A study of leptospirosis in South African horses and associated risk factors

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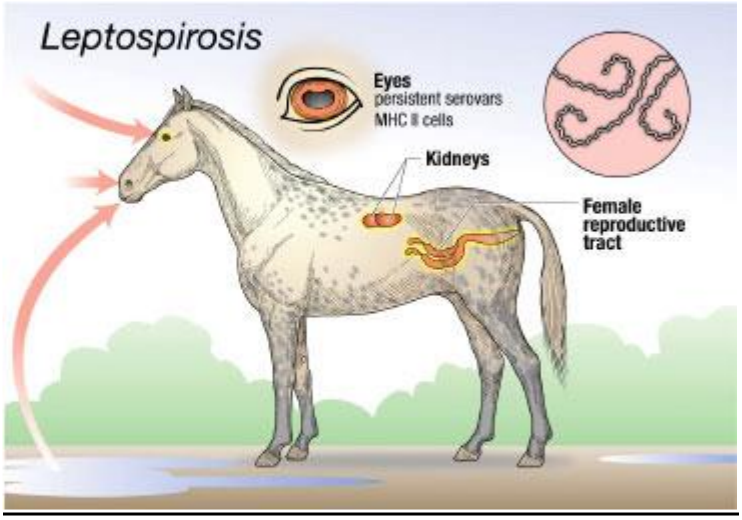
Graphical abstract

Summary:

Little has been published on prevalence of equine leptospirosis in South Africa and the disease status is not known. Literature available on the prevalence of the disease in horses is scant and its epidemiology is not known. The climate in South Africa, together with various risk factors, is conducive for the propagation, maintenance and survival of leptospires in the environment. This raises the interest of investigating the status of the disease in South African horses as well as its risk factors.

Pictogram: Equine leptospirosis



Source:
https://www.google.co.za/search?q=leptospira+pictures&hl=en&biw=1366&bih=667&site=webhp&tbm

Highlights

- Little has been published on equine leptospirosis in South Africa.
- This is the first comprehensive review of equine leptospirosis in South Africa
- High proportion of horses in South Africa are exposed to a wide range of serovars.
- The most frequent serovars found in this study are Bratislava, Djasiman, Arborea and Tarassovi.
- Risk factors related to seropositive horses are identified.

Abstract

Most leptospiral infections in horses are asymptomatic; however, acute disease manifestations as well as reproductive failure and recurrent uveitis have been reported. In South Africa, the epidemiology of the disease in horses is not well documented. A serosurvey to determine what serovars were present in horses from Gauteng, KwaZulu-Natal and Western Cape Provinces and to get an estimate of the seroprevalence of leptospirosis was carried out from January 2013 until April 2014 with the assistance of four large equine hospitals located in these provinces. Furthermore, associations between potential risk factors and both seropositive horses to the predominant serovar Bratislava and to *Leptospira* spp were statistically evaluated using univariate analysis and multivariable logistic regression models. A total of 663 horse sera were collected and tested against a panel of 24 leptospiral serovars using the microscopic agglutination test. The most predominant serovars in Gauteng were Bratislava [32%, 95% CI: 29-35%]; Djasiman [10.4%, 95% CI: 8-12%] and Arborea [8.9%, 95% CI: 7-11%], in the Western Cape Province, Bratislava [27.35%, 95% CI: 23-32]; Djasiman [15.4%, 95% CI: 12-19%] and Arborea [14.5%, 95% CI: 11-18%] and in KwaZulu-Natal, Bratislava [39.4%, 95% CI: 34-44%]; Arborea [9.6%, 95% CI: 7-13%]; and Tarassovi [7.7%, 95% CI: 5-10%] respectively. Twenty one serovars representing 17 serogroups were detected with serovar Bratislava being the most serodominant. The apparent prevalence to one or more serovars of *Leptospira* spp at a serum dilution of 1:100 in Gauteng, KwaZulu-Natal and Western Cape Provinces were 49%; 37% and 32% respectively. The true prevalence was calculated for each province taking into account the clustering effect during the sampling and was found to be between 24-74% in Gauteng; 26-39% in the Western Cape and 20-54% in KwaZulu-Natal. Nooitgedacht (South African horse breed) horses were found to be at greater risk of being seropositive to both serovar Bratislava (OR=5.08) and *Leptospira* spp (OR=6.3). Similarly, horses residing on properties with forestry in the vicinity were found to be at greater risk of being seropositive to both serovar Bratislava (OR=9.3) and *Leptospira* spp (OR=5.2). This study has shown that a high proportion of horses in South Africa are exposed to a wide range of serovars, inferring a complex epidemiology. It also describes for the first time new serovars of *Leptospira* in South African horses that have not previously been reported.

Keywords:

Horses; leptospirosis; serovars; prevalence; risk factors; South Africa.

1. Introduction

Leptospirosis is a widespread zoonotic disease caused by infection with spirochetes belonging to the genus *Leptospira* (Levett, 2001). Leptospirosis, in addition to being the most common bacterial zoonosis worldwide (Lau et al., 2010), is also classified as an emerging infectious disease (Leon et al., 2006). The infection usually results from direct transmission via contaminated urine, placental fluids, and tissues of affected animals or indirectly from a contaminated environment (Baverud et al., 2009). Leptospires can also penetrate the accidental host through mucous membranes or skin abrasions (Thiermann, 1984).

The disease in animals may present in various forms, depending on host species, environmental conditions and the infecting serovar (Pinna et al., 2014). Incidental serovars usually cause acute clinical syndromes including haematuria, fever, jaundice, anorexia and respiratory distress (Yan et al., 2010) but host adapted serovars cause chronic or subclinical infections in reservoir hosts, becoming an important source of infection for humans or other animals (Suepaul et al., 2011).

Although most leptospiral infections in horses are asymptomatic (Hathaway et al., 1981), the most frequently recognized clinical manifestations of leptospirosis in horses are recurrent uveitis and periodic ophthalmia (Levett, 2001; Verma and Stevenson, 2012). The disease has been associated with reproductive tract infection (Divers and Chang, 2009) in pregnant mares which can result in placentitis, abortion or stillbirths (Timoney et al., 2011), renal disease in young horses (Quinn et al., 2011) and hepatic dysfunction (Hathaway et al., 1981).

Titers to a wide variety of serovars have been reported in horses, and although there is a variation between studies, titers to *Leptospira interrogans* serovars Icterohaemorrhagiae, Bratislava, Pomona, Ballum, and Grippotyphosa tend to be most common (Hines, 2014).

