Systematic review and meta-analysis of seroprevalence studies of West Nile virus in equids in Europe between 2001 and 2018

Running title: WNV seroprevalence in equids in Europe

Authors: Marine B. C. Metz[1], Olaolu T. Olufemi[2], Janet M. Daly[2], Marta Barba[1*]

[1]Agentes Microbiológicos asociados a la Reproducción Animal (ProVaginBio), Veterinary Faculty, Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain.

[2]School of Veterinary Medicine and Science, University of Nottingham, United Kingdom.

*Corresponding author email address: martabrvet@gmail.com

Summary

There is some evidence that West Nile virus (WNV), which causes encephalomyelitis in equids, is an emerging disease in Europe. The aim of this study was to perform a systematic review and meta-analysis to analyse seroprevalence studies of West Nile virus in equids in European countries between 2001 and 2018. Two electronic databases, PubMed and Scopus, were searched for relevant publications published from 2001 to 2018 using predetermined keywords. A total of 1484 papers was initially found. After applying the eligibility criteria, 39 papers were finally included in the systematic review. Analysis of 28,089 equids from 16 European countries revealed a pooled seroprevalence of 8% (95% CI 5–12%, P<0.001, I^2 = 99.3%) in Europe. The pooled seroprevalence was slightly higher in Mediterranean basin countries than other countries and when calculated for samples collected between 2001 and 2009 compared to 2010 to 2018. Differences in study design (e.g. sampling associated with recent outbreaks of WNV) contributed to a high degree of variability among studies. Further studies with harmonized study design and reporting of the results are recommended to better estimate and monitor European seroprevalence of West Nile virus in equids.

Keywords: horses, equids, Europe, Mediterranean basin, seroprevalence, WNV.

Introduction

West Nile virus (WNV) is a mosquito-borne zoonotic virus from the genus *Flavivirus* and family *Flaviviridae* (Anon, 2017). Its transmission cycle involves birds and mosquitoes, especially from the *Culex* species, which act as vectors of the virus. Several vertebrate species can be infected by the virus, but mammals, particularly humans and equids, are considered "dead-end" hosts as they do not usually develop sufficiently high levels of viraemia for transmission to blood-feeding mosquitoes (Komar, 2000). The virus was first isolated from a human being in 1937 in the West Nile region of Uganda (Smithburn et al., 1940). Since the first reported case of West Nile virus in horses in Egypt in 1963 (Schmidt & Mansoury, 1963), the disease has expanded in range causing significant human and animal health issues. An example of these are the outbreaks reported in EU member countries since the start of the 2020 transmission season and as of 10 September 2020, 173 human cases of WNV infection and 15 deaths (Greece, Spain, Italy and Romania) and 60 outbreaks among equids (Spain, Italy, France, Portugal and Germany) have been reported through the European Animal Disease Notification Systemervice (ADNS).

Several genetic lineages of the virus have been found, but isolates from lineages 1 and 2 have mainly been responsible for the disease in humans and equids in European countries, with lineage 1 predominant until the mid-2000s (Ciccozzi et al., 2013; Long, 2014). As a neurotropic virus causing encephalomyelitis, clinical signs in horses include ataxia, paralysis of the limbs, prolonged recumbency, muscle fasciculations, and abnormal mentation (Long, 2014). The mortality rate in horses has been estimated at 35–45% (Long, 2014). However, studies suggest that only around 10% of infected horses present neurological signs (Gardner et al., 2007). For diagnosis, laboratory testing is necessary to confirm the infection as the neurological signs are not pathognomonic for the disease. Treatment is mostly supportive as there are no known effective antiviral medications (Long, 2014). An equine WNV vaccine was first licensed in the USA in 20035, and further types of WNV vaccines have since been approved for use in horses, but an equine WNV vaccine was not licensed for use in Europe until 2009. Due to the low viral titres in horses, *ante mortem* PCR-based detection of viral RNA is unreliable (Kleiboeker et al., 2004). Therefore, suspected cases of WNV infection are usually confirmed by IgM capture ELISA and/or measuring seroconversion using a plaque-reduction neutralization test (PRNT). Equine WNV-specific IgM antibodies are usually detectable from

