# Establishing post-outbreak freedom from African horse sickness virus in South Africa's surveillance zone

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# Abstract

An African horse sickness (AHS) outbreak occurred in South Africa's AHS controlled area in autumn 2016. A freedom from disease survey was performed to establish the likelihood of ongoing circulation of the associated virus during the same period the following year. A single-stage surveillance strategy was employed with a population-level design prevalence of 1% to establish a survey population sensitivity of 95% (probability that one or more positive horses would be detected if AHS was present at a prevalence greater than or equal to the design prevalence). In March 2017 a total of 262 randomly selected horses from 51 herds were sampled from the 2016 outbreak containment zone. Three within-herd and herd-level design prevalence scenarios were used in evaluating the post-survey probability of freedom. Depending on the underlying design prevalence scenarios, effectively ranging between 0.8% and 6.4%, and the use of informed or uninformed priors, the probability of freedom derived from this surveillance ranged between 73.1% and 99.9% (uninformed prior) and between 96.6% and 100% (informed prior). Based on the results the authors conclude that it is unlikely that the 2016 AHS virus was still circulating in the autumn of 2017 in the 2016 outbreak containment zone. The ability to perform freedom from disease surveys, and also to include risk-based methods, in the AHS controlled area of South Africa is influenced by the changing underlying population at risk and the high level of vaccination coverage in the horse population. Ongoing census post-outbreak must be undertaken to maintain a valid sampling frame for future surveillance activity. The seasonality of AHS, the restricted AHS vaccination period and the inability to easily differentiate infected from vaccinated animals by laboratory testing impact the ability to perform a freedom from disease survey for AHS in the 12 months following an outbreak in the controlled area.

## Keywords

African horse sickness type 1; Surveillance evaluation; Freedom from disease

# 1. Introduction

African horse sickness virus (AHSV) is an orbivirus causing African horse sickness (AHS) in equids. It is transmitted by *Culicoides* spp. vectors and results in significant clinical disease and equine losses in sub-Saharan Africa (Coetzer & Guthrie, 2004). The disease has impacted the international trade of horses from Southern Africa due to its occurrence within South Africa's AHS controlled area (Grewar, 2016). This controlled area consists of an inner AHS free zone, a surveillance zone and a protection zone (Figure 1), and was established, based on historical risk profiling and the nature of the equine population in the zone, to allow direct trade of equines between South Africa and the European Union (EU) (Bosman, Brückner, & Faul, 1995). Animal health control and regulatory measures relating to AHS are in place in the controlled area and include restrictions on ownership, movement and vaccination of equines associated with the controlled area (Animal Diseases Act (Act No.35), 1984). Movement control is primarily focussed on horses originating from the AHS infected zone and movi

ng into the AHS controlled area, and prerequisites for movement include: positive identification; pre-movement health and vaccination status attestation by a veterinarian; and the issuing of permits by the Veterinary Services based on a low risk AHS profile of the area from where the horse originates. Vaccination against AHS in the free and surveillance zone is specifically prohibited unless authorised by the State Veterinary Authority, and authorised vaccination is restricted to the June - October period (winter and spring) each year to minimize the risk of vector transmission of live attenuated vaccine virus. The

vaccination coverage within the horse population in the AHS free and surveillance zone remains high however (70% of horses surveyed during the 2004 outbreak were previously vaccinated) due to compliance with the movement protocol between AHS control zones in the country (Sinclair, Bührmann, & Gummow, 2006).