Favourable environmental conditions such as warm temperatures, moisture, neutral soil pH (Dickeson and Love, 1993) and standing surface water support the survival of pathogenic leptospires in the environment for a long period, hence increasing infection opportunities.

The climate in South Africa, together with various other risk factors, is conducive for the propagation, maintenance and survival of leptospires in the environment. Despite this, a paucity of information exists regarding the leptospiral serovars in the equine population of South Africa. The reported predominant serovar was found to be Pomona in horses that were tested in South Africa (Anon, 1986-1987) but the epidemiology of this disease in South Africa has not yet been fully investigated. The present study was carried out to: (i) identify the common leptospiral serovars existing in the horses that were serviced by four large equine hospitals in South Africa and to (ii) carry out an epidemiological study to examine risk factors associated with equine leptospirosis in South Africa.

2. Materials and methods

2.1. Serological survey and study area

Ethical approval was obtained from the University of Pretoria animal use and care committee (V040/12) to carry out a serological survey from January 2013 until April 2014 in three provinces of South Africa with the assistance of four large equine hospitals: Two in the Western Cape Province, one in KwaZulu-Natal and one in Gauteng. Three different horse populations were considered in this survey, each one residing in a province with a different ecosystem: The Western Cape Province has a Mediterranean climate with cool, wet winters and warm, dry summers. KwaZulu-Natal Province experiences sunshine year-round with a temperate, sub-tropical climate. Gauteng is primarily a Moist Highveld Grassland climate region with its extreme northern parts classified as Central Bushveld climate (Kruger, 2004).

2.2. Sample collection, processing and storage

A total of 663 horse sera was collected using a non-probability sampling after owners gave consent (Table 1). Adult horses (>2years) admitted to each equine hospital and horses seen on ambulatory service for various purposes were included in the study. To control for potential bias, severely-ill, chronically infected, and horses with underlying immunosuppressive conditions were excluded from sampling. Horses that had not been on the farm for at least 12 months prior to the time of blood collection were also excluded from sampling.

Horses were bled via jugular venipuncture and blood was collected into 10 ml vacutainer tubes with clot activator. After centrifugation, the serum was transferred into two cryotubes and stored at -20ºC. A unique code was given to each of two duplicate cryotubes for identification of the horse bled. The sera were then stored in each hospital before shipping frozen on dry ice to the ARC-Ondersterpoort Veterinary Institute (ARC-OVI) in Pretoria. Pertinent data was captured and maintained in Microsoft Excel 2013 for analysis.

2.3. Questionnaire interview and data collection

A questionnaire was made available for the owners of horses in the study in order to identify risk factors related to seropositive horses. All owners of horses bled were informed about the project and consented to participate prior to bleeding. The questionnaire followed a classic epidemiological approach taking into account population (e.g. age, sex, breed), spatial (e.g. where the horses were kept) and temporal (e.g. clinical signs in the last twelve months) variables as well as management factors (e.g. water source, other animals sharing the same pasture with horses, presence of rodents on the farm etc.) that could be related to leptospirosis in horses. Owner details were also recorded.

Other environmental information regarding the annual average temperature and rainfall which correspond to each horse property was obtained from the South African Weather Service (http://www.weathersa.co.za). This information was used to determine a possible association between those two factors and the leptospiral positive properties.

2.4. Serology

The Microscopic Agglutination Test (MAT) was carried out at the ARC-OVI in Pretoria which consists of two tests in succession namely a screening test to determine if the sera react to the antigen followed by titrations of the reacting sera to determine if they are positive at a final dilution of 1:100. All sera were initially screened at a dilution of 1:80 against antibodies to 24 serovars (19 serogroups) of leptospires using live antigens. These serovars are the most commonly isolated in the tropics and the most commonly reported in different surveys across the globe (Hines, 2014). Live cultures with densities of approximately 2×10^8 leptospires per ml were used as the antigens. An agglutination of 50-100% was taken as a reaction and reacting sera were considered for the titration test where sera were retested to determine an endpoint using doubling dilutions of sera resulting in final dilutions of 1/100 through to 1/3200. The endpoint was defined as the dilution of serum that shows 50% agglutination, leaving 50% free leptospires compared with a control culture diluted 1:2 in phosphate buffered saline (OIE, 2008).

2.5. Data analysis

MAT titers for each serovar in each province were summarized in tables. The apparent prevalence for each of the 24 serovars of leptospires per province was calculated based on the frequency of positive titers to that specific serovar. The predominant serovar was the one with the greatest frequency of titres. A sensitivity of 92% and a specificity of 95% for the MAT was used to calculate the 95% confidence interval for the true prevalence (Hickey, 2010) using Equation 1 and 2 adopted from Cameron (1999).

$$\text{Var (AP)} = \frac{\text{AP}(1-\text{AP})}{\text{n}(\text{Se}+\text{Sp})^2} \qquad \text{(Equation 1)}$$

Where: Var (AP) = estimate of variance for the apparent prevalence, AP = apparent prevalence, Se = sensitivity of the MAT (92%) and Sp = specificity of the MAT (95%).

95% Confidence Interval (CI) for the true prevalence = AP - ($Z_{\alpha/2}$ x $\sqrt{\text{Var(AP)}}$); AP + ($Z_{\alpha/2}$ x $\sqrt{\text{Var(AP)}}$) (Equation 2)

Where, $Z_{\alpha/2}$ at a 95% confidence level is 1.96.

The overall seroprevalence of leptospirosis in each province was calculated taking into account clustering within the data using Equation 3 and Equation 4 (Thrusfield, 2005). A seropositive horse to one or more serovars was considered seropositive to *Leptospira*.

$$\hat{P}\text{-}1.96\left\{\frac{C}{T}\sqrt{\frac{V}{C(C-1)}}\right\}, \hat{P}\text{+}1.96\left\{\frac{C}{T}\sqrt{\frac{V}{C(C-1)}}\right\}, \qquad \text{(Equation 3)}$$

Where: C=number of clusters in the sample, T=total number of animals in the sample and

$$\text{V}= \hat{P}^2(\sum n^2) - 2\hat{P}(\sum nm)+(\sum m^2), \qquad \text{(Equation 4)}$$

Where: V=between-cluster variance. n=number of animals sampled in each cluster, m=number of diseased animals sampled in each cluster.

