Evidence of multiple intraspecific transmission routes for *Leptospira* acquisition in Norway rats (*Rattus norvegicus)*

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Summary

Infectious diseases frequently have multiple potential routes of intraspecific transmission of pathogens within wildlife and other populations. For pathogens causing zoonotic diseases, