 months and 30 years, with a mean of 10.54 years (sd: ±5.88, variance: 34.56). The sex of the horses distributed equally between male and female, with 98 mares (49 per cent), 98 geldings (49 per cent) and 4 stallions (2 per cent). Most of the horses (149, 74.5 per cent) were mixed breeds, and the rest were of various breeds including Quarter horses (24, 12 per cent), Arabians (6, 3 per cent), ponies (6. 3 per cent), Tennessee walking horses (4, 2 per cent), Paint horses (3, 1.5 per cent), Appaloosas (2. 1 per cent), Missouri Fox Trots (2, 1 per cent), warmbloods (2, 1 per cent), one Shire (0.5 per cent) and one Thoroughbred (0.5 per cent). The management varied at different farms. Some of the farms in the north and Golan Height kept the horses in pastures (86 horses, 43 per cent), and the rest of the horses were housed in paddocks (59 horses, 29.5 per cent) or stalls (55 horses,

27.5 per cent). All of the horses were apparently healthy during sampling and their body temperature was within normal limits (36.5–38.5°C).

Serum antibodies detected by iELISA

Nineteen horses (9.5 per cent) tested positive by iELISA for one or both *S equi* antigens (A or C). In total, 10 horses were positive for antigen A, 11 were positive for antigen C and 2 tested positive for both. Positive horses were identified at 6/20 farms (30 per cent), between 1 and 7 horses (10–53.8 per cent) in each farm (Fig 1).

All of the horses were apparently healthy, and none displayed clinical signs of strangles or other respiratory disease before sampling. All horses had rectal temperature within normal limits (36.5–38.5°C) at the time of sampling. None of the veterinarians treating the farms with positive horses reported clinical cases, suspected of being strangles, within these farms for at least two years.

Univariable statistical analysis for potential risk factors revealed the farm (P<0.001), the geographical area (P<0.001) and the presence of competing horses at the farm (P=0.003) to be significantly associated with *S equi* exposure. Horses in farms in central Israel were significantly more likely to be serologically positive than horses in other parts of the country (OR 7.63, 95 per cent CI 2.83 to 20.57). Factors including horse sex, age and breed were not found to be significantly associated with the serological status. Horses that were housed in stalls were 2.64 times more likely (95 per cent CI 1.02 to 6.86) to be seropositive than horses in paddocks or pastures (P=0.057). Horses at farms which participated in competitions were 4.65 more likely (95 per cent CI 1.61 to 13.42) to be seropositive than horses in farms that did not participate in equine sport events. This factor (the presence of competing horses at a farm) was found to be associated with the farm, geographical area and housing management (P<0.001). Participation of the farm in equine sport events was the only factor that was significantly associated with *S equi* seropositivity in multivariable statistical analysis (P=0.022). No associations were identified in the analysis using GEE.

Long-term surveillance of positive horses

Horses that tested positive (OD_{450nm} of ≥0.5) by the iELISA test were retested three and six months after the initial survey. Also, 12 of the 16 positive horses (75 per cent) remained positive throughout this time period. One positive horse (6 per cent) gradually seroconverted to negative, and in three horses, antibody levels decreased after three months, but increased again after six months.

S equi strains identified by sequencing SeM alleles

Analysis of the SeM gene sequences of 16 isolates that were recovered between 2008 and 2012 identified two SeM alleles. Allele 2 was the most prevalent and was identified in 11/16 isolates (69 per cent). The remaining five isolates (31 per cent) were classified as allele 28.

Horse sex, age and breed, farm location and date of submission were analysed as potential risk factors, but due to the small sample size, and lacking information in some of the forms submitted by the referring veterinarians, no significant correlations were found.

Antibiotic susceptibility tests were performed in 12 cases at the time of submission (data not shown). No significant correlation was found between the SeM allele and the antibiogram.

DISCUSSION

Surveillance and monitoring of *S equi* persistence in healthy horse populations is challenging, and the role of carrier horses has been recognised as an important factor in maintaining a pathogen reservoir and source of infection between outbreaks (Newton and others 2000).

In recent years, only a few cases of strangles were reported by Israeli veterinarians. As cases do not occur often, horses are not tested for *S equi* carriage before their introduction to new farms. Furthermore, currently vaccines against strangles are not available in Israel. The purpose of this study was, therefore, to evaluate the level of exposure of clinically healthy horses to *S equi* using the Animal Health Trust iELISAs test (Robinson and others 2013). By using the iELISAs, the authors found a seroprevalence, representing recent exposure to *S equi*, of 9.5 per cent across 20 farms located throughout Israel. Further investigation of all 20 farms did not identify any new outbreaks occurring three months before or six months following the initial survey. Unfortunately, although it was recommended that seropositive horses were examined by guttural pouch endoscopy, none were examined further by the attending veterinarian. Therefore, the carriage status of seropositive horses could not be determined. However, 12 of 16 horses remained seropositive at three or six months postinitial testing, suggesting that at least some of the population of horses at these farms were persistently infected with *S equi*.

This is the first report of long-term surveillance of the strangles serology of healthy horses. The data suggest that serological screening may be useful to detect individuals with persistently high serology that should be further examined by endoscopy to determine whether they represent an infection risk to in contact animals.