Data from the questionnaire interviews were captured and transferred to a spreadsheet (Microsoft Excel 2013) and analysed with NCSS 9 statistical software. This information was useful to group horses into different categories and to analyse different factors that may be associated with leptospirosis namely demographic (breed, sex, age); geographic and environmental (temperature and rainfall received by horse properties, agricultural activities in the vicinity of horse properties) as well as management (water source, other species of animals present on the property and in the neighbourhood, presence of rodents) factors. Clinical signs (ocular disease, high fever, abortion and still births) related to leptospirosis were also considered for the analysis. The proportion of *Leptospira* seropositive horses in different categories and groups was calculated. Similarly, the proportion of seropositive horses to the predominant serovar (Bratislava) in different categories and groups was also calculated. A univariate analysis for the association between Bratislava seropositive horses and various risk factors was carried out using the Chi-square test. A parallel univariate analysis for the association between *Leptospira* seropositive horses and various risk factors was also carried out. Risk factors with $P<0.15$ in the univariate analysis were included in a multivariable logistic regression model (Katz, 1999). Two models were developed; the first looking at serovar Bratislava (the most prevalent serovar) positive horses and the second looking specifically at *Leptospira* seropositive horses irrespective of serovar. A reference value was specified for each categorical variable (Hintze, 2013).

For the logistic regression models a hierarchical forward switching algorithm was used to examine for interactions and determine the best model fit. With this algorithm, the term with the largest log likelihood is entered into the regression model. The term which increases the log likelihood the most when combined with the first term is entered next. Then, each term in the current model is removed and the rest of the terms are checked to determine if, when they are used instead, the likelihood function is increased. If a term can be found by this switching process, the switch is made and the whole switching operation is begun again. The algorithm continues until no term can be found that improves the likelihood. This model then becomes the best two-term model. Next, the subset size is increased by one, the best third term is entered into the model, and the switching process is repeated. This process is repeated until the maximum subset size is reached. Hence, this model finds the optimum subset for each subset size. One run is made to find an appropriate subset size by looking at the change in the log likelihood, then the maximum subset size is reset to this value and the model rerun. With hierarchical models only those terms that will keep the model hierarchical are candidates for addition or deletion. The final logistic regression models after hierarchical forward selection were then reduced by backward elimination, removing each independent variable with $P_{Wald}>0.05$ until all the remaining variables were significant ($P_{Wald}<0.05$). The results were reported as odds ratios with 95% confidence intervals and P-values (Hintze, 2013).

3. Results

3.1. Apparent prevalence (AP) of specific serovars of Leptospira in three provinces.

Of the 24 serovars (19 serogroups) used in the MAT, 21 serovars representing 17 serogroups were detected. Titers to two or more serovars were detected in the majority of horses (85%). Distribution of the MAT titers ranged from 100 to 3200. The most predominant serovars in Gauteng were Bratislava [32%, 95% CI: 29-35%]; Djasiman [10.4%, 95% CI: 8-12%] and Arborea [8.9%, 95% CI: 7-11%], in the Western Cape Province, Bratislava [27.35%, 95% CI: 23-32%]; Djasiman [15.4%, 95% CI: 12-19%] and Arborea [14.5%, 95% CI: 11-18%] and in KwaZulu-Natal, Bratislava [39.4%, 95% CI: 34-44%]; Arborea [9.6%, 95% CI: 7-13%]; and Tarassovi [7.7%, 95% CI: 5-10%] respectively. Details of the serovars identified are shown in Table 2. Serovars Panama, Kremastos and Hebdomadis were not found in this study.

3.2. Apparent prevalence (AP) of Leptospira spp in three provinces.

The apparent prevalence of *Leptospira* spp in general at a serum dilution of 1:100 in Gauteng, KwaZulu-Natal and Western Cape Provinces were 49%; 37% and 32% respectively (Table 3, Table 4 and Table 5). The true prevalence was calculated for each province taking into account the clustering effect during the sampling and was found to be between 24-74% in Gauteng; 26-39% in the Western Cape and 20-54% in KwaZulu-Natal. A cluster was considered as a group of horses coming from one property or a stable with several owners.

3.3. Analysis of risk factors for association with serovar Bratislava and Leptospira spp

From the prevalence results, Bratislava was found to be the most prevalent serovar. It was therefore decided to focus the risk analysis on this serovar. In addition, the number of seropositive horses to other serovars was low, making the multivariable logistic regression unreliable for these serovars.

The seroprevalence of serovar Bratislava by demographic, geographic, environmental and management factors is presented in Table 6. Horses were grouped into six categories of breeds. Horses that were crossbreeds and the breeds which were underrepresented were grouped under an "Others" category. Univariate analysis showed that breed was significantly associated with seropositive horses to serovar Bratislava (χ^2 = 72.33, P < 0.001) (Table 6) and *Leptospira* spp (χ^2 = 81.16, P < 0.001) (Table 7). Nooitgedacht horses were found to be at greater risk of being seropositive to both serovar Bratislava (OR=5.08) and *Leptospira* spp (OR=6.3) (Table 8 and Table 9 respectively).

Age of sampled horses ranged from 1 to 30 years, with a median of 11 years old. Although the results showed that seroprevalence of serovar Bratislava increased with age, no significant association was observed between seropositivity and age groups (χ^2 = 2.60, P = 0.27). Similarly, no significant association was observed between seropositivity to *Leptospira* spp and age groups (χ^2 = 1.26, P = 0.53).

The difference between mares, stallions and geldings was significant on univariate analysis for serovar Bratislava (χ^2 = 11.43, P < 0.001) and *Leptospira* spp (χ^2 = 13.68, P = 0.001). However, when included in the multivariable logistic regression models, the variable "gender" was no longer significant for both serovar Bratislava and *Leptospira* spp. and an association could not be proved.

Annual average temperature of the properties in the three provinces ranged from 21.5°C to 26.7°C with a median of 26°C. Horses were grouped into three categories based on the annual average temperature their properties received. The group with annual average temperature ranging from 21.5 to 23.9°C was considered as a low temperature group whereas the groups with annual average temperature ranging from 24-25.9 and ≥ 26°C were considered as medium and high temperature groups respectively. The proportion of seropositive horses to serovar Bratislava was calculated for those categories. There was no significant difference between the three categories of temperature on univariate analysis (χ^2 = 0.18, P = 0.91).