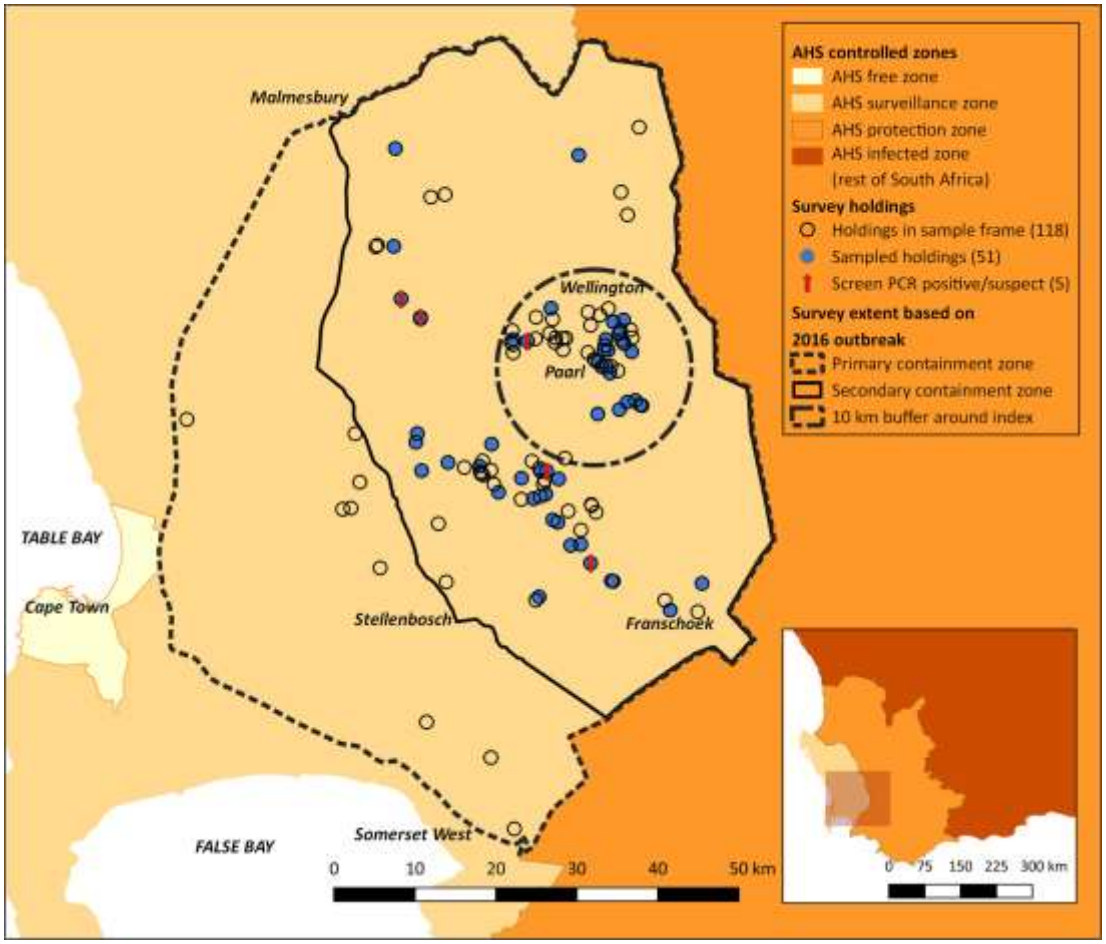


**Figure 1**. Herds associated with the 2017 African horse sickness freedom from disease survey. Black-rimmed circles indicate all herds within the sampling frame, blue-filled circles show herds where sampling took place (n=51). Upward red arrows show the five herds where screening PCR results were suspect or positive and where follow-up investigation was performed. Extents of the 2016 outbreak are shown with the black dashed polygon indicating the primary outbreak containment zone, the solid black line indicating the secondary containment zone and the dash-dot line indicating the area within 10 km of the index case. African horse sickness controlled zones are indicated by varying shades of orange-brown, from the free zone in Cape Town in the south-west, the surveillance zone within which the survey was conducted, and the protection zone which acts as a further buffer from the infected zone which consists of the rest (and majority) of South Africa.

Surveillance in the AHS controlled area consists of both active and passive components. The active sentinel surveillance program targets 150 horses a month with proportional sampling based on the underlying horse distribution in the surveillance and free zones. All sentinels are tested for AHSV RNA, with 60 unvaccinated horses included which are also tested using serology for AHSV group-specific antibodies (Grewar et al. 2017).

Freedom from disease surveys in animal populations are undertaken for a variety of reasons. Global and regional standard-setting organisations such the World Organisation for Animal Health (OIE) and the European Commission institute specific requirements for freedom from disease surveillance, with outcomes ranging from the herd, region, country and ultimately global population level. A freedom from disease status is beneficial to either promote trade of animals and animal products between countries (Vallat, 2006) or to provide confidence in the safety of food, for example, *Trichinella* surveillance in the pig industry in EU (EU, 2015). Beyond categorising populations as free of disease, freedom from disease surveys are also undertaken to allow areas to return to a freedom status after an incursion of a disease. Requirements to return to freedom in the post-AHS outbreak period in the controlled area of South Africa is an example of such a scenario, with these requirements included in the EU legislation regulating the importation of horses from South Africa (EU, 2008).