Two endemic strains, SeM-2 and SeM-28, were identified in Israel. The MLST database includes SeM-2 isolates from the USA (Kelly and others 2006), Canada (Kelly and others 2006), Japan (Waller and Jolley 2007) and New Zealand (Patty and Cursons 2014). SeM-28 isolates were recovered from the USA (Waller and Jolley 2007), the UK (Ivens and others 2011) and Dubai (http://pubmlst.org/szooepidemicus/seM/, last accessed January 2016). The two strain types found here were both recovered from different years and geographical locations. Interestingly, the phenotypic antibiogram of both strains is inconsistent, with occasional resistance to some antibiotics in strains of

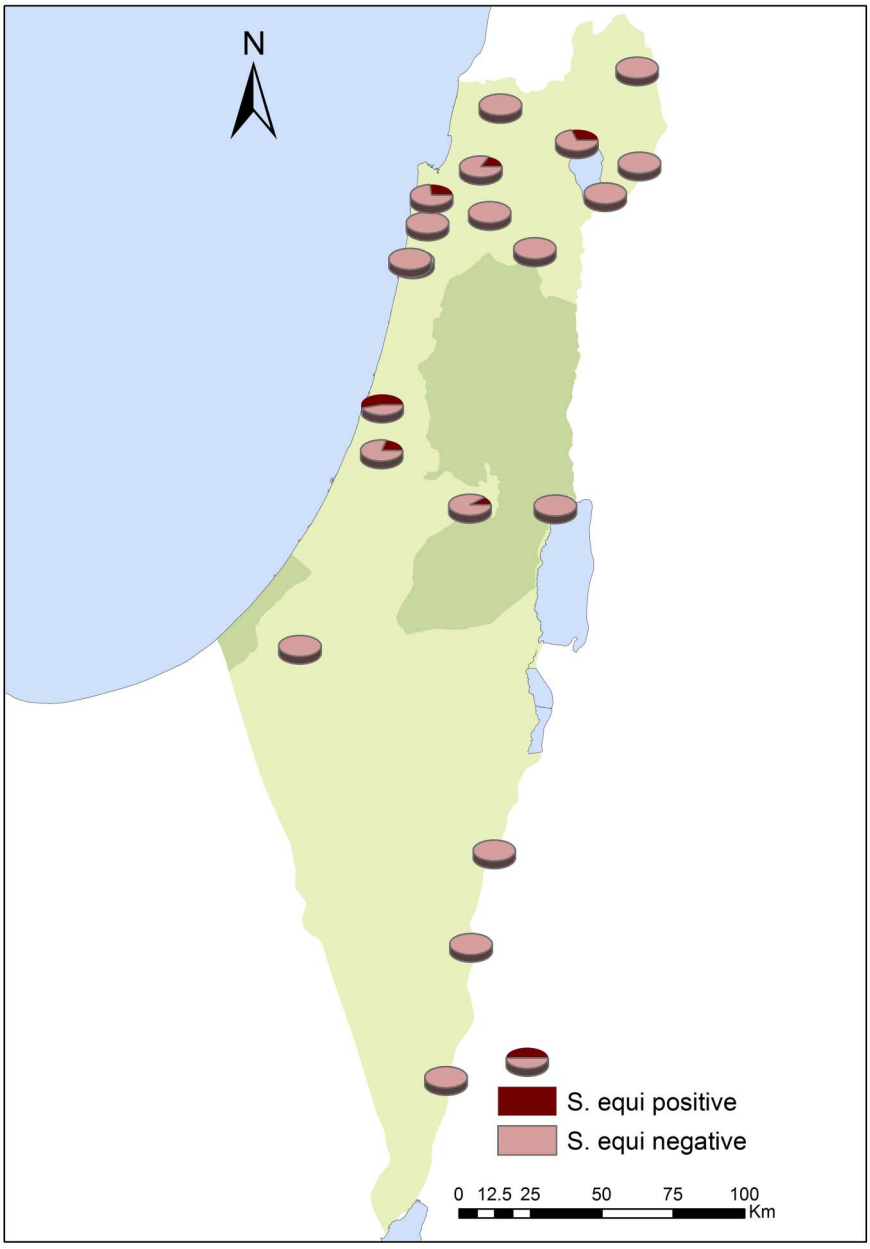

FIG 1: Prevalence and geographical distribution of *Streptococcus equi* in horse farms sampled in Israel during November–December 2014. The number of horses sampled in each farm is represented by the size of each mark. The relative number of horses in each farm found with high titres of *S equi* antibodies (as detected by indirect ELISA) is represented by pie charts

either SeM type. Recently, the *S equi* genome was fully sequenced from different strains revealing high plasticity in many loci, which may lead to changes in virulence to adapt to a persistent state (Harris and others 2015). These potential changes may explain the phenotypic change in antibiotic resistance within a specific strain in isolates from clinical cases. The molecular characterisation of *S equi* isolates from clinical and carrier cases is important to better understand both the epidemiology and evolution of this important bacterium, and to assist in the construction of an effective vaccine.

In conclusion, serological surveys to detect recent exposure to *S equi* antigens in clinically healthy horses may be a useful tool to detect potential exposure or carriage and may help treat and prevent the spread of bacteria and future outbreaks. This is the first large-scale and long-term survey of a healthy population in a non-outbreak period that may point out potential reservoirs of this important pathogen. Further studies are needed, and a combination of serology and bacteriology is highly recommended in order to identify persistently infected horses. This is also the first epidemiological survey of the prevalence and molecular characterisation of *S equi* in Israel. This molecular information is important for molecular epidemiology during outbreaks of strangles and to trace the introduction of new strains in Israel.

Competing interests None declared.

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