Horses were grouped into three categories based on the average annual precipitation their properties received. Average annual rainfall received by the properties in the three provinces ranged from 395.6 mm to 1050.6 mm with a median of 625.6 mm. The three categories were classified as low average annual rainfall (0-600 mm), medium average annual rainfall (600-1000 mm) and high average annual rainfall (>1000 mm). The seroprevalence to serovar Bratislava was calculated for these three groups and analysed. The variable "average annual rainfall" was associated with horses that had antibodies to serovar Bratislava (Yates' $\chi^2 = 28.14$, $P < 0.001$) using the Chi-square test. Similarly, this variable was significantly associated with horses being seropositive to *Leptospira* spp ($\chi^2 = 22.74$, $P < 0.001$). However, the multivariable logistic regression analysis did not reveal any significant association between this variable and seropositive horses to serovar Bratislava and *Leptospira* spp.

The presence of cattle, pigs, dogs, small ruminants on the property or in the neighbourhood, and their influence on the status of the horses being seropositive to serovar Bratislava and *Leptospira* spp, were also analysed. Presence of cattle (Yates' $\chi^2 = 2.45$, $P = 0.12$), small ruminants (Yates' $\chi^2 = 8.45$, $P = 0.003$), dogs (Yates' $\chi^2 = 4.55$, $P = 0.03$) and pigs (Yates' $\chi^2 = 21.24$, $P < 0.001$) was significantly associated with seropositivity to serovar Bratislava in horses. Similarly, these species were also significantly associated with seropositivity to *Leptospira* spp. However, in the multivariable logistic regression analysis, none of these species had a significant association with seropositivity of horses to serovar Bratislava and *Leptospira* spp.

Agricultural activities in the vicinity of horse properties were considered as another variable. The different categories that formed part of this variable were: Forestry, fruit trees, sugarcane and other. This variable "agricultural activities" was found to be significantly associated with horses seropositive to serovar Bratislava after analysis using the Chi-square test (Yates' $\chi^2 = 50.09$, $P < 0.001$). Similarly, this variable was found to be associated with horses seropositive to *Leptospira* spp ($\chi^2 = 25.57$, $P < 0.001$). Horses neighbouring "forestry" as the main agricultural activity had the highest seroprevalence (80%) compared to other categories. In the multivariable logistic regression analysis, horses residing on properties with forestry in the vicinity were found to be at greater risk of being seropositive to both serovar Bratislava (OR=9.3) and *Leptospira* spp (OR=5.2) compared to other groups.

The difference in the proportion of seropositive horses whose owners claimed to have rodents on the property compared with properties without rodents was not significant for Bratislava (Yates' $\chi^2 = 0.43$, $P = 0.51$) but significant for *Leptospira* spp ($\chi^2 = 2.26$, $P = 0.13$) on univariate analysis. However, the multivariable logistic regression analysis did not show any significant association between the presence of rodents on the properties and seropositive horses to *Leptospira* spp. Similarly, the univariate analysis did not reveal any significant association between seropositivity of horses to both serovar Bratislava and *Leptospira* spp and the source of drinking water for the sampled horses.

3.4. Clinical history and association with serovar Bratislava and Leptospira spp.

Owners were asked if their horses had exhibited clinical signs related to leptospirosis in the past 12 months. Analysis of the data on ocular disease, high fever, abortion and still births did not show any significant association with seropositivity to serovar Bratislava and *Leptospira* spp.

4. Discussion

4.1. Predominant serovars in three provinces

Twenty four serovars representing 19 serogroups were used in the MAT. Out of these serovars, 21 serovars from 17 serogroups were detected in this survey. The most predominant serovars in Gauteng were Bratislava; Djasiman and Arborea, in the Western Cape Province, Bratislava; Djasiman and Arborea and in KwaZulu-Natal, Bratislava; Arborea and Tarassovi respectively. Serovars Panama, Kremastos and Hebdomadis were not detected in the three provinces. Serovars which were reported for the first time in South African horses in this study were Bratislava, Arborea, Tarassovi, Djasiman, Topaz, Canicola, Javanica, Robinson, Cynopteri, Grippotyphosa, Ballico, Icterohaemorrhagiae, Bulgarica, Celledoni, Hardjo, Zanoni, Swajizak, Bataviae, Mednensis and Shermani. This confirms that South African horses in this study were exposed to a wide range of serovars.

The three most predominant serovars per province were almost the same except for KwaZulu-Natal where serovar Tarassovi had the third highest prevalence. The most common serovar in all three provinces was Bratislava. This is not surprising since various studies across the globe have reported the seropredominance of this serovar in their surveys in horses (Baverud et al., 2009; Ebani et al., 2012; Turk et al., 2013). However in South Africa, this seems to be the first report in horses. The findings of this study are also in agreement with other studies where horses may be the maintenance hosts for serovar Bratislava (Rocha et al., 2004). The antibody titers of seropositive horses to this serovar ranged from 100 to 800 except for KwaZulu-Natal where the range was between 100 and 3200. This high range in KwaZulu-Natal may indicate more active infection in this province.

Serovar Arborea was originally described in Italy in 1955 (Kmety and Dikken, 1993). Since then, infection has been reported in humans and animals in different parts of the world including Argentina (Vanasco et al., 2000), New Zealand (Subharat et al., 2011) and Australia (Slack et al., 2006; Slack et al., 2010). Rodents, particularly *Mus domesticus* and *Rattus rattus* are believed to be the predominant animal reservoirs for this serovar (Lau et al., 2015). In Australia, it was found to be the most predominant serovar in a recent survey in horses (Wangdi et al., 2013). This serovar was detected in humans in Australia in 1998 and was isolated from rodents from two sites in Queensland (Slack et al., 2006). In his study, Wangdi et al. (2013) found that pigs and high rainfall were significant risk factors for seropositive horses to serovar Arborea in Australia. It was speculated that pigs may have been exposed through mud and flood water due to their poorly developed body heat regulatory mechanisms (Wangdi et al., 2013). It was also reported that serovar Arborea had been emerging in Queensland (Australia) since 2001, with increase in human case numbers as well as expansion of its geographic distribution. Climatic factors (especially flooding) and environmental change were suspected to be the main cause of this emergence (Lau et al., 2015).