around 8 days post-infection (so most horses with encephalitis test positive at the time that clinical signs are first observed) and remain detectable for up to 3 months (Beck et al., 2017). Neutralising (IgG) antibodies are detectable in equine serum by 2 weeks post-infection and can persist for more than 1 year. The OIE Terrestrial Manual (OIE, 2018) suggests that IgG indirect and competitive ELISAs, virus neutralisation test (VNT) or PRNT are suitable methods for determining prevalence of infection. However, as ELISA methods are less specific, where related flaviviruses co-circulate with WNV, positive results obtained should be confirmed by PRNT or VNT and testing against other flaviviruses in parallel. Other flaviviruses detected in Europe include Bagaza virus (BAGV), louping ill virus (LIV), tick-borne encephalitis virus (TBEV) and Usutu virus (USUV) (Llorente et al., 2015; Long, 2014).

The aim of this study was to conduct a systematic review and meta-analysis to analyse the prevalence reported on –WNV studies in equids in Europe from the year 2001 to 2018 inclusive, to compare the prevalence in countries of the Mediterranean basin with other European countries and to evaluate the prevalence of two periods: from 2001 to 2009 and from 2010 to 2018.

Materials and methods

Search strategy

A systematic search strategy was performed in the databases PubMed and Scopus to identify all published studies reporting the prevalence of WNV in equids in Europe from 1 January 2001 to 20 March 2019 (the date the search was performed). The following key words and Boolean operators ("AND" and "OR") were used: (prevalence OR incidence OR frequency OR occurrence OR detection OR identification OR isolation OR characterization OR investigation) AND (WNV OR West Nile virus OR Flavivirus) AND (horse OR equine OR equid OR donkey OR mule OR foal). In Scopus, the search terms were applied to the title, abstract and the keywords. In PubMed, the search terms were applied in all fields. No language restrictions were applied. Retrieved searches were entered into a Microsoft Excel (2018) file.

The reference lists of the selected publications were reviewed manually to identify all potential studies that could have been missed in the two databases.

Eligibility criteria

Inclusion criteria were divided into two categories: inclusion criteria related to the literature search and inclusion criteria inherent to the studies. First, the studies had to be published

between 1 January 2001 and 20 March 2019 and the full text had to be available in English, Spanish or French. In addition, studies had to be prospective or retrospective serosurveys with animal level prevalence and animals of the genus *Equus* (excluding zebra) reported, carried out in a European country and have performed a VNT and/or PRNT to confirm the specificity of antibodies detected by ELISA.

Studies were excluded if the titles and abstracts were not relevant to the subject of interest, did not fulfil the above eligibility criteria, had data missing or duplicated data published in another included study.

Study selection and data extraction

In the first screening of all searched studies, duplicates were eliminated. The titles and abstracts of all retrieved studies were then independently screened by two authors (MBCM and MB) to identify potentially relevant studies. When the study could not be assessed from the title and abstract, the full text was screened. The full text of the studies retained after the first screening were further scanned independently and in a standardized manner by two authors (MBCM and MB) applying the eligibility criteria.

After the eligibility assessment process, data were extracted independently by two authors (MBCM and MB) and classified in three categories: general data related to the study, data related to the diagnostic techniques and data related to the animals. Any disagreements that arose between the authors was resolved through discussion with a third author (JMD). All the extracted data were summarized in a Microsoft Excel (2018) file.

The general data related to the study were: title, first author's name, name of the journal, year of publication, database where the study was identified (PubMed or Scopus), type of study (i.e. prospective or retrospective serosurvey), language, country and region, sampling protocol (e.g. convenience or random sample), year and season of testing. The data related to the diagnostic techniques were: initial serological test to detect the presence of antibody (immunoglobulin G), type of confirmatory test, strain of the confirmatory test and additional serological tests performed. The data related to the animals included: number of equids, mean age, sex, breed, vaccination, clinical signs.

The total number of equids tested and the number testing positive specifically for WNV antibodies (and without a reported history of vaccination) were also extracted independently by two authors (JMD and OTO) and any disagreement confirmed by a third author (MB).

