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Prevalence of Leptospira spp. and Seoul hantavirus in brown rats (Rattus norvegicus) in four regions in the Netherlands, 2011-2015

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ABSTRACT
Background: Brown rats (Rattus norvegicus) may carry pathogens that can be a risk for public health. Brown rats in the Netherlands were tested for the zoonotic pathogens Leptospira spp. and Seoul hantavirus (SEOV), in order to obtain insight in their prevalence. Methods and results: Cross-sectional studies were performed at four locations from 2011 to 2015. The rats were tested for Leptospira spp. using real-time PCR and/or culture resulting in a prevalence ranging between 23% and 57%. Testing for SEOV was done through an adapted human Seoul hantavirus ELISA and real-time RT-PCR. Although at several locations the ELISA indicated presence of SEOV antibodies, none could be confirmed by focus reduction neutralization testing. Conclusion: The results indicate a widespread presence of Leptospira spp. in brown rats in the Netherlands, including areas with a low leptospirosis incidence in humans. No evidence for circulation of SEOV was found in this study.

Introduction
Rodents are known to carry pathogens that may be a risk for public health [1–4]. Despite this, little is known about the prevalence and geographical distribution of zoonotic rodent-borne pathogens in the Dutch rodent population. This therefore limits opportunities for preventive measures and complicates the risk-assessment of transmission to humans.

Leptospirosis is (re-)emerging globally and numerous outbreaks have occurred worldwide during the past decade [5], resulting in a significant health burden, especially in resource-poor tropical countries [6]. In the Netherlands, human leptospirosis has been noticeable since 1928 [7]. In 2014 and 2015, 60 and 44 autochthonous human cases were notified respectively, representing a four-fold increase compared with 2010–2013 (mean: 13, range: 9–17 cases) [8–10]. Since rodent and Leptospira spp. survival are facilitated by warm weather, the increase in human leptospirosis cases could possibly be explained by 2014 and 2015 being the warmest years on record [11,12]. Areas with a high incidence of human leptospirosis can be found in the central and northern parts of the Netherlands. Most of the autochthonous Leptospira spp. cases are related to recreational, e.g. swimming, or occupational activities, mostly among farmers [8,9]. About half of the autochthonous cases from 2013–2015 for whom the probable infecting serogroup could be deduced were infected with serovars belonging to the serogroup Icterohaemorrhagiae. For this serogroup, rats are considered the main reservoir. Rats are considered a major source of Leptospira infection to humans through direct and indirect transmission, e.g. through contact of mucous membranes or broken skin with water or moist soil which is contaminated with the urine of Leptospira infected animals. However, reports on the presence of pathogenic Leptospira spp. and the various serovars in rats in the Netherlands are scarce. One report from 1934 quotes prevalences in rats ranging from 11–56%, emphasizing local differences [13]. In muskrats (Ondatra zibethicus), leptospires were isolated from 24 of 327 (7%) muskrats caught in the Netherlands [14].

In addition to pathogenic Leptospira spp., Rattus spp. may also carry Seoul virus (SEOV). SEOV is a hantavirus, a genus of mainly zoonotic RNA viruses that are carried, amongst others, by rodents [15]. Human SEOV infections often lead to mild or no disease [16] but some people may develop a form of...
haemorrhagic fever with renal syndrome, which has a case-fatality rate of 1–2% [17] Compared to Puumala virus, another hantavirus which is presumably responsible for a higher number of clinical cases in the Netherlands, the clinical manifestations of SEOV are more severe and give a higher mortality [17]. In Europe, a few severe clinical human cases of SEOV infections have been confirmed in the UK and France, where the source of infection was established as pet rats [18,19]. Furthermore, a serosurvey among farmers exposed to wild rats showed a seroprevalence of 8% in the UK [20]. Since 2000, the presence of SEOV in wild brown rats was demonstrated in Belgium, France and the UK [18,21–23]. In the Netherlands, SEOV infection was detected in wild brown rats captured in the area around Doetinchem in 2013 [24]. There has been no evidence for autochthonous human cases from wild rats yet [25], while some cases of SEOV in humans have occurred since 2016, with pet rats or feeder rats as the source of infection. Human hantavirus infections have been notifiable in the Netherlands since 2008.