Serovar Djasiman was first described in Indonesia and it was believed to be restricted to South-East Asia. Several cases have been reported in overseas tourists returning from South-East Asia (WHO, 2011). However, a new serovar of serogroup Djasiman has been isolated from an aborted dog foetus in Argentina (Rossetti et al., 2005). The high level of circulating antibodies to this serovar found in the present study, ranging from 100 to 1600 indicate that this serovar may not be exotic to South Africa. Further studies regarding the significance of this serovar in South Africa are needed. It may also be useful to raise the awareness of health service practitioners in South Africa with regard to the clinical disease caused by this serovar; a recent case of massive intra-alveolar haemorrhage caused by serovar Djasiman in a 38 years old man returning from Laos is an indication of the severe clinical disease this serovar can cause (Hery et al., 2015).

Serovar Tarassovi which is thought to be maintained by pigs (Alonso-Andicoberry et al., 2001), has been isolated from dogs (Myburgh et al., 1993) and cattle (Hunter, 2004; Hesterberg et al., 2009) in

South Africa. However this serovar was not detected in a study of South African pigs (Potts et al., 1995). In Northeastern Mexico, this serovar was found to be the most predominant in cattle (Carvajal-de la Fuente et al., 2012).

Some horses had titers to multiple serovars. This may represent multiple infections or different cross-reactions between serovars from the same serogroups. As an example, serovars Ballum and Arborea both belong to the same serogroup (Ballum) and they react similarly in the MAT (Slack et al., 2006). However, serovar Ballum has never been isolated from any source in South Africa nor was it included in the panel of antigens used. Pending further studies to detect serovar Ballum in South Africa, it was assumed that Arborea seropositive horses were probably only exposed to serovar Arborea.

Low titers to serovar Bratislava and Australis (strain Ballico) were found in this study. These two serovars belong to the same serogroup Australis. Although serovar Australis was isolated from dogs in South Africa (Roach et al., 2010), the authors believe that this was due to a cross-reaction since it is believed that cross-reactivity occurs predominately in lower titers (Barwick et al., 1998). High titers to both serovars Tarassovi and Topaz belonging to the same serogroup Tarassovi were also found in this study, suggesting a multiple exposure for some horses. The isolation of local strains of *Leptospira* in South African horses is needed to give better clarification of the serological results.

4.2. Apparent prevalence of Leptospira spp in three provinces

The apparent prevalence of *Leptospira* found in this study appears to be within the same range as other reported findings across the globe. In the present study, the reported apparent prevalence of *Leptospira* at a serum dilution of 1:100 in Gauteng, KwaZulu-Natal and Western Cape Provinces was 49%; 37% and 32%, respectively. Studies conducted in different parts of the world have reported a prevalence of *Leptospira* in horses ranging from 12.8% to 79% (Barwick et al., 1998; Rocha et al., 2004; Jung et al., 2010). Within the same region or countries, variations of the prevalence have been also reported, like in Brazil (Coiro et al., 2012; de Oliveira Filho et al., 2014), USA (Barwick et al., 1998) and Australia (Dickeson and Love, 1993; Wangdi et al., 2013). The difference in prevalence between studies may be due to the study area, study population, sampling techniques, environmental conditions, panel of serovars used in the MAT and the interpretation of results in relation to the cut-off used. In the present study, although there appears to be a difference in apparent prevalence between the provinces, a closer examination of the 95% CI for true prevalence for each province (GP=24-74; WCP=26-39; KZN=20-54) which accounts for clustering bias shows a large overlap in true prevalence between the provinces. This implies that more work needs to be done before one can confirm that there is a difference in prevalence between the provinces. The coastal region of KZN would have been expected to have the highest prevalence due to its subtropical climate with high humidity and rainfall. The warm and humid climate along with different other risk factors contribute to the propagation, maintenance and survival of leptospires in the environment. Factors like flooding that occurs from time to time over Gauteng Province (Dyson et al., 2015) and presence of rodents are also associated with higher prevalences, with flooding allowing closer contact between the bacteria and the accidental host (Levett, 2001; Lau et al., 2010; Bandino et al., 2015). A further survey with a good representative sample across each of the provinces will help to clarify the true prevalence of leptospirosis in these provinces.

4.3. Risk factors and their association with serovar Bratislava and Leptospira spp

4.3.1. Demographic factors

Seroprevalence to serovar Bratislava varied according to breed. Breed was also significantly associated with seropositity of horses to *Leptospira* spp. In Sweden, ponies and coldbloods had a lower odd ratio of seropositivity to serovar Icterohaemorrhagiae infection compared with other breeds (Baverud et al., 2009). This is also in agreement with the findings from Korea where a low seroprevalence was found in Thoroughbred horses used for racing due to the high level of management and care associated with this breed (Jung et al., 2010). In this study, the high seroprevalence of serovar Bratislava was found among Nooitgedacht horses (67%). This breed also had the highest seroprevalence of *Leptospira* spp compared with other breeds (89%). Interestingly, this breed was also found to be at greater risk of being seropositive to serovar Bratislava (OR=5.08) and to *Leptospira* spp (OR=6.3) in both models. Nooitgedacht horses are a rare South African breed with only about 400 pure bred in existence. It was created to preserve the genetics of the Basuto pony and has some Arab blood in its line. It is used predominantly for gymkhana, polo, endurance riding and hacking. Due to the small population there is evidence of inbreeding in these horses and perhaps this contributed to them being more susceptible to leptospirosis (Wikipedia, 2016). The high seroprevalence of both *Leptospira* spp and Bratislava found in this breed may also be due to the fact that all these horses were in a single cluster, which could have had a single source of exposure and they may not be representative of the whole population. Further studies are therefore necessary to find out why this breed is at high risk.

This study also found a significant association between gender and seropositivity to serovar Bratislava using the Chi-square test. Gender was also significantly associated with seropositivity of horses to *Leptospira* spp. In Australia, the difference between variable “gender” with regard to seropositivity to serovar Arborea was demonstrated (Wangdi et al., 2013). The odds of geldings being seropositive to this serovar were 3.3 times more than non-geldings in the final model. However, in South Africa, the factor gender was not significant in the multivariable logistic regression for both Bratislava and *Leptospira* spp. models.

This current study showed that seropositity to serovar Bratislava increased with the increase in age. The highest prevalence (29%) was found in the oldest age group (16+). Similarly, the oldest age group (16+) had the highest seroprevalence to *Leptospira* spp (46%). Other authors have also reported the same increase of seroprevalence with increase of age in horses due to the long period of exposure (Williams et al., 1994; Baverud et al., 2009). However, the difference in seroprevalence between different age groups was not statistically significant in the Chi-square test. This was probably due to the lack of a good representative sample of horses with different ages from each province.

