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Seroprevalence of segmented flavi-like Alongshan virus in horses

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ABSTRACT

The emergence of new pathogens poses a significant threat to global health, exacerbated by climate change, biodiversity loss, and increased globalization. Ticks, as vectors for various pathogens, contribute to the rising incidence of diseases. Surveillance programs are crucial for identifying and controlling emerging pathogens. This study focuses on the Alongshan virus (ALSV), a segmented flavi-like virus first identified in humans in China in 2017. Despite its ability to infect a wide range of mammals, the natural hosts and transmission pathways of ALSV remain poorly understood. Therefore, we investigated the prevalence of ALSV in equine cohorts from different geographical regions to assess its spread and potential risk.

Using commercial serum pools from the Americas, Europe, and Oceania, we detected anti-ALSV antibodies in 12 out of 13 pools, indicating a broader geographical distribution of ALSV than previously known. In a cohort of 473 Thoroughbred horses from Germany, 87.1 % tested positive for ALSV antibodies, with a significant correlation between antibody positivity and age. Longitudinal tracking of 124 horses over five years revealed dynamic changes in antibody levels, with 71.77 % testing positive at some point during the study.

Our findings demonstrate a high prevalence of ALSV antibodies in equine populations across diverse regions, suggesting frequent exposure to the virus. The study highlights the importance of horses as sentinel models for monitoring tick-borne pathogens and underscores the need for further research to understand the potential risk of ALSV exposure to human and animal health.

1. Introduction

The emergence of new diseases poses an increasing threat to human, animal and plant health worldwide. In particular, climate change and biodiversity loss, can facilitate the spread of infectious diseases by creating favorable conditions for host species that carry pathogens (Baker et al., 2022; Keesing et al., 2010). In addition, globalization and expanding trade and travel increase the risk of pathogens being introduced into new areas. The recent decades have shown that many known human pathogens originate from wild or farmed animals, with zoonotic transmission to humans mediated by arthropod vectors. These include mosquitoes or ticks, which are blood-feeding ectoparasites, serve as vectors for various pathogens, including protozoa, bacteria and viruses that infect humans, domestic animals and wildlife. The incidence of tick-borne diseases is steadily increasing and represents a growing global health risk (Kilpatrick and Randolph, 2012; Madison-Antenucci et al., 2020). Surveillance programs are therefore essential for the implementation of effective control measures against emerging pathogens.

In recent years, such surveillance programs have identified and characterized many emerging pathogens. These include the viruses of the Jingmenvirus group, which were first discovered in ticks as part of a metagenomic study (Qin et al., 2014). Although Jingmenviruses, which includes the Alongshan virus (ALSV), share homology to the *Flaviviridae* family their genome architecture is considerably different. Instead of having a single non-polyadenylated RNA molecule encoding a single

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open reading frame that is post-translationally cleaved into mature proteins, ALSV has its genome separated on four segments. Segment 1 and segment 3 encode non-structural proteins (NSP1 and NSP2) with homology to NS3 and NS5 of traditional flaviviruses, respectively. In contrast, segments 2 and 4 encode structural proteins (VP1, VP2 and VP3) with no sequence homology to other flaviviruses. The majority of segmented Jingmenviruses have been identified in a range of arthropods and mammals, but not in humans (Colmant et al., 2022). One exception is the ALSV, which was first identified in northeastern China in 2017 in individuals presenting with a febrile illness and non-specific symptoms following a tick bite (Wang et al., 2019a).

Although ALSV can infect humans and a wide range of mammals, our understanding of its natural hosts and transmissions pathways remains limited. To date, ALSV has been detected in arthropods and various mammals including horses in East Asia, Russia and Europe (Gömer et al., 2024). It is likely that the geographic range of ALSV and other segmented flavi-like viruses extends beyond these regions, but this has not yet been reported. Furthermore, the frequency with which ALSV circulates at the human-animal interface remains under-investigated. The primary aim of this study was to examine the seroprevalence and geographical distribution of ALSV in equine populations across different continents, including the Americas, Europe, and Oceania. To this end, a cohort of commercial equine serum pools from the Americas, Europe and New Zealand was investigated to assess ALSV sero-reactivity on different continents. Additionally, a well-characterized cohort of Thoroughbred racehorses, and a longitudinally-sampled equine cohort was followed for five years, allowing the study of antibody positivity over time. Together, these data layer spatial and temporal resolution onto ALSV circulation patterns to inform risk-assessment for zoonotic transmission to humans. They also expand the viruses reported geographical range.