Data analysis and presentation

Statistical meta-analysis of the proportion of WNV antibody-positive animals was conducted and a forest plot generated using *metaprop* in STATA 16 (Nyaga et al., 2014). Subgroup meta-analysis was done for the Mediterranean and non-Mediterranean countries of Europe to investigate if greater prevalence was reported in Mediterranean countries where the climate is more favourable for mosquitoes. As well, analysis of two balanced 9-year-period subgroups (2001-2009 and 2010-2018) were performed. This allowed the impact of the introduction of a WNV vaccine on seroprevalence studies to be determined. Estimates from individual studies were transformed using the Freeman-Tukey double arcsine transformation to stabilize the variance. Heterogeneity was assessed using the I squared statistic (I^2). A funnel plot to assess publication bias was generated and outliers identified using R (R Core Team, 2014).

Maintenance of study standard

This study has been performed in accordance with guidelines for meta-analysis of observational studies (MOOSE statement) and preferred reporting items for systematic reviews and meta-analyses (PRISMA statement) (Moher et al., 2015; Stroup et al., 2000).

Results

Search results and study selection

From the initial database search, 1484 potentially relevant publications were identified of which 663 were found in Scopus and 821 in PubMed. After removing the duplicates, the title and abstract of 950 studies were screened. Of the 104 studies that remained, 65 studies were excluded for reasons listed in Figure 1. The lack of a confirmatory test to measure neutralising antibodies was one of the main reasons for exclusion (n=13). The other exclusion factors were: review articles, type of study, language other than English, French or Spanish, insufficient data, full text not available, duplicated data, type of study and year of study.

Finally, a total of 39 publications satisfied the inclusion criteria and were included in the systematic review, of which 38 were in English and one in French. Of the 39 publications, 3 were found in Scopus, 8 in PubMed and 28 in both databases. No additional studies that satisfied the inclusion criteria were found in the reference lists of selected studies. Table 1 presents the studies included in the systematic review.

Study characteristics

Of the 39 studies included in the systematic review, the majority (n=36) were prospective serosurveys; 3 studies were retrospective. In 14 studies (35.9%), it was stated that the equine serum samples were taken randomly. It was assumed that in the other studies, convenience samples were obtained. In total, 28,089 equids were tested, of which 375 were donkeys or mules. The prevalence was described only in horses in 34 studies, only in donkeys and mules in one study (García-Bocanegra et al., 2012c) and in both horses and donkeys in 3 studies (Bosiljka et al., 2013; Ozkul et al., 2013; Raleigh et al., 2012) The mean number of equids sampled in each study was 720 with a wide range (68 to 5178).

Of the 16 European countries in which studies were conducted, 7 (Albania, Croatia, Spain, France, Italy, Portugal and Turkey) are part of the Mediterranean basin (Figure 2). The highest number of studies was found for Spain (n=9), followed by France and Serbia (n=4). Prevalence data were available for both date ranges in the following ten countries: Croatia, Czech Republic, France, Germany, Ireland, Poland, Portugal, Serbia, Spain and Turkey (34 studies). Twelve of the studies were carried out in the first date range (2001–2009) and 19 in the second period (2010–2018). In one study (Raleigh et al., 2012), prevalence data were separated in the two periods of time. There were five studies that started in the first period and finished in the second period and two for which the year(s) of sampling was not specified. In the majority of studies (n=24), samples were first screened by ELISA and some or all of the positive-testing samples were confirmed by testing for WNV-specific neutralising antibodies. In 10 studies, a neutralisation test was performed without prior screening by ELISA and in one study both ELISA and VNT were used to screen the samples. In three studies, the initial screening test was either agar gel immunodiffusion (AGID), immunofluorescence antibody test (IFAT), or multiplex immuno-assay (MIA). Additional tests performed included western blot and haemagglutination inhibition (HI) test.

The virus strain used for the VNT or PRNT was described in 24 studies. The strain ‘Eg101’ was used in 12 studies, ‘New York (NY99)’ in 7 studies and ‘Israel 1998 (IS98-ST1)’ in one study. These three strains belong to genetic lineage 1. Only five studies used genetic lineage 2 strains: ‘Austrian’ (n=3), ‘Hungary 578/2010’ (n=1) or unspecified (n=1). Of the five studies that used genetic lineage 2 strains, one study also used a strain from genetic lineage 1. The remaining studies (n=15) did not specify the strain used.

In 18 of the 39 studies, samples were additionally screened for neutralising antibodies to other flaviviruses; TBEV only in 2, USUV only in 6, USUV and TBEV in 9 and Bagaza virus in 1. Positive titres were detected against TBEV or USUV in five and seven studies, respectively. In four studies, some samples had similar titres against both WNV and USUV.