To obtain insight in *Leptospira* spp., brown rats from four geographic areas in the Netherlands were tested for its presence. This allows comparison between prevalence in rats within areas of high and low incidence of human leptospirosis (Figure 1), as well as the potential differences between urban and rural locations. To study the anticipated presence of SEOV in the Netherlands, these rats were also tested for SEOV. This paper describes the results of these studies.

**Methods**

**Monitoring studies**

From 2011–2015, four cross-sectional studies on *Leptospira* spp. and SEOV in brown rats were performed. From December 2011 to May 2012 the presence of *Leptospira* spp. and SEOV was studied in Limburg, from May 2012 to July 2013 in Friesland, from January 2014 to August 2015 in Amsterdam city center and from June to November 2015 in a rural area between Nijmegen and Doetinchem. Based on

![Figure 1. Incidence of autochthonous human leptospirosis cases in the Netherlands 2011–2015 by public health service region. Study areas are indicated with blue circles: 1: Limburg; 2 Friesland; 3 Amsterdam; 4 Nijmegen/Doetinchem.](image)
Rats

Within the study area, locations were selected based on known presence of rats. Because the total rat populations were unknown, the capturing of rats was continued for each study area until about 50 rats (for Amsterdam 30 rats) were captured or when capturing efforts were not effective anymore. Rats were captured outside buildings by employees of a professional rat control agency using live traps (Killgerm Inc., Turnhout, Belgium) baited with food, though rat infestations were not necessarily present. Rats were transported alive to either the National Institute of Public Health and the Environment (RIVM) (rats from Limburg, Amsterdam, Doetinchem) or Wageningen Bioveterinary Research (WBVR) (rats from Friesland). Procedures at WBVR were similar to those at RIVM. Rats were anesthetized using isoflurane (RIVM) or CO₂ (WBVR), weighed, and blood was collected, after which they were euthanized by cervical dislocation or isoflurane overdosing. Rats were classified in age groups (adult, subadult, juvenile) according to their appearance and weight. During post mortem inspection, kidney and urine samples were collected to test for *Leptospira* spp. and lungs were collected to test for SEOV. All procedures were approved by the Dutch Animal Ethics Committee (DEC project numbers 200900164, 201200208, and 201500089).

*Leptospira* spp. detection

Rats were tested by real-time PCR and/or culture, and rats were considered positive if one of the tests was positive. The diagnostic methods differed slightly between the four field studies: culture was introduced after the first study, because it enabled additional serotyping. In Limburg (2011–2012), kidneys were collected and were stored in 90% ethanol and sent to the National Collaborating Centre for Reference and Research on Leptospirosis (NRL) in Amsterdam for real-time PCR testing for leptospires. In Friesland (2012–2013), after euthanization of the rat, one of its kidneys, and if possible also urine, was immediately added to liquid EMJH culture medium [26]. The next day, the culture tubes were sent to the NRL for further incubation. Additionally, of 18 rats, a kidney sample in 90% ethanol was sent to the NRL for real-time PCR testing for leptospires. In Amsterdam (2014–2015) and Nijmegen-Doetinchem (2015), kidneys from all captured rats were both tested by culture as well as by real-time PCR at the NRL. At the NRL, culturing, real-time PCR analyses and *Leptospira* species determination were performed by previously described methods [27]. Prevalence rates and confidence intervals were determined using R version 3.2.0 [28].

**Seoul hantavirus diagnostics**

For the detection of SEOV IgG in rat sera, a human SEOV ELISA (Hantavirus Dobrava/Hantaan IgG Elisa, Progen Biotechnik GmbH, Heidelberg, Germany) was adapted to enable detection of rat IgG. Rabbit-a-rat IgG horseradish peroxidase labeled (Sigma–Aldrich Chemie B.V. Zwijndrecht, The Netherlands) was used as conjugate at a 1:5,000 dilution. Samples were tested in duplicate and the average optical density (OD) value of the samples was used for analyses. The frequency distribution of the ODc-values of field samples was analyzed and the optimal cut-off value was determined by use of a binary mixture model [29]. This model used ELISA results of 161 brown and 61 black rats captured from 2008–2010, of which a subset was confirmed by an in-house ELISA performed at the Belgian Reference Laboratory for Vector-borne diseases, by focus reduction neutralization test (FRNT) [30] performed at the Swedish Institute for Communicable Disease Control, by immuno-fluorescence assay (IFA) based on SEOV infected cells and/or by real-time RT-PCR for SEOV on total RNA isolated from lung tissue, as previously described [31,32].