During April and May 2016, an outbreak of AHSV type 1 occurred in South Africa's AHS surveillance zone near the town of Paarl, extending the already imposed ban of direct trade of horses between South Africa and the EU (Grewar et al., 2018). This study describes the freedom from disease survey undertaken to assist in classifying the AHS status of the AHS

controlled area affected by the 2016 AHS outbreak and provide evidence of AHS freedom in order to regain AHS free status and promote resumption of trade. It details the influence that changing equine populations can potentially have on the definition of an appropriate sampling frame and the impact that AHS vaccination has on the timing of surveillance activity, particularly within a disease control area where legislation prescribes a seasonal vaccination protocol. Furthermore, the interpretation of surveillance results and the ability to perform representative sampling from strata of different risk in order to increase the efficiency of the surveillance design are challenging where registered vaccines and available diagnostic tests do not allow for the differentiation between infected and vaccinated animals (DIVA).

# 2. Materials and Methods

## *2.1 Ethics approval*

Ethics approval for this study was obtained by the Western Cape Department of Agriculture's (WCDOA) Departmental Ethics Committee for Research on Animals (Reference DS17/119). Informed written consent was obtained from each participating herd owner/manager.

## *2.2 Sample size and surveillance strategy*

The sampling frame (1813 horses in 118 herds) was established using population data obtained during the 2016 Paarl AHS outbreak. These data were primarily obtained from the outbreak epicentre and all herds within 5 km of infected herds in the outbreak (Grewar et al., 2018), and as shown in Figure 1, the majority of herds within the sampling frame (and all the herds involved in the final sampling) were found within the secondary containment zone

of the 2016 outbreak. The sampling frame was dominated by Thoroughbred horses (59%) with American Saddlebred (9%), South African Warmblood (3.8%), Arab (3.5%), Boerperd (2%) and Friesian (2%) making up the majority of the remaining known purebred horses, while crossbred or unknown breeds made up the remaining horses (20.7%). The outbreak data were collected in April and May 2016 and updated animal-level census data in that area were not available when the freedom from disease survey took place in March 2017.

A single-stage surveillance strategy was chosen and calculations for the total number of horses to sample were made using previously described methods (Cameron & Baldock, 1998a) implemented in EpiTools (Ausvet (Pty) Ltd: http://epitools.ausvet.com.au/), using the '*Sample size to achieve specified population level sensitivity*' option. The overall crude AHS affected proportion within the horse population during the 2016 Paarl AHS outbreak was 0.01 (Grewar et al., 2018), and this was used as the design prevalence to be detected through the surveillance. The AHSV real-time reverse transcription quantitative PCR (RT-qPCR) screening assay used in the survey had an estimated median sensitivity of 0.978 and a median specificity of 0.999 and has been proposed as highly useful for discriminating between AHSV-infected and non-infected horses (Guthrie et al., 2013). Serological testing was not considered an option for screening in the survey, since the goal was a point-in-time estimate of probability of freedom from the previously circulating 2016 AHSV, which the RNA-based testing could provide with a single test rather than the paired testing required for determining seroconversion; the latter would be required since the vaccination coverage in the region is high and there was no DIVA serological test available. Overall specificity of 100% was assumed since follow-up to a final negative endpoint was performed for each horse that tested suspect or positive on RT-qPCR. A type one error rate of 5% was used

reflecting a 95% probability of detecting AHS should it exist within the survey parameters. The population size was known (N=1813) and the sample size calculation used the hypergeometric approximation. Based on these parameters a sample size of 271 horses was established, and a random list of horses to be sampled was extracted from the population data, without replacement, using the '*Random sampling from a sampling frame*' option in EpiTools. Due to the time period between the 2016 outbreak and the survey in March 2017, during which changes occurred in the equine population, a random replacement list was drawn up for each herd using the population dataset to replace horses selected to be sampled that were unavailable on the day of sampling. A single round of random herd selection was required to replace two herds which were unavailable for the survey. An updated aggregated census was obtained when each sampled herd was visited to allow for accurate post-surveillance evaluation.