Of the 39 selected studies, only 3 described minimal demographic data (age, sex, breed) and 8 reported whether or not any of the tested equids were vaccinated or showed any clinical signs.

More than half of the studies (n=23) reported the season of year when the animals were sampled; the majority of the studies were carried out in autumn (September to November).

Meta-analysis of West Nile virus seroprevalence

The pooled seroprevalence was 8% (95% CI 5–12%) with substantial heterogeneity (I^2 = 99.3%) (Figure 3). Pooled seroprevalence was slightly higher in Mediterranean (9%, 95% CI 5–14%) than non-Mediterranean countries (7%, 95% CI 3–14) and in the first sampling period (8%, 95% CI 2–17%) than in the second sampling period (7%, 95% CI 4–10%) (Table 2). A funnel plot (Figure 4) did not identify significant publication bias. However, two studies (Calistri et al., 2010; Petrović et al., 2014) were identified as outliers in the Studentized residual test.

Discussion

This systematic review sought to highlight important trends in WNV seroprevalence in equids in Europe; prevalence data of WNV reported in 39 studies of equids in Europe were analysed from the year 2001 to the year 2018. The pooled seroprevalence obtained was 8% (95% CI 5–12%). However, few studies reported using random sampling methods, therefore caution must be applied when generalising the seroprevalence estimates to the target population. The substantial heterogeneity (I^2 = 99.3%) meant that meaningful conclusions could not be drawn about differences in seroprevalence between Mediterranean and non-Mediterranean countries of the two periods evaluated.

There was no evidence that small studies with small effect sizes were missing. However, the two studies that were identified as outliers (Calistri et al., 2010; Petrović et al., 2014) were also the two studies with the highest seroprevalence: 39% and 49%, respectively. The study by Calistri et al. (2010) was associated with investigation of an outbreak of WNV. Similarly, in the study by Petrović et al. (2014), samples were collected from horses in November and December 2012 after the first human outbreak of WNV reported in Serbia, which started in

August 2012. Other studies with high seroprevalence were also associated with recent outbreaks.

The quality of data reporting varied between studies, for example, only three studies provided information on animal characteristics such as age, sex and breed, each of which could influence risk of exposure to and/or susceptibility to WNV infection for example, older animals are more likely to have been exposed to virus. Reporting these demographic data is important for comparisons are to be made between the outcomes of different studies. Recruitment criteria are important in understanding disease transmission in mobile animal populations such as horses where animals may have been exposed to the virus somewhere other than the study location. Furthermore, vaccination status became important after an equine WNV vaccine was first licensed in Europe in 2009. For example, Ziegler et al. (2012) found four samples positive for WNV antibodies in a study conducted in Germany, but three of these were from vaccinated horses (and were therefore removed from the seroprevalence estimation in this study) and one was from a horse from Hungary. However, vaccination status was only reported in 8 studies although 19 were conducted on samples collected after 2009. ELISA is often the assay of choice for conducting seroepidemiological studies because it is simple, sensitive, rapid and often commercially available. However, due to extensive cross-reactivity between antibodies raised against different flaviviruses, the ELISA can yield false positive results where different flaviviruses co-circulate (Beck et al., 2013). Therefore, this systematic review only included studies that used virus / plaque reduction neutralisation tests to confirm positive samples, however 21 out of 39 studies did not test in parallel other flaviviruses, which could have introduced errors in their prevalence estimation. The issue of cross-reactivity in ELISA was illustrated in some of the studies, for example Berxholi et al. (2013) found that two of seven samples that were positive in ELISA but negative in WNV VNT were positive for TBEV antibodies. Similarly, Ziegler et al. (2013a) found that four samples that were positive by ELISA but negative by WNV VNT were positive for TBEV (but not for USUV). One of the included studies (Lupulovic et al., 2011) was the first to report neutralising antibodies to USUV in horses, however, as the PRNT titres were 120 and 90 for WNV and USUV, respectively, they were not able to conclude whether this represented cross-reactive antibodies or prior exposure to both viruses. Calistri et al. (2010) mention that USUV was circulating in Italy in the year before samples were obtained in their study, but they did not