In the current study, if a positive ELISA result was obtained, lung tissue was tested by a SEOV–specific real-time RT-PCR [33]. For the rats from Nijmegen-Doetinchem, this real-time RT-PCR was performed on all samples, to be confident that no early-stage infections were missed. This was only performed for the rats of this location, because of the findings of SEOV in rats in this area by Verner-Carlsson et al. [24]. Three samples from Limburg with positive ELISA results were tested by FRNT.

**Results**

**Rats**

The numbers of live rats captured were as follows: 42 in Limburg (2011–2012), 24 in Friesland (2012–2013), 31 in Amsterdam (2014–2015) and 53 in Nijmegen-Doetinchem (2015).

**Leptospira** spp. detection

In Limburg, 14 of 42 (33%) rats tested positive for *Leptospira* spp. by real-time PCR. Sequence analysis
Brown rats were captured at four locations in the Netherlands to obtain information about the prevalence and geographical distribution of *Leptospira* spp. and SEOV. The prevalence of *Leptospira* spp. ranged between 33.5% for *Leptospira interrogans* in Limburg, one rat each from Amsterdam and Nijmegen-Doetinchem, and 57% for *Leptospira interrogans* in Nijmegen-Doetinchem.

It was unexpected to find the same prevalence (33%) in rats from Limburg and Friesland, since Friesland has a much higher leptospirosis incidence. Possible explanations are environmental differences.

Table 1. Results of *Leptospira* spp. and SEOV diagnosed in cohorts of rats in four geographical regions in the Netherlands. *Leptospira* spp. results are based on combined results of culture and RT-PCR. The SEOV results are based on ELISA and RT-PCR of seropositive animals. Of the Nijmegen-Doetinchem study, all animals were tested by SEOV RT-PCR. Seropositive results for SEOV could not be confirmed by RT-PCR or a neutralization test, and were therefore considered negative.

| Study area        | Number of brown rats tested | Culture positive (prevalence; 95% confidence interval) | PCR positive (prevalence; 95% confidence interval) | Serovar species (number) | EUSA positives/number tested (average OD values of positive samples) | RT-PCR positives/number tested |
|-------------------|-----------------------------|-------------------------------------------------------|--------------------------------------------------|--------------------------|--------------------------------------------------------------------|------------------------------|
| Limburg           | 42 (34 in 2011, 8 in 2012)  | 14/42 (33%; 21–49%)                                    | Not done                                         | *L. interrogans* (3)     | 0/42 (0%; 0–8%)                                                    | 0/6                          |
| Friesland         | 24 (7 in 2012, 17 in 2013)  | 8/24 (33%; 18–53%)                                     | 7/24                                             | serovar Icterohaemorrhagiae (7) (serogroup Icterohaemorrhagiae) | 0/24 (0%; 0–14%)                                                    | 0/1                          |
| Amsterdam         | 31 (9 in 2014, 22 in 2015)  | 12/31 (39%; 24–56%)                                    | 12/31                                            | serovar Icterohaemorrhagiae (6) and serovar Copenhageni (5) (serogroup Icterohaemorrhagiae) | 0/31 (0%; 0–11%)                                                    | 0/31                        |
| Nijmegen-Doetinchem | 53 (2015)                  | 30/53 (57%; 43–69%)                                    | 27/53                                            | *L. interrogans* (9/27) and serovar Copenhageni (18/27) (serogroup Icterohaemorrhagiae) | 0/53 (0%; 0–7%)                                                    | 0/53                        |

* Combined prevalence, culture and PCR results if available.
* Three positive amplicons were further sequenced as being derived from *L. interrogans*.