The sampling time-frame for whole blood samples stored in EDTA is shown in Figure 2. The RT-qPCR used as a screening test was not DIVA capable and the survey time period was selected in order to decrease the likelihood of false positive RT-qPCR screening results due to recent vaccination in 2016 in the area where the survey was to take place. The latest AHS vaccination date was obtained for each sampled horse. Sampling took place during a similar time of year to when the Paarl 2016 outbreak occurred while still leaving enough time to do follow-up investigations before the start of the next vaccination period which started in June 2017.

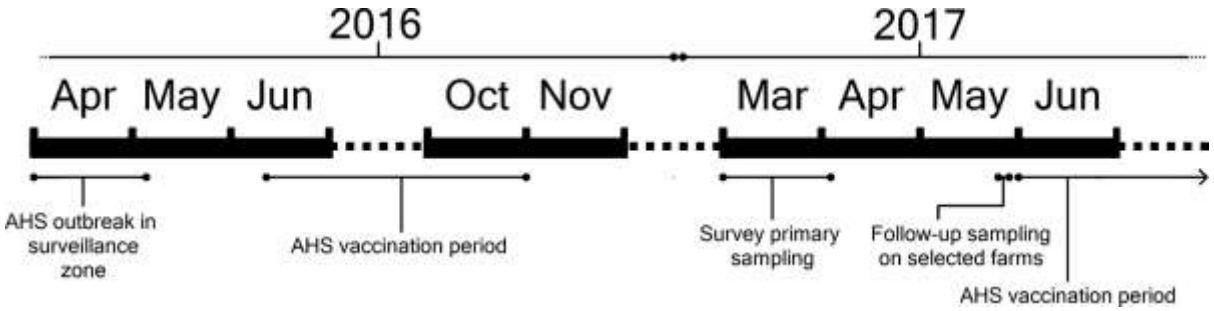


**Figure 2.** Sampling time-frames associated with the 2017 African horse sickness freedom from disease survey. The 2016 section indicates the period in which the 2016 outbreak took place as well as the subsequent vaccination period where African horse sickness vaccination could take place in the AHS controlled area. The primary and follow-up sampling periods for the survey took place prior to the start of the vaccination period in 2017.

## *2.3 Surveillance case definition*

The goal of the surveillance was to establish the probability of freedom from the AHSV that was responsible for the Paarl 2016 outbreak. The surveillance case definition used was based on a combination of the isolation and RNA detection clauses of the OIE's AHS case definition (OIE, 2016), specifically:

> *"AHSV has been isolated and identified from an equid or a product derived from that equid; or*
>
> *antigen or ribonucleic acid specific to AHSV has been identified in samples from an equid showing clinical signs consistent with AHS, or epidemiologically linked to a suspected or confirmed case"*

Screening of primary samples was performed using the qRT-PCR as previously mentioned where the sensitivity of the test influenced the sample size. The lack of DIVA capability and the group-specific nature of the screening PCR resulted in the requirement for further diagnostic testing to establish a final case classification. AHSV typing, using a type-specific

RT-qPCR (Weyer et al., 2015), and virus isolation (VI), was performed on all suspect samples. The latter assisted in establishing the likelihood of suspect results originating from live virus circulation rather than residual RNA from prior vaccination. Sequencing of any VI positive cultures was planned in order to genetically link any positive results to the 2016 outbreak; however, there were no VI positives which precluded the use of sequencing.

All suspect or positive RT-qPCR samples were re-extracted and re-tested. In herds where suspect or positive RT-qPCR results were obtained a follow-up sample was collected, from all previously sampled horses, for further RT-qPCR testing.