2. Results

2.1. Anti-ALSV antibodies are frequently present in commercial equine serum pools from the Americas, Europe and Oceania

The current reported geographical range of ALSV encompasses regions from East Asia to Central Europe, but the virus has not been detected on other continents. To this end, we used equine serum pools that have previously been used to determine the prevalence of several other diseases, including equine parvovirus (Meister et al., 2019a). These serum pools were collected for commercial purposes from equidae from different geographical locations in the Americas, Europe and Oceania (Fig. 1A). We performed the luciferase immune precipitation assay (LIPS) to quantify the seroprevalence of anti-ALSV core antibodies as previously described (Ebert et al., 2023). Of the 13 commercial serum pools tested, twelve were found to contain antibodies indicative of previous ALSV infection (Fig. 1B, Table S1). This extended the known geographical range in which ALSV has been detected by the Americas and Oceania. Only one of the serum pools tested was negative. This sample came from Brazil and was collected from fetal equids. We then performed a PCR specific for segment 3 of ALSV, encoding the nonstructural protein NS5, to detect viral RNA (Ebert et al., 2023). Using this PCR, all 13 commercial serum pools tested negative. In summary, anti-ALSV antibodies in commercial equine serum pools could be detected for the first time in North America and New Zealand.

2.2. High anti-ALSV positivity in German Thoroughbred breeding horses

We then quantified ALSV antibodies using LIPS in a cohort of thoroughbred horses from northern and western Germany, including the states of Lower Saxony and North Rhine-Westphalia. In total, the cohort consisted of 473 animals, of which 87.1 percent tested positive for anti-ALSV antibodies (Fig. 2A, B, Table S2). Furthermore, a unique and valuable feature of this cohort is that it has already been closely monitored and information on age, gender transport, vaccination as well as the location are available. These data were used to assess whether the number of antibody-positive animals was evenly distributed across the sampling region or whether there was evidence of infection hotspots (Fig. 2C). Overall, the number and percentage of positive animals were found to be evenly distributed, as no clusters appeared on the map shown in Fig. 2C. We then correlated various parameters including age, sex, place of birth, vaccination status, number of animals on a farm and travel history (defined as horses crossing country borders) with antibody positivity to gain a deeper insight into the factors involved in the transmission of ALSV (Fig. 3, Table S3-S7). We first analyzed whether age was an important factor in the presence of anti-ALSV antibodies by plotting the age of the animals against their serostatus. We also used a density function to calculate the proportion of animals at each age with antibodies against ALSV. This analysis showed that the number of anti-ALSV antibody positive animals was significantly higher in older compared to younger animals using a logistic regression model (p-value 0.0315, odds ratio 3.93, mean age negative = 9.75 y, positive = 11.1 y, Fig. 3A, B). Correlation analysis was also performed between anti-core positivity and sex, place of birth, vaccination, farm size and travel history of the animals (Table S3-S7). However, no significant correlation was observed between these factors and anti-ALSV positivity. In addition to the antibody prevalence analysis, we performed an RT-qPCR screen on these serum samples to determine the number of RNA positive animals. All serum samples tested negative for ALSV RNA. In conclusion, antibodies to ALSV were found to be highly prevalent in this cohort, while ALSV RNA was not detected. In addition, antibody positivity showed a positive correlation with age.