**Seoul hantavirus**

Based on the cut-off (0.74) determined with the binomial mixture model, six rats from Limburg, one rat from Friesland and three rats from Nijmegen-Doetinchem were serologically positive for SEOV by the adapted human SEOV IgG ELISA. No rats from Amsterdam were seropositive for SEOV. Because extensive cross-reactivity exists within the genus hantavirus and due to limited validation of the in-house adapted SEOV ELISA on wild rats, serum samples from three seropositive rats from Limburg were tested further by FRNT in Uppsala, Sweden, for the presence of SEOV neutralizing antibodies. The presence of SEOV could not be confirmed by FRNT and none of the lung tissues from these rats tested positive in the real-time RT-PCR for SEOV RNA.

Discussion

Brown rats were captured at four locations in the Netherlands to obtain information about the prevalence and geographical distribution of *Leptospira* spp. and SEOV. The prevalence of *Leptospira* spp. ranged between 33% in Friesland, 57% in Nijmegen-Doetinchem, and 33% in Limburg. Serovars Copenhageni and Icterohaemorrhagiae were isolated, which are known to belong to species *Leptospira interrogans* in the Netherlands.

It was unexpected to find the same prevalence (33%) in rats from Limburg and Friesland, since Friesland has a much higher leptospirosis incidence. Possible explanations are environmental differences.
might be (1) more awareness amongst Frisian physicians for human leptospirosis than in Limburg, (2) a higher exposure risk because of water recreational differences between Friesland and Limburg or (3) differences in environmental conditions, such as type of ground soil, which could influence Leptospira spp. survival. Also, it needs to be taken into account that sample sizes differed between locations and that there may have been seasonal or yearly variation in Leptospira spp. circulation. Furthermore, in Limburg rats were only tested by real-time PCR of the kidney, whereas in the other studies culture was also performed, which could potentially influence the prevalence found.

The relatively high prevalence of Leptospira spp. found in different areas in the Netherlands could be relevant for public health. General practitioners and other medical professionals need to be aware of possible human cases of leptospirosis. This knowledge also emphasizes the importance of personal protection for people that occupationally come into contact with rats or frequent wet outdoor environments. Since the increase in human leptospirosis cases in 2014, various actions have been undertaken to enhance awareness and ensure early detection and reporting of new cases. For example, alerts at the start of leptospirosis season (July-September) were sent in a weekly report by the Netherlands Early Warning Committee to professionals working in the field of infectious diseases in the Netherlands. In addition, veterinarians were informed through a Dutch veterinary journal and microbiologists through Labinf@ct, a message service for professionals working in medical microbiological laboratories.

Although currently most human leptospirosis cases contract the disease outside cities, the Leptospira spp. prevalence of 39% in rats in Amsterdam suggests there is also a risk of infection within cities. This may be particularly relevant for (large) swimming events in the city, which are becoming increasingly popular, and for smaller swim play areas that are present in the city.

Although results of the in-house adapted SEOV ELISA suggest the presence of SEOV antibodies in sera of rats captured in Limburg, Friesland and Nijmegen-Doetinchem, the presence of SEOV could not be confirmed by genome detection or virus neutralization tests. The latter two tests are considered as confirmation of an infection, although they depend on samples taken within a limited time-frame from the start of infection. Because of this, there is no confirmation of SEOV infection in the brown rats in this study.

Recently, the first detection of SEOV in wild brown rats in the Netherlands was reported, with 3 of 16 (19%) rats testing positive for SEOV specific antibodies and viral RNA [24]. The rats in our study in 2015 were captured in a larger area, encompassing the area where these three rats were captured. However, we were unable to detect SEOV. One potential explanation is that SEOV is only present in very focal areas, as patchy distributions are also known for other hantaviruses depending on habitat connectivity of their hosts [34].

All four studies were cross-sectional studies, which limits further analyses on for example the influence of seasonal and yearly changes in Leptospira spp. prevalence in rats. However, the results do indicate a high prevalence of Leptospira spp. in brown rats throughout the Netherlands. SEOV was found to be either absent, present at low prevalence, or present in specific hot-spots only.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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