test for USUV antibodies. In most cases, neutralisation tests were positive for one virus only or titres were markedly higher (e.g. at least 2-fold) for one virus. However, neutralisation tests were not always discriminatory, particularly where VNT titres were low (Jiménez-Clavero et al., 2007). Furthermore, Vanhomwegen et al. (2017) concluded that of 21 samples that were positive for flavivirus antibodies, 11 were specifically positive for WNV, 2 for USUV and 1 for TBEV while 8 were positive for an unidentified flavivirus (1 of which they reported as positive for both WNV and an unidentified flavivirus). Ziegler et al. (2013a) reported four samples that were positive by WNV ELISA but VNT negative with WNV and USUV, ~~which~~although this could ~~also~~ be ~~related with~~ due to the lower sensitivity of VNT compared to ELISA (Beck et al., 2017). Furthermore, a high prevalence of tick-borne encephalitis virus (TBEV) has been reported in other studies in some European countries (Rushton et al., 2013). Therefore, there is evidence of non WNV flaviviruses circulating in Europe, which should be taken into account when performing a serosurvey study.

Although most studies did not specify if seropositivity was caused by lineage 1 or 2 WNV strains, this is probably not critical, because it is not possible to tell by which virus lineage the immune response was elicited as the two strains differ only in three amino acids within domain III of the E protein, against which most neutralizing antibodies are directed (Berxholi et al., 2013).

Most studies were performed in autumn, probably after the beginning of the transmission season and occurrence of outbreaks in the summer. This should not affect the results of the meta-analysis other than the already mentioned bias caused by association of a serosurvey study after regional outbreaks, which could falsely increase the prevalence estimation.

Despite the fact that WNV transmission and outbreaks in equids occurs in EU countries, the limitations detected in this study precluded the evaluation of an increase in seroprevalence over time. An analysis of the number of reported outbreaks could be useful to determine the re-emerging status of this virus, however this was not the scope of this study.

Horses have been suggested as useful sentinels for WNV surveillance. However, the true seroprevalence of WNV in European equids remains uncertain due to variation in study design and reporting, and difficulty discriminating between cross-reactive antibodies. Standardised seroprevalence studies are critical to better understand the current epidemiological status of WNV in Europe and to monitor future changes.

Conflict of interest statement

No conflict of interest to declare by the authors.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is a review article with no original research data.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Table 1. Characteristics of studies included in the systematic review

Publication	Country[1]	No. positive	No. tested	Seroprev-alence (%)	Year	Screening test	Tests for other flaviviruses
1. Abad-Cobo et al. 2017	Spain (ES)	5	369	1.36	2011–2013	ELISA	USUV
2. Alba et al. 2014	Spain (ES)	0	178	0	2011	ELISA	
3. Bakonyi et al. 2013	Hungary (HU)	79	276	28.62	2009	IFAT	
4. Barbić et al. 2012	Croatia (HR)	72	2098	3.43	2010–2011	ELISA	TBEV + USUV
5. Barbić et al. 2013	Croatia (HR)	48	1380	3.48	2011	ELISA	TBEV + USUV
6. Barros et al. 2011	Portugal (PT)	40	1313	3.05	2004–2010	ELISA	
7. Barros et al. 2017	Portugal (PT)	18	989	1.82	2011–2016	ELISA	
8. Bażanów et al. 2018	Poland (PL)	62	411	15.09	2012–2013	VNT	USUV
9. Berxholi et al. 2013	Albania (AL)	37	167	22.16	N.S.	ELISA & VNT	TBEV
10. Bosiljka et al. 2013	Serbia (RS)	45	1199[2]	3.75	2008–2012	AGID	
11. Busani et al. 2011	Italy (IT)	348	2528	13.77	2008 & 2009	ELISA	TBEV + USUV
12. Busquets et al. 2019	Spain (ES)	9	138	6.52	2017 & 2018	ELISA	BAGV
13. Cabre et al. 2005	France (FR)	0	94	0	2003	ELISA	
14. Calistri et al. 2010	Italy (IT)	794	2030	39.11	2008	PRNT	
15. Csank et al. 2018	Slovakia (SK)	10	145	6.90	2013	ELISA	TBEV + USUV
16. Durand et al. 2005	France (FR)	304	906	33.55	2003	ELISA	
17. Ergunay et al. 2014	Turkey (TR)	48	389	12.34	2011–2013	PRNT	
18. García-Bocanegra et al. 2012a	Spain (ES)	36	510	7.06	2010	ELISA	
19. García-Bocanegra et al. 2012b	Spain (ES)	12	109	11.01	2010–2011	ELISA	
20. García-Bocanegra et al. 2012c	Spain (ES)	12	165[3]	7.27	2011	ELISA	
21. Hubálek et al. 2008	Poland (PL)	0	78	0	2006	PRNT	USUV