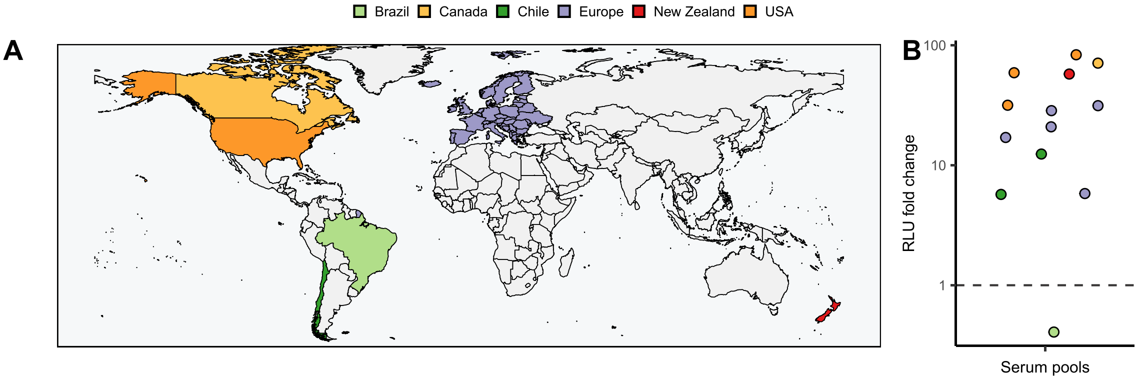

Fig. 1. Prevalence of anti-ALSV core antibodies in commercial serum pools. Thirteen commercial serum pools were tested for anti-ALSV core antibodies. (A) Geographical origin of the serum pools. (B) LIPS assay of serum pools. RLU normalized as fold change over background (set to 1). European samples originate from different countries including Germany, Italy and France.

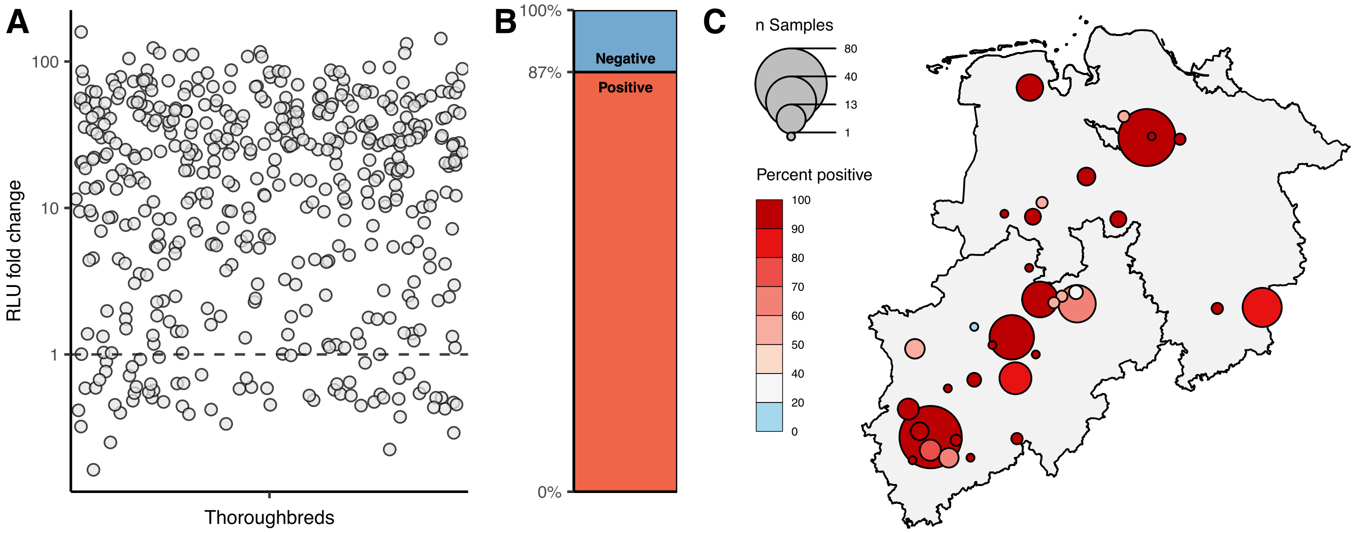

Fig. 2. Seroprevalence of anti-ALSV core antibodies in a well-characterized Thoroughbred cohort. (A) Fold change in anti-ALSV core antibody levels compared to the background control (set to 1) for individual horses as measured by LIPS. (B) Animals with an RLU fold change greater than 1 were considered antibody positive. (C) Geographical origin of animals in the Thoroughbred cohort. Dot size represents the number of samples from each location. Color corresponds to percentage of positive animals.

2.3. *Longitudinal tracking of anti-ALSV core antibodies in horses*

The third cohort consisted of horses monitored longitudinally from October 2013–2018. During this period, we assessed the prevalence of ALSV antibodies at five different time points in 124 horses, resulting in 419 measurements to understand the fluctuations in anti-core antibody levels over time (Fig. 4A, Table S8). A total of 89 individuals tested positive for ALSV antibodies during this period (71.77 %), which was comparable to the percentage of positive animals in the Thoroughbred cohort. From these 89 individuals we had 195 positive measurements (46.53 %) over the monitoring timeframe. In October and December 2013, the percentage of positive animals remained relatively stable at 52 % and 38 %, respectively. Similarly, in 2015, 2016, and 2018, the percentages of ALSV antibody-positive animals were 43 %, 58 %, and 41 %, respectively (Table S8). During the monitoring period, we identified 39 animals that were negative for ALSV antibodies at baseline (October 2013). Of these, 23 horses (59 %) acquired anti-ALSV antibodies during the study, while only three horses remained antibody-negative at all five time points. The remaining 13 horses tested also negative, but not all time points were available for serology. In addition, 28 animals lost their antibody status, being positive at one time point and negative at a subsequent time point. We also performed qPCR to detect RNA-positive animals, but similar to the previous cohorts, all serum samples tested RNA-negative. Despite the absence of active infection signs, we investigated potential correlations between liver enzyme levels (AST, GGT, GLDH), albumin, bilirubin, and bile acid levels with antibody status (Figure S1). None of these parameters were significantly different between anti-ALSV antibody-positive and -negative animals.