4.3.2. Geographic and environmental factors

The present study failed to demonstrate a significant association between temperature and seropositivity of horses to both serovar Bratislava and *Leptospira* spp. This was probably due to the fact that the temperature data from different meteorological stations in each province showed little or no variation between them, making the range very small.

The variable “annual average rainfall” was significantly associated with horses being seropositive to both serovar Bratislava and *Leptospira* spp in the Chi-square test. This is in agreement with Ward (2002) who reported a strong association between periods of high rainfall and the incidence of leptospirosis.

In Australia, Wangdi et al. (2013) also found that the odds that horses on a horse property with an average annual rainfall of >2000 mm becoming seropositive to serovar Arborea were 6.1 times higher compared to horses residing in horse properties with an average annual rainfall of <1000 mm. Longer periods of rainy season and floods increase the exposure to leptospires by releasing the leptospires from the soil and bringing them to the surface in standing water. In this study, in contrast, the highest prevalence of serovar Bratislava and *Leptospira* spp was found among the horses whose properties received a low average rainfall (0-600 mm). This is not surprising since a decreased rainfall was proven to eliminate food sources and forces carrier animals (including rodents) into human habitats or surroundings, to scavage for food, thereby increasing their contact with accidental hosts (Gubler et al., 2001). In the multivariable logistic regression however, this variable was no longer significant for both models. Poor sampling technique was believed to have contributed to this finding.

When analysed using the Chi-square test, the difference in proportion of seropositive horses to both serovar Bratislava and *Leptospira* spp among the categories of agricultural activities surrounding the horse properties was significant. The seroprevalence of both Bratislava and *Leptospira* spp was the highest for horses residing on the properties surrounded by “forestry.” In the multivariable logistic regression analysis, the two models (Bratislava and *Leptospira* spp) showed that horses surrounded by “forestry” were at greater risk of being seropositive to both serovar Bratislava (OR=9.3) and *Leptospira* spp (OR=5.21). Forests harbour different animal reservoirs of *Leptospira* spp including rodents and small mammals which play an important role in transmission of leptospirosis to other species including humans (Millan et al., 2009). This is also in agreement with a recent study in Europe where leptospirosis was found to be a major zoonotic occupational disease in forestry workers due to the close contact between them and wild animals (Richard and Oppliger, 2015). In addition, another recent study confirmed that serovars from serogroup Australis (including Bratislava) are maintained in wild life animal species (Milas et al., 2013). However, there is still no serological evidence of any serovar from serogroup Australis being reported in South African wildlife. An isolation of serovar Bratislava (or any other serovar from this serogroup) and other leptospiral serovars in wildlife surrounding these horse properties would give more insight into the possible transmission of leptospirosis from wildlife animal species to horses.

4.3.3. Management factors

Presence of other animals on the same pasture with horses or in the vicinity of horse properties had a significant influence on the status of the horses being seropositive to serovar Bratislava and *Leptospira* spp. In the Chi-square test, presence of pigs and cattle on the same pasture with horses or in the vicinity of horse properties had the highest prevalence among other categories of animals. Pigs are thought to be maintenance hosts of serovar Pomona (Adler and Faine, 2006). Antibodies to serovar Pomona were detected in South African pigs in various studies (Potts et al., 1995; Gummow et al., 1999) and the isolation of this serovar was made (Delange et al., 1987; Hunter et al., 1987; Gummow et al., 1999). A previous survey conducted on horses in South Africa has detected and reported a prevalence of 27% for serovar Pomona (Anon, 1986-1987). However, the details with regard to the study area, sample size and panel of antigens used in the survey are scant. In this current study, the apparent prevalence of this serovar in horses was 1.1% in Gauteng Province; 2.56% and 5.8% in the Western Cape and KwaZulu-Natal Provinces respectively. The small sample size related to this variable because of the low prevalence in this study, makes it difficult to establish the potential role of pigs in transmission of serovar Pomona to horses. A similar situation was also seen for cattle which are known to be the maintenance hosts of serovar Hardjo (Adler and Faine, 2006). This serovar was only detected in Gauteng Province and the apparent prevalence was 0.4%. It appears that horses rarely come into contact with reservoirs of the serovar Hardjo.

Concerning the serovar Bratislava, it was found in pigs (Potts et al., 1995) and cattle in KwaZulu-Natal (Hesterberg et al., 2009) and Gauteng Provinces (Personal communication, Madoroba, OVI, 2014). However, for the same reason mentioned above, the role of pigs and cattle in transmission of serovar Bratislava to horses or vice versa needs further investigation. It is probably due to the small sample size that the variable “other species in the vicinity of horse properties” was not significant in the multivariable logistic regression models.

Different types of water sources did not have any influence on the status of horses being seropositive to both serovar Bratislava and *Leptospira* spp. Wangdi et al. (2013) also did not find any significant difference among different sources of drinking water in his study.

Variable "rodents" did not have any significant association for seropositivity to serovar Bratislava using the Chi-square test, but was significantly associated with *Leptospira* spp. However in the multivariable logistic analysis, this variable did not come up significant on the Wald test. Some owners reported the presence of house mice (*Mus musculus*) on their properties. Rodents including house mice were reported to harbour serovar Bratislava. In Canada, the combined results from MAT and FAT suggested that natural infection with serovar Bratislava occur in house mice (Smith et al., 1992). Other studies also reported the isolation of serovar Bratislava from house mice (Galton, 1966) and Norway rat (*Rattus norvegicus*) (Hathaway et al., 1983). In South Africa, rodents were also reported to be the main risk factor of human leptospirosis. In a study published in 2012, it was found that communities in informal settlements in urban areas are at risk as infected rodent populations are a continuous source of transmission (Saif et al., 2012). The apparent incidence of leptospirosis in the South African human population was also found to be moderately high (Saif et al., 2012). The failure of this study to demonstrate the role of rodents in transmission of leptospiral serovars to South African horses is probably due to the lack of a good representative sample of horse properties with rodents across each of the provinces. In the previous paragraph, the authors discussed the difficulty in explaining the role of the pigs in transmission of serovar Bratislava to horses due to the small sample size of horse properties with pigs. Pigs have been found to attract house mice (or rodents) because house mice are frequently abundant where pigs are raised due to ready access to food and shelter (Smith et al., 1992). It appears that the number of rodents that could be attracted by pigs was small since the number of pigs on the properties or in the vicinity of horse properties was also small. The role of the rodents in the epidemiology of leptospirosis in both humans and animals in South Africa needs to be investigated thoroughly. An attempt was made to isolate *Leptospira* serovars from rodents in South Africa but only a small number of rodents were trapped and neither the isolation nor serology could yield any positive result (Gummow et al., 1999). However, the isolation of serovar Bratislava is known to be difficult due to its fastidious nature (Smith et al., 1992).