## *2.4 Post-surveillance evaluation*

Population sensitivity and confidence of freedom were calculated using previously described methods (Martin, Cameron, Barfod, Sergeant, & Greiner, 2007) and since the population size was known for each sampled herd the hypergeometric approximation for determining population sensitivity was used (MacDiarmid, 1988; Cameron & Baldock, 1998a). Population sensitivity (*SeP*) was initially calculated assuming the entire population was a single herd as defined in the single-stage surveillance strategy using Eq. 1:

$$SeP = 1 - (1 - SeU \times \frac{n}{N})^d \quad (1)$$

where *SeU* is the sensitivity of the RT-qPCR assay, *n* is the number of animals tested, *N* is the number of animals in the population and *d* is the number of expected diseased animals, a product of the animal level design prevalence and the number of animals in the population.

Separately $SeP$ was calculated assuming a 2-stage design using herd-level data, for varying animal and herd-level design prevalence values. Population sensitivity was estimated as Eq. 2:

$$SeP = 1 - (1 - \frac{\sum SeH_i}{n} \times \frac{n}{N})^d \quad (2)$$

Here $SeH$ is estimated separately for each herd sampled, using Eq.1. $N$ is the number of herds in the entire population, $n$ is the number of herds tested and $d$ is the number of herds expected to be infected, a product of the herd-level design prevalence and the number of herds in the population.

Three combinations of within-herd and herd-level design prevalence were used to reflect varying scenarios established from: the Paarl 2016 outbreak; AHS outbreaks in the same AHS controlled zone in South Africa in 1999, 2004, 2011, 2014 and 2016 (Grewar et al. 2018; Weyer et al. 2016; Sergeant et al., 2016; Sinclair, Bührmann, & Gummow, 2006; Western Cape Department of Agriculture (WCDOA, unpublished data); and a generic option to reflect the overall design prevalence (0.01) used in the initial sample size calculation (Table 1). Since an effective design prevalence of 0.01 can be obtained through combination of a range of within-herd and herd-level values, a separate evaluation was made of the resulting probability of freedom for combinations of these prevalences. For this analysis, within-herd prevalence values between 0.02 and 0.5 were used, with corresponding herd-level prevalences between 0.5 and 0.02 respectively, such that the product of the two values was fixed at 0.01.

**Table 1**

Mean herd-level surveillance sensitivity, population surveillance sensitivity and overall confidence of freedom from African horse sickness infection for the population using uninformed and informed priors. Columns reflect the varying design prevalences used as inputs to analyse the surveillance outcomes for both single-stage and two-stage analysis.

| | Descriptions and values of design prevalences based on varying data sources | | | |
|---|---|---|---|---|
| | Design prevalence used in survey design (single-stage analysis) | Generic prevalences to reflect an effective overall design prevalence used in survey design (two-stage analysis)† | Prevalences from Paarl 2016 outbreak data | Prevalences from historical AHS surveillance zone outbreak data |
| | Input design prevalence | | | |
| Within-herd animal level prevalence ($P^*_U$) | 0.01 | 0.2 (0.02 – 0.5) | 0.128 | 0.278 |
| Herd-level prevalence ($P^*_c$) | N/A | 0.05 (0.5 – 0.02) | 0.067 | 0.233 |
| Effective population prevalence ($P^*_U \times P^*_c$) | 0.01 | 0.01 | 0.008 | 0.064 |
| | Resulting outcome | | | |
| Mean herd-level surveillance sensitivity (*MeanSSH*) | N/A | 0.515 (0.25 – 0.69) | 0.448 | 0.586 |
| Population surveillance sensitivity (*SeP*) | 0.945 | 0.779 (0.632 – 0.999) | 0.821 | 0.999 |
| Confidence of population freedom – uninformed prior (*PFreeU*) | 0.948 | 0.819 (0.731 – 0.999) | 0.848 | 0.999 |
| Confidence of freedom – informed prior (*PFreeI*) | 0.995 | 0.979 (0.966 – 1) | 0.983 | 0.999 |

† The range and resulting outcomes for combinations of design prevalences reflecting an effective population prevalence of 0.01 are included in parentheses

The confidence of freedom estimates ($P_{free}$), equivalent to the negative predictive value of the surveillance program, for both an uninformed (0.5) and informed (0.912) prior confidence of freedom ($PriorP_{free}$) were established using Eq. 3:

$$P_{free} = \frac{PriorP_{free}}{(1 - SeP \times (1 - PriorP_{free}))} \quad (3)$$

where population sensitivity (*SeP*) is determined by Eq.1 or Eq.2 for single or two-stage evaluation respectively. The AHS surveillance zone in the Western Cape of South Africa undergoes active monthly sentinel surveillance. An evaluation of the program between September 2016 and August 2017 showed a final posterior probability of freedom of 95.9%. The posterior probability of freedom at the end of February 2017 from that analysis was 91.2% (Grewar et al., 2017), and this was used as the informed prior estimate of confidence of freedom.