In conclusion, over the five-year period, the number of positive animals was comparable to the other cohorts investigated. Additionally, we observed dynamic changes in antibody positivity within the cohort, including fluctuations in anti-ALSV antibody positivity over time.

3. Discussion

In this study, a sero-survey in horses was conducted to investigate the prevalence of anti-ALSV antibodies, an emerging segmented flavi-like virus with the capacity to infect a wide range of mammals and arthropods. The survey was carried out on three different equine cohorts: a commercial serum pool cohort, a thoroughbred cohort and a cohort of animals followed longitudinally. Equine cohorts were used for this analysis because these animals are exposed to tick bites and therefore provide valuable insight into the prevalence of tick-borne viruses such as ALSV.

It could be demonstrated that anti-core ALSV IgG antibodies are commonly detectable in commercially available horse sera from a diverse range of geographical origins, which suggests a global dissemination of ALSV. Only the pooled fetal serum sample tested negative for anti-ALSV antibodies. There are several factors that could account for the absence of detectable antibodies. Foals are born with negligible serum immunoglobulin concentrations because IgG does not cross the equine placenta and only after ingestion of colostrum, foal IgG levels increase rapidly. Consequently, the detection of antibodies in fetal serum is not feasible, and serological detection is only possible after colostral intake (Erhard et al., 2001). Secondly, the sampled population may have experienced little to no exposure to infected ticks, particularly if the animals were kept in controlled environments or during periods of low vector activity. In this study, we report for the first time the detection of ALSV antibodies in specimen from North and South America, as well as from Oceania. The identification of ALSV in commercial serum samples is of significant importance for two reasons. Firstly, it provides an insight into the circulation of these viruses. Secondly, horse sera are commonly used for the production of antisera that are licensed for use in a variety of animal species and humans, including snake venom immunoglobulin and botulism antitoxin (Chandriani et al., 2013; Divers et al. n.d.; Aleman et al., 2005). Moreover, equine serum can be used as a supplement to cell cultures, which could interfere with experimental results or contaminate the production of live vaccines (Franke et al., 2014).

In addition to the economic role of equine sera, the role of horses as companion animals for humans is very important. Horses are widely distributed throughout the world for domestic use or their use as sport horses. As such their health is closely monitored. The Thoroughbred cohort from northern and western Germany that we analyzed here is a good example of this: According to the registry of the German Association for Breeding and Racing of Thoroughbreds (Cologne), this cohort represents approximately one quarter of the actively bred Thoroughbreds in Germany. Therefore, the results obtained from this cohort can be considered representative for the region. In addition, further research in other countries, both within and outside Europe, would be beneficial to provide a more detailed insight into the prevalence of ALSV. The