4.4. Disease history and its association with antibodies to both serovar Bratislava and Leptospira *spp.*

Analysis of the limited data in this study showed that none of the clinical signs were associated with seropositivity of horses to serovar Bratislava and *Leptospira* spp suggesting that most leptospiral infections in horses are asymptomatic.

In this study, many samples were collected by private veterinarians on a convenience basis instead of following the required protocol. This often resulted in many samples coming from a single property instead of having a few samples from many different properties which is what was originally intended when the clinics were engaged.

5. Conclusion

Leptospiral infection is wide spread among South African horses from three provinces. The apparent prevalence to one or more serovars of *Leptospira* spp at a serum dilution of 1:100 in Gauteng, KwaZulu-Natal and Western Cape Provinces were 49%; 37% and 32% respectively. The true prevalence in the three provinces was calculated taking into account the clustering effect during the sampling and was found to be between 24-74% in Gauteng; 26-39% in the Western Cape and 20-54% in KwaZulu-Natal.

As expected, antibodies to a wide range of leptospiral serovars were detected in three provinces.

The predominant serovars circulating in the horse population of the three provinces are Bratislava, Arborea, Djasiman and Tarassovi.

The breed “Nooitgedacht” and the presence of “Forestry in the vicinity of horse properties” were the main risk factors for seropositivity to serovar Bratislava and *Leptospira* spp. In the populations surveyed.

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Table 1 Sampling sites and number of horses per site

Province	Origin of the horse	Number of horses sampled
Gauteng	Pretoria North	89
	Pretoria East	197
	North West	2
	Not known*	9
KwaZulu-Natal	Summerveld	78
	Greytown	60
	Empangeni	23
	Mereense	11
Westen Cape	City of Cape Town	41
	Swartland	2
	Robertson	35
	Paarl	56
	Tulbagh	11
	Piketbearg	35
	Ceres	14
	TOTAL	**663**

*The origin of the horses was missing

Table 2 Apparent Prevalence (AP) of specific serovars in three provinces of South Africa, 2014.

Serovar	Gauteng (n*=269)		Western Cape (n*=117)		KwaZulu-Natal (n*=104)	
	No of titers (≥100)	AP (%) (95% CI)	No of titers (≥100)	AP (%) (95% CI)	No of titers (≥100)	AP (%) (95% CI)
Bratislava[a]	86	32 (29-35)	32	27.35 (23-32)	41	39.4 (34-44)
Djasiman	28	10.4 (8-12)	18	15.4 (12-19)	7	6.7 (4-9)
Arborea	24	8.9 (7-11)	17	14.5 (11-18)	10	9.6 (7-13)
Topaz[b]	23	8.5 (7-10)	6	5.1 (3-7)	6	5.8 (3-8)
Canicola	14	5.2 (4-7)	9	7.7 (5-10)	5	4.8 (3-7)
Icterohaemorrhagiae	14	5.2 (4-7)	1	0.85 (0-2)	2	1.9 (1-3)
Zanoni	13	4.8 (3-6)	0	0 (0-0)	0	0 (0-0)
Robinsoni	13	4.8 (3-6)	2	1.7 (0-3)	4	3.8 (2-6)
Ballico[a]	9	3.3 (2-4)	0	0 (0-0)	2	1.9 (1-3)
Javanica	8	2.97 (2-4)	11	9.4 (7-12)	5	4.8 (3-7)
Cynopteri	8	2.97 (2-4)	2	1.7 (0-3)	4	3.8 (2-6)
Tarassovi[b]	7	2.6 (2-4)	8	6.8 (4-9)	8	7.7 (5-10)
Bataviae	6	2.2 (1-3)	0	0 (0-0)	0	0 (0-0)
Mednensis[c]	5	1.86 (1-3)	0	0 (0-0)	0	0 (0-0)
Pomona	3	1.1 (0-0.02)	3	2.56 (1-4)	6	5.8 (3-8)
Swazijak	3	1.1 (0-0.02)	0	0 (0-0)	0	0 (0-0)
Bulgarica	3	1.1 (0-0.02)	5	4.3 (2-6)	1	0.96 (0-2)
Hardjo[c]	1	0.4 (0-0.01)	0	0 (0-0)	0	0 (0-0)
Shermani	1	0.4 (0-0.01)	0	0 (0-0)	0	0 (0-0)
Grippotyphosa	0	0 (0-0)	2	1.7 (0-3)	2	1.9 (1-3)
Panama	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)
Celledoni	0	0 (0-0)	1	0.85 (0-2)	1	0.96 (0-2)
Kremastos	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)
Hebdomadis	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)

*Number of positive titers (from 100 to 3200)
[a, b, c] Serovars in the same serogroup cross-react.
CI: Confidence Interval

Table 3 Prevalence of *Leptospira* spp in surveyed horses from Gauteng Province, South Africa, 2014.

Cluster	**No of horses sampled**	**No of positive horses**	**Apparent Prevalence (%)**
Cluster 1	22	17	77
Cluster 2	54	48	89
Cluster 3	2	0	0
Cluster 4	38	27	71
Cluster 5	59	17	29
Cluster 6	100	24	24
Cluster 7	1	1	100
Cluster 8	1	1	100
Cluster 9	1	1	100
Cluster 10	1	1	100
Cluster 11	1	1	100
Cluster 12	1	1	100
Cluster 13	1	1	100
Cluster 14	1	0	0
Cluster 15	1	0	0
Cluster 16	1	0	0
Cluster 17	1	1	100
Cluster 18	1	1	100
Cluster 19	1	1	100
Cluster 20	9	2	22
TOTAL	**297**	**145**	**49***
95% CI for true provincial prevalence: 24-74% after accounting for clustering			

Table 4 Prevalence of *Leptospira* spp in surveyed horses from the Western Cape Province, South Africa, 2014.