# 3. Results

Of the targeted 271 randomly selected horses to sample, 262 were sampled from 51 herds, of which 166 (63%) were selected in advance from the sampling frame, with the remainder being randomly selected replacement horses (Figure 1). Tables S1 and S2 provides a summary of the demographics of the sampled horses. Overall the prior vaccination status against AHS was 97.5% (n= 237 of 243 participants with a known vaccination history).

Five horses from five different herds tested suspect or positive on RT-qPCR on the primary round of sampling. All previously sampled horses in each of the five herds were then re-sampled (n=76). After both rounds of sampling, a total of 8 horses from 5 herds had tested suspect or positive on the group-specific RT-qPCR (Table 2). All samples tested negative on VI and this precluded the sequencing of any of these samples. Positive AHSV typing results were found in 3 of the 8 horses. Horse 1883 was AHSV type 1 positive on its screening sample, and it tested negative on the group-specific RT-qPCR on follow-up sampling. Horse 479 was AHSV type 3 positive on both the screening and follow-up sampling rounds. Horse 141 was typed as AHSV type 1 on follow-up sampling after being negative on the initial screening round. None of the 8 suspect horses fulfilled the positive case definition of the surveillance protocol as a result of a combination of their prior AHS vaccination history (all were vaccinated in 2017), the quantitation cycle values of their group-specific screening RT-qPCR results, their AHSV typing and virus isolation results, as well as a lack of any clinical signs associated with AHS detected during sampling.

**Table 2**

Demographic and testing results for all screened and follow-up RT-qPCR suspect and positive horses showing their African horse sickness virus type specific and virus isolation results.

| Herd ID/Horse ID | Age† | Days between positive result and last AHS vaccination | Primary round of surveillance | | | Follow-up round of surveillance | | |
|---|---|---|---|---|---|---|---|---|
| | | | Minimum qRT-PCR Cq Value | AHS type specific qRT-PCR result | VI result | Minimum qRT-PCR Cq Value | AHS type specific qRT-PCR result | VI result |
| 14/141 | 2 | 217 | Negative | N/A | N/A | 35.1 | AHSV1 | Negative |
| 14/307 | 20 | 316 | Negative | N/A | N/A | 36.7 | Negative | Negative |
| 14/316 | 2 | 146 | 35.94 | Negative | Negative | Negative | N/A | N/A |
| 24/479 | 13 | 211 | 31.4 | AHSV 3 | Negative | 33.5 | AHSV 3 | Negative |
| 66/1396 | 9 | 238 | 34.6 | Negative | Negative | Negative | N/A | N/A |
| 149/1869 | 2 | 205 | Negative | N/A | N/A | 34.9 | Negative | Negative |
| 149/1883 | 2 | 128 | 31.42 | AHSV 1 | Negative | Negative | N/A | N/A |
| 6469/6230 | 4 | 219 | 35.44 | Negative | Negative | 37.6 | Negative | Negative |

AHS: African horse sickness

Cq: quantitation cycle

N/A: Not applicable

qRT-PCR: Real-time reverse transcription quantitative PCR

VI: Virus isolation

†Years old– rounded to the nearest year

Evaluation of the system sensitivity and probability of freedom for three different scenarios with respect to within and herd-level design prevalences are shown in Table 1. The graphical surveillance outcomes, obtained from varying combinations of within-herd and herd-level prevalences resulting in an overall effective design prevalence of 0.01, is shown in Supplementary figure 1, with the range of outcomes included in Table 1. For effective design prevalences (the product of the within-herd and herd-level prevalences) ranging between 0.8% and 6.4%, established during the Paarl 2016 outbreak and averaged from prior outbreaks in the AHS controlled area between 1999 and 2016 respectively, the sensitivity of the surveillance system, i.e. its probability of detecting a positive case given the population had been infected, ranged between 63.2% and 99.9%. The confidence of freedom differed

when using an uninformed prior compared to an informed prior, ranging between 73.1% and 99.9% in the former and between 96.6% and 100% in the latter.