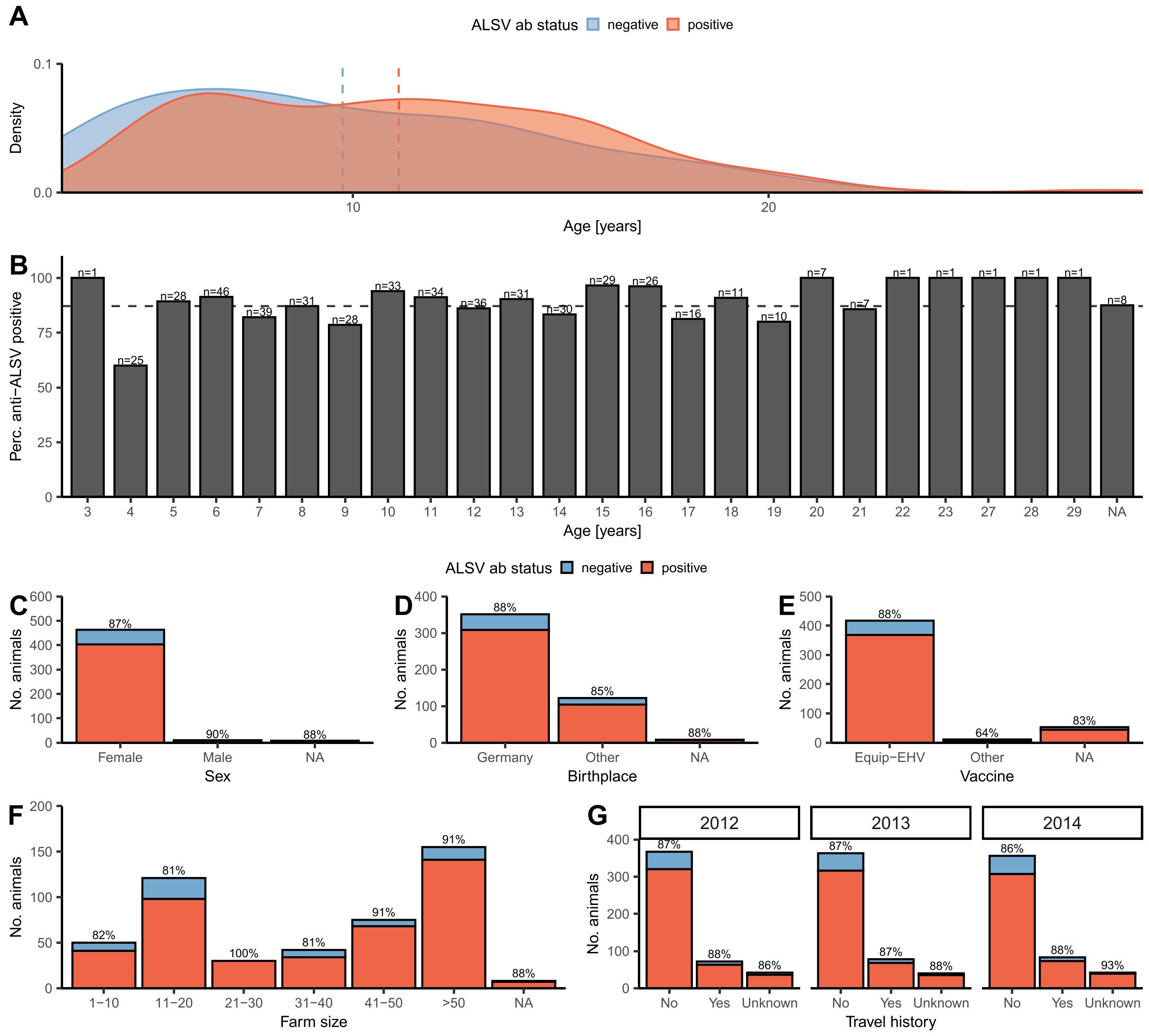

Fig. 3. Factor analysis influencing seropositivity in Thoroughbreds. (A) Age-dependent ALSV antibody positivity as a density function and as a binned bar chart for each age category. Dotted line represents mean age for antibody negative (9.75 y) and positive (11.1 y) animals. Age differences between the ALSV antibody-positive and -negative groups were assessed using a generalised linear model (GLM). The GLM output was used to determine statistical significance, with age as the dependent variable and ALSV antibody status as the predictor variable. (B) The numbers above the bars indicate the number of samples per age category. (C-G) Number and percentage of positive animals (number above bar) for animal sex, birthplace, vaccination status, farm size and travel history. Farm size is defined as the total number of animals kept on the farm and is categorised into bins. Travel history indicates whether a horse has been transported across national borders and subsequently returned to Germany ('Yes') or has remained within the Germany ('No').

detailed monitoring of this cohort allows an in-depth analysis of multiple risk factors for acquiring ALSV. Indeed, a significant correlation of age and anti-ALSV antibodies was detected, while other factors were not significantly enriched. Similarly, the antibody prevalence of the equine Hepacivirus (EqHV) and equine Parvovirus has been shown to increase with age, but not with farm size, vaccination status or sex as has been shown for this cohort previously (Reichert et al., 2017; Meister et al., 2019b). It is worth noting that the route of transmission which may be different for equine Parvovirus or EqHV compared to ALSV, plays an important role in the correlation with age.