Cluster	No of horses sampled	No of positive horses	Apparent Prevalence (%)
Cluster 1	41	13	32
Cluster 2	2	1	50
Cluster 3	35	13	37
Cluster 4	16	6	38
Cluster 5	11	2	18
Cluster 6	35	7	20
Cluster 7	14	4	29
Cluster 8	40	17	43
TOTAL	**194**	**63**	**32***
95% CI for true provincial prevalence: 26-39% after accounting for clustering			

Table 5 Prevalence of *Leptospira* spp in surveyed horses from KwaZulu-Natal Province, South Africa, 2014.

Cluster	**No of horses sampled**	**No of positive horses**	**Apparent Prevalence (%)**
Cluster 1	78	21	27
Cluster 2	6	4	67
Cluster 3	9	5	56
Cluster 4	25	20	80
Cluster 5	20	4	20
Cluster 6	8	2	25
Cluster 7	11	2	18
Cluster 8	15	5	33
TOTAL	**172**	**63**	**37***
95% CI for true provincial prevalence: 20-54% after accounting for clustering			

Table 6 Seroprevalence of serovar Bratislava in South Africa, 2014, by demographic, geographic, environmental and management factors

Variable	No. of horses tested	No. of positive horses	
		Titre ≥1:100	AP (%)
Gender			
Mares	364	101	28
Stallions	10	5	50
Geldings	241	43	18
Age (years)			
Group 1 (1-6 years)	103	28	27
Group 2 (7-15 years)	359	80	22
Group 3 (16+ years)	140	40	29
Breed[a]			
Arabian	32	5	16
Nooitgedacht	63	42	67
Pony	57	11	19
Thoroughbred	332	65	20
Warmblood	47	5	11
Others	84	22	26
Temperature[b]			
Low	179	43	24
Medium	82	21	26
High	311	80	26
Avg annual rainfall[c]			
Low	162	62	38
Medium	431	86	20
High	34	2	6
Agricultural activity[d]			
Forestry	25	20	80
Fruit trees	100	22	22
Sugarcane	104	13	13
Other	363	90	25
Other species of animals in the vicinity			
Cattle	329	102	31
Small ruminants	42	4	10
Pigs[e]	116	53	46
Dogs	17	1	6
Water source			
Bore hole	314	80	25
Dam and bore hole	12	3	25
Municipal	88	17	19
River	201	50	25
Dam water	14	1	7
Rodent problem[f]			
No	54	11	20
Yes	575	140	24

[a] Yates' Chi-square= $\chi^2 = 72.33$, df = 5, $P < 0.001$, [b] Yates' Chi-square= $\chi^2 = 0.18$, df = 2, $P = 0.91$, [c] Yates' Chi-square= $\chi^2 = 28.14$, df = 2, $P < 0.001$, [d] Yates' Chi-square= $\chi^2 = 50.09$, df = 3, $P < 0.001$, [e] Yates' Chi-square= $\chi^2 = 21.24$, df = 1, $P < 0.001$, [f] Yates' Chi-square= $\chi^2 = 0.43$, df = 1, $P = 0.51$. AP, apparent prevalence.

Table 7 Seroprevalence of *Leptospira* spp in South Africa, 2014, by demographic, geographic, environmental and management factors

Variable	No. of horses tested	No. of positive horses	
		Titre ≥1:100	AP (%)
Gender			
Mares	365	172	47
Stallions	10	6	60
Geldings	241	79	33
Age (years)			
Group 1 (1-6 years)	103	41	40
Group 2 (7-15 years)	359	148	41
Group 3 (16+ years)	141	65	46
Breed[a]			
Arabian	32	14	44
Nooitgedacht	64	57	89
Pony	57	21	37
Thoroughbred	332	105	32
Warmblood	47	15	32
Others	84	46	55
Temperature[b]			
Low	179	67	37
Medium	82	33	40
High	312	146	47
Avg annual rainfall[c]			
Low	163	92	56
Medium	431	157	36
High	34	9	26
Agricultural activity[d]			
Forestry	25	20	80
Fruit trees	100	36	36
Sugarcane	104	29	28
Other	364	161	44
Other species of animals in the vicinity			
Cattle	329	161	49
Small ruminants	42	11	26
Pigs[e]	116	82	71
Dogs	17	3	18
Water source			
Bore hole	315	138	44
Dam and bore hole	12	3	25
Municipal	88	31	35
River	201	83	41
Dam water	14	4	29
Rodent problem[f]			
No	54	17	31
Yes	576	242	42

[a] Yates' Chi-square= $\chi^2 = 81.16$, df = 5, $P < 0.001$, [b] Yates' Chi-square= $\chi^2 = 4.35$, df = 2, P = 0.18, [c] Yates' Chi-square= $\chi^2 = 22.74$, df = 2, $P < 0.001$, [d] Yates' Chi-square= $\chi^2 = 25.57$, df = 3, $P < 0.001$, [e] Yates' Chi-square= $\chi^2 = 38.08$, df = 1, $P < 0.001$, [f] Yates' Chi-square= $\chi^2 = 2.26$, df = 1, P = 0.13. AP, apparent prevalence.

Table 8 Multivariable logistic regression analysis for Bratislava model

Independent variables	Regression coefficient	Odds ratios	Lower 95% confidence	Upper 95% confidence	Wald P-value
Forestry	2.23	9.3	4.25	20.36	0.00
Fruitrees	-0.58	0.56	0.31	0.99	0.05
Sugarcane	-0.78	0.46	0.26	0.82	0.01
Arabian	0.26	1.3	0.45	3.74	0.62
Nooitgedacht	1.62	5.08	2.84	9.09	0.00
Pony	-0.21	0.81	0.29	2.24	0.68
Thoroughbred	-0.82	0.44	0.26	0.75	0.00
Warmblood	-1.06	0.35	0.12	0.99	0.05

Table 9 Multivariable logistic regression analysis for *Leptospira* model

Independent variables	Regression coefficient	Odds ratios	Lower 95% confidence	Upper 95% confidence	Wald P-value
Arabian	0.46	1.58	0.56	4.47	0.39
Nooitgedacht	1.84	6.31	3.04	13.13	0.00
Pony	-0.70	0.49	0.20	1.22	0.13
Thoroughbred	-1.24	0.29	0.18	0.46	0.00
Warmblood	-0.48	0.62	0.29	1.29	0.20
Forestry	1.65	5.21	2.42	11.23	0.00
Fruitrees	-0.28	0.75	0.46	1.23	0.26
Surgacane	-0.68	0.50	0.30	0.83	0.01