# 4. Discussion

To our knowledge, this is the first published freedom from disease survey for AHS in a post-outbreak scenario. Although freedom from disease surveillance methodologies are well described we found the practical application in the AHS and South African context challenging when designing and evaluating the program. Challenges arose due to the requirement to perform this surveillance in a zone within an AHS infected country where a high proportion of horses were vaccinated against AHS, with legislation prescribing a seasonal vaccination protocol; moreover the vaccine used was live-attenuated and the routine diagnostic tests available cannot differentiate between infected and vaccinated animals. These factors dictated the time period appropriate to perform the surveillance (early autumn) and, as a result of the seasonal nature of AHS infection, this time period is likely to be the same for future post-outbreak surveillance programs of a similar nature. Challenges were compounded because of the seasonal nature of the majority of horse breeding in the area which changed the equine population between outbreak and survey, making both establishing an animal-level sampling frame and using a risk-based surveillance approach difficult.

Overall, 97.5% of all sampled horses were previously vaccinated, which is substantially higher than the overall vaccination status of all horses in the population at the time of the Paarl 2016 AHS outbreak (74.3% - Grewar et al., 2018). This difference is likely due to a change in the vaccination status of individual horses following the Paarl 2016 outbreak, and the lack of an updated sampling frame in 2017 that included new unvaccinated horses. The

well-vaccinated population, combined with the use of a vaccine that did not allow DIVA diagnostics, lowered the appropriate design prevalence of the surveillance system, hence increasing the required sample size and associated cost. It also precluded the use of serological testing as an option for screening or confirmatory testing, and increased the possibility, as experienced in this survey, of detecting false positive reactors most likely due to residual vaccine RNA. These factors resulted in a complex case definition where follow-up strategies required re-sampling, virus isolation, typing assays and/or genome sequencing to confirm the diagnosis for screened suspect cases.

Depending on the chosen underlying design prevalence and the use of informed or uninformed priors, the confidence of freedom from this once off surveillance event ranged between 73.1% and 100%. The evaluation of the surveillance program in both a single-stage (animal level only) and two-stage fashion (both animal and herd level) provides estimates of the probability of freedom based on the implemented sampling strategy and also accounts for any clustering of infection in herds. This illustrates the importance of reporting disease outbreak prevalences, both at animal and herd level, where freedom from disease surveillance may be contemplated during the post-outbreak period. A two-stage sampling strategy will generally provide less information (lower sensitivity estimates) than a one-stage strategy for equivalent sample sizes. This is evident from the results where the evaluation of the survey in a two-stage manner gave lower sensitivity and probability of freedom estimates for equivalent effective population prevalences. The exception is as shown in Supplementary figure 1, with very high underlying herd-level prevalence in conjunction with low within-herd prevalence (effectively no clustering of infection in herds). The main reason for this effect is that when sampling from a population, each additional

animal sampled from a herd that has already been sampled provides progressively less information about population status than an additional animal sampled from a previously unsampled herd. While the OIE is prescriptive in the required design prevalence to establish freedom for certain diseases like brucellosis and bovine spongiform encephalopathy (OIE, 2018a; OIE, 2018b) it is not explicit when describing the required design prevalence for AHS freedom (OIE, 2016). The clearest indication of trade acceptable design prevalence for AHS freedom comes from the surveillance requirements of the EU for AHS in the South African sentinel surveillance program, where the required sample size corresponds to an animal level design prevalence of 5% (EU, 2008). Animal level design prevalences selected for freedom from disease surveys for other arboviral diseases range between 1% and 5% (Camphor, 2014; Diarmita, 2018; Grigore, 2018; Tratalos et al., 2018) but can be as low as an effective animal level prevalence of 0.5% (Stokes, Baylis, & Duncan, 2016). In our case the design prevalence used to determine the overall sample size assumed that the AHSV associated with the Paarl 2016 outbreak was circulating in 1% of the population; decreasing this below 1% would have been cost-prohibitive. Our choice of a simple random survey treating the entire population as a single homogenous population stemmed from the fact that we had an individual animal sampling frame, the target population was contained within a relatively small geographic area and the hazard surveyed for was midge-borne, making infection clustering less likely compared to a contact transmitted agent. Since the area surveyed was relatively small, the added cost of sampling additional herds was not considered prohibitive and the advantage of making use of a two-stage survey design, where herd and animal sample sizes can be manipulated to reduce costs while still maintaining appropriate outcomes (Cameron & Baldock, 1998b), was not considered.