To better understand the importance of age as a risk factor for acquiring ALSV, a more detailed analysis including younger animals will be important. In addition, follow-up studies could investigate whether the length of time foals or young horses spend in paddocks correlates with an increased risk of infection. In addition to age, we analyzed whether seropositive animals showed signs of liver inflammation by measuring liver enzymes. Although without significant differences, it will be important for future studies to analyse clinical signs in the presence of acute infection, which will be particularly important for horses participating in sport competitions.

The overall prevalence of anti-core ALSV antibodies in our cohorts, particularly among Thoroughbreds, appears to be higher than that reported in a comparable equine study, in which only around 15 % of animals tested positive for antibodies (Ebert et al., 2023). The same study also showed that in Lower Saxony a high number of ticks collected from animals were found to be ALSV RNA positive (46.43 %) (Ebert et al., 2023). Similarly, the high prevalence of 61.8 % of EqHV in this Thoroughbred cohort could suggest that there are overlapping risk factors with ALSV, which may be linked to management practices or tick exposure (Pfaender et al., 2015; Reichert et al., 2017; Meister et al., 2019b; Reinecke et al., 2021). Due to the high seroprevalence in this cohort, one could argue either high exposure to tick-borne viruses such as ALSV or antibody cross-reactivity with members of the *Flaviviridae* family or other closely related viruses - a possibility that cannot be ruled