22. Hubálek et al. 2013	Czechia (CZ) & Slovakia (SK)	19	395	4.81	2008–2011	PRNT	TBEV + USUV
23. Jiménez-Clavero et al. 2007	Spain (ES)	13	157	8.28	2005	VNT	USUV
24. Jiménez-Clavero et al. 2010	Spain (ES)	0	68*	0	2008	VNT	USUV
25. Lupulovic et al. 2011	Serbia (RS)	42	349	12.03	2009–2010	ELISA	USUV
26. Madić et al. 2007	Croatia (HR)	4	980	0.41	2010–2011	ELISA	
27. Maquart et al. 2017	France (FR)	9	96	9.38	2014	ELISA	USUV
28. Medić et al. 2014	Serbia (RS)	72	252	28.57	2007–2011	ELISA	
29. Monaco et al. 2010	Italy (IT)	271	770	35.19	2008	VNT	TBEV + USUV
30. Ozkul et al. 2006	Turkey (TR)	36	299[4]	12.04	N.S.	PRNT	
31. Ozkul et al. 2013	Turkey (TR)	57	180	31.67	2011	PRNT	
32. Petrović et al. 2014	Serbia (RS)	64	130	49.23	2012	ELISA	
33. Pradier et al. 2014	France (FR)	143	1159	12.34	2007–2008	ELISA	
34. Raleigh et al. 2012	Ireland (IE)	0	490[5]	0	2005–2006 (n=90) & 2010 (n=400)	ELISA	
35. Vanhomwegen et al. 2017	Spain (ES)	11	172	6.40	2011–2012	MIA	TBEV + USUV
36. Weissenböck et al. 2003	Austria (AT)	0	350	0	2001	PRNT	
37. Ziegler et al. 2012	Germany (DE)	1	1282	0.08	2007–2009	ELISA	TBEV + USUV
38. Ziegler et al. 2013a	Germany (DE)	2	5178	0.04	2010–2012	ELISA	TBEV + USUV
39. Ziegler et al. 2013b	Ukraine (UA)	42	310	13.55	2010–2011	ELISA	TBEV

[1]Two letter ISO country code

N.S., not specified

AGID, agar gel immunodiffusion; IFAT, immunofluorescence antibody test; ELISA, enzyme-linked immunosorbent assay; MIA, multiplex immuno-assay; PRNT, plaque reduction test; VNT, virus neutralization test.

BAGV, Bagaza virus; TBEV, tick-borne encephalitis virus; USUV, Usutu virus

[2]1133 horses and 66 donkeys; [3]82 donkeys and 83 mules; [4]259 horses and 40 mules; [5]386 horses and 104 donkeys

*Only results from samples collected in 2008 were included in the meta-analysis

Table 2. Pooled seroprevalence of WNV in Europe

		No. positive	No. tested	% (95% CI)	D.F.	I^2 (%)
Region	Mediterranean	2327	17,244	9 (5–14)	24	99.1
	Non-Mediterranean	438	10,845	7 (3–14)	13	99.0
Sampling period	2000–2009	1953	9788	8 (2–17)	12	99.4
	2010–2018	521	14,327	7 (4–10)	19	98.3
Overall		2765	28,089	8 (5–12)	38	99.3

Figure legends

Figure 1. Flow diagram of article selection for West Nile prevalence in equids in Europe

Figure 2. Map showing European countries for which data were included in the systematic review. Created using https://mapchart.net/europe.html with different colour shading used for Mediterranean and non-Mediterranean countries and depth of shading indicating number of studies performed in each country.

Figure 3. Forest plot showing the pooled estimated seroprevalence (ES) of West Nile virus among equids in Europe. Horizontal lines represent 95% confidence intervals (Cls). Each square box denotes the seroprevalence rate point estimate and the area is proportional to the weight of the study.

Figure 4. Funnel plot of standard error by Freeman-Tukey double arcsine transformed proportion for all studies (n=39)