The total cost of the survey amounted to R210, 000 with the majority of cost associated with laboratory testing (51%) and personnel time (35%). As a component of total costs associated with disease control and return to freedom this is relatively minor. Not only do outbreaks in the AHS controlled area incur substantial direct costs (Grewar et. al., 2013) but the annual industry-wide revenue loss of a direct export market outside of Africa and ongoing AHS control and surveillance in the controlled area is estimated at R500 million and R6 million per year respectively (A. Todd – South African Equine Health and Protocols NPC, personal communication). Using a lower design prevalence would therefore not substantially inflate the overall cost of control; however, because the impact of AHS outbreaks in the controlled area in South Africa is long lasting (at least two years loss of direct trade opportunity to major trade partners) and due to the high level of compliance required for direct trade, it is difficult to estimate the actual benefit that a single survey, such as the one described here, would have on re-opening trade. Thus the choice of design prevalence was based on likely disease parameters.

It should be considered that the horse population changes over a one year period, and generating a sampling frame in 2017, from 2016 outbreak census data, results in sample selection bias towards horses associated with the outbreak. While we do not expect this made a practical difference to the outcome of the survey, this bias would be mitigated through either maintaining a thorough census after outbreaks or generating an up to date census prior to selecting horses to sample. During the Paarl 2016 outbreak, the risk of unvaccinated horses being diagnosed with AHS was 2.3 times higher than in previously vaccinated horses (WCDOA, unpublished data). A component of opportunistic risk-based surveillance (separate sampling of unvaccinated horses) was incorporated into the

surveillance plan in addition to the single strata design, but the results thereof could not be statistically evaluated along with the non-risk based data due to a lack of representativeness of the sampling. Demographic data showed a clear bias towards young horses (and hence breeding establishments on a herd level) being more likely to be unvaccinated. The foaling season for Thoroughbred horses, the breed most represented in the sampling frame for this surveillance (59%), runs from August through early December each year (Schulman, Marlow, & Nurton, 2012), so there is a large influx of unvaccinated foals into any potential population at risk in the AHS surveillance zone during this period. This fluctuation in the individual horse level demographic and vaccination status made using risk-based surveillance a logistical challenge, particularly in this high-density population. Risk-based surveillance strategies can improve both the effectiveness and cost implications for freedom from disease surveillance (Stärk et al., 2006); however, for it to be feasible in future surveys of this nature ongoing census, demographic and risk factor data collection would need to take place in the population at risk between the cessation of the outbreak and the freedom from disease survey.

For the described surveillance event the result was not only specific for the virus associated with the prior outbreak, but the sampling frame was based on the prior outbreak controlled area, which was focused primarily on herds surrounding infected herds (Grewar et al., 2018). This limited the geographic extent of the surveillance outcome. A point-in-time freedom from disease survey forms just part of an overall surveillance strategy for a scenario where establishment of zonal freedom is attempted in a country with endemic disease.

# 5. Conclusion

This study showed that it was unlikely that the AHSV responsible for the Paarl 2016 outbreak in the Western Cape was still circulating the following autumn in the area defined by the outbreak containment zone. Post-outbreak capture of census, demographic and risk factor data in populations at risk that will be targeted for freedom from disease surveys is critical to inform future survey design. This is especially true where factors such as disease seasonality, use of a live attenuated virus vaccine, a seasonal vaccination policy and a lack of available tests with DIVA capabilities preclude the possibility of performing this surveillance immediately after the cessation of an outbreak.

# 6. Acknowledgements

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# 7. Conflict of Interest

The Equine Health Fund, a division of Wits Health Consortium (Pty) Ltd, is funded by private donors (http://www.equinehealthfund.co.za/Home.aspx). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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