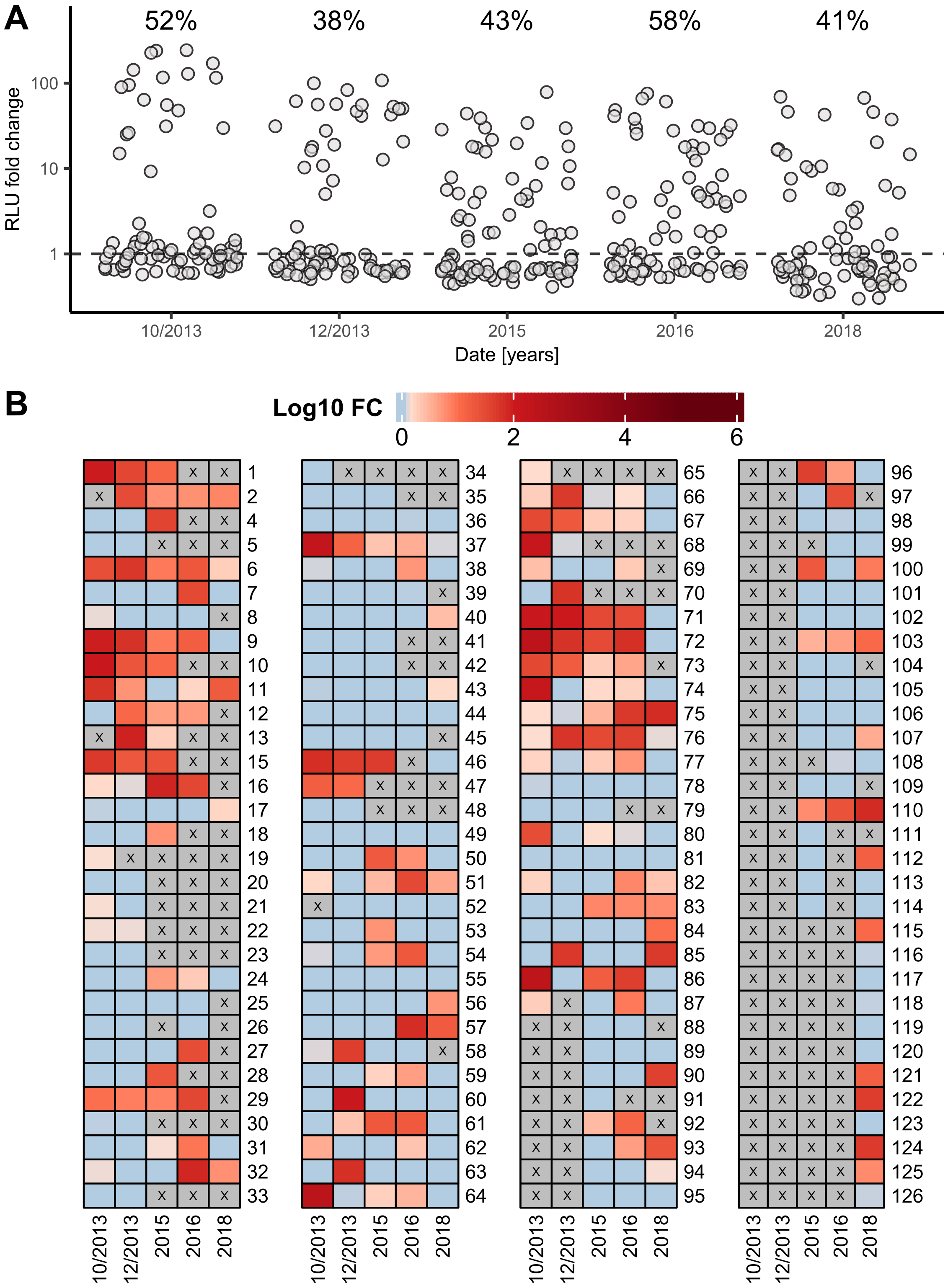

Fig. 4. Anti-ALSV core antibody prevalence in a longitudinal cohort. (A) Fold change in anti-ALSV core antibody levels for consecutive years: October 2013, December 2013, 2016, and 2018. Fold changes were normalized to the background control (set to 1). Text shows the percentage of horses considered ALSV antibody positive, as determined by a fold change greater than 1. (B) Time-resolved antibody levels for all individual horses. Color gradient displays log 10 RLU fold change. Boxes with an X represent samples that could not be measured as they have been used up in prior studies.

out at this time. Of note, to our knowledge, these cohorts have not been vaccinated against West Nile virus. Also arguing against the possibility of cross-reactivity, the core protein used as the serological target in the LIPS assay does not show high homology between segmented flaviviruses and traditional flaviviruses (Garry and Garry, 2020). To further demonstrate this, a phylogenetic analysis of amino acid sequences from different orthoflaviviruses and members of the Jingmenvirus group was performed (Fig. S2). This analysis indicates that the genetic relatedness between the core proteins of segmented flavi-like viruses and canonical flaviviruses is relatively low, a finding consistent with previous reports (Garry and Garry, 2020). As an additional evaluation for the reactivity of the anti ALSV core LIPS, we performed serology testing on human serum pools from blood donors from northern and western Germany, collected between February and March 2024 (Fig. S3). These serum pools consisted of 96 serum samples each. Using LIPS, we did not detect a positive signal for ALSV antibodies in human blood donors and thus no evidence of past infection with ALSV. Additionally, we evaluated whether patients that were positive for other arboviruses including Dengue, West Nile, Chikungunya or Tick-borne encephalitis virus. Similarly, we were not able to detect ALSV in this cohort either (Fig. S3). Given the limited genetic relatedness to canonical members of the flavivirus family and other members of the Jingmenvirus group as well as the reactivity profile in human serum, a cross-reaction with other members of the same family is not very likely, but cannot be ruled out completely.

There are several possible explanations for the absence of ALSV RNA detection in this study. For instance, Ebert et al. screened 536 serum samples from game animals and identified just one RNA-positive case in a roe deer (Ebert et al., 2023). As with other tick-borne flaviviruses, such as tick-borne encephalitis virus (TBEV), ALSV probably causes brief, low-level viremia that can easily be overlooked unless sampling occurs during the short initial phase of infection, which often precedes the appearance of clinical symptoms (Holzmann, 2003; Ruzek et al., 2019). Furthermore, as described for Powassan virus and TBEV, ALSV may replicate preferentially in sites other than peripheral blood, such as the central nervous system or the skin at the tick attachment site, which would further reduce RNA detectability in serum. Therefore, the use of serum as diagnostic material for tick-borne viruses with neurological manifestations is inherently limited by low and transient viremia. Cerebrospinal fluid (CSF) often yields higher viral loads in neurotropic flavivirus infections and may therefore provide greater diagnostic sensitivity. Similarly, testing ticks collected from seropositive animals can enhance detection, as viral replication in the vector can result in higher RNA concentrations. Although ALSV RNA has been detected in serum from infected patients and animals (Wang et al., 2019a, 2019b), the duration and consistency of viremia in natural hosts remains poorly characterized. Notably, Ebert et al. reported that 46.43 % of ticks collected from animals tested positive for ALSV RNA, highlighting their potential as a valuable diagnostic source. Future studies should therefore examine a broader range of sample types, including CSF, tissue biopsies and co-fed ticks, to better define the temporal dynamics and tissue distribution of ALSV infection.

In conclusion, this study offers valuable insights into the prevalence and distribution of ALSV, an emerging segmented flavi-like virus, across various equine cohorts. The findings reveal a high seroprevalence of ALSV antibodies in equidae from diverse geographical regions and local breeding cohorts. Despite this widespread antibody presence, no evidence of acute ALSV infection was detected. Additionally, a correlation between antibody positivity and age suggests that prolonged tick exposure may influence seroprevalence. These results underscore the importance of equids as a valuable sentinel model for monitoring tick-borne viral pathogens.

4. Material and methods

4.1. Cohorts

4.1.1. Commercial serum cohort

A total of 13 different horse serum pools were collected from a variety of providers (Table S1). The samples were shipped and stored at −20 °C until further analysis. Thaw and freeze cycles were kept at a minimum.

4.1.2. Thoroughbred cohort

Breeding horses from north-western Germany that were registered at the German Thoroughbred Studbook Authority (Cologne) were examined in autumn 2014 (Pfaender et al., 2015). In this region, all horses presented for the annual fertility monitoring were investigated, if the owners confirmed participation in the study and the horses were obedient. Although only horses from northern and western Germany were assessed, the study population represented 49 % of all registered brood mares and 29 % of all registered stallions. The samples were transferred to the laboratory within 12 h after collection. Serum was prepared by centrifugation (Universal 320®, Hettich, Tuttlingen, Germany) for 6 min at 3000 rounds per minute. All samples were stored at −80 °C until further analysis.

4.1.3. Longitudinal cohort

Serum samples of 124 horses were collected for diagnostic purposes from an isolated cohort in Germany between 2013 and 2018 (Reinecke et al., 2021). Serum samples were stored at −80°C.

4.2. LIPS assay

The serostatus of equine samples was analyzed using an anti-core ALSV luciferase immunoprecipitation system (LIPS), initially described by Burbelo et al. and later adapted by Ebert et al. to measure ALSV antibodies (Ebert et al., 2023; Burbelo et al., 2012). Relative light units (RLU) were measured using a plate luminometer (LB 960 XS3; Berthold, Bad Wildbad, Germany). All measurements were conducted in duplicates. The background cut-off limit was defined as the mean RLU value plus three standard deviations of ALSV core antibody-negative horse serum, which was included on each plate to account for inter-batch variation. RLU values were expressed as fold changes (FC) relative to this threshold. Samples were classified as ALSV antibody-positive if FC > 1.0, and as negative if FC < 1.0."The specificity and dynamic range of the assay were evaluated using an anti-FLAG tag antibody, which binds to the FLAG tag within the fusion antigen. IgG-depleted human serum and mock-transfected cells were also used to assess background signal (Figure S4). Intra- and inter-assay variability were also determined, with variations remaining below 10 %.

4.3. qPCR

RNA from horse sera was isolated using QIAamp DNA Blood Mini Kit as recommended by the manufacturer. Subsequently, qPCR was performed with primers published by Ebert et al. (5'-ATAATCCAGTACCTCCCAGCCG-3', 5'-CCCCGATGAAACCTGTCCTCTG-3'), which target the NS5-like gene of ASLV using the Promega One-Step RT-PCR kit according to the manufacturer's instructions. qPCR was performed using a Rotor-Gene 3000 (Qiagen) instrument. Reverse transcription was carried out at 45 °C for two minutes, followed by a 45-cycle amplification step consisting of a combined elongation and extension step at 60 °C for one minute. The detection limit was determined to be 100 RNA copies based on an RNA standard curve included in each run.

4.4. Statistics and data visualization

All statistical analyses were performed in R (version 4.3.1; R Core

Team, 2023), with data manipulation and visualisation conducted using the tidyverse package collection (Wickham et al., 2019). Differences in age between the positive and negative groups were assessed using a generalised linear model (GLM) fitted using the glm function from base R, examining the relationship between age and ALSV antibody status.

CRediT authorship contribution statement

Eike Steinmann: Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Gömer Andre:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Daniel Todt:** Writing – review & editing, Validation, Investigation. **Richard J.P. Brown:** Writing – review & editing, Validation, Investigation. **Jessika Cavalleri:** Writing – review & editing, Investigation, Funding acquisition. **Tanja Vollmer:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Ricarda Plümers:** Writing – review & editing, Investigation, Data curation. **Anna Sophie Ramsauer:** Writing – review & editing, Formal analysis. **Saskia Janshoff:** Writing – review & editing, Visualization, Investigation, Formal analysis.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used DeepL in order to improve grammar. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Eike Steinmann reports financial support was provided by German Centre for Infection Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2025.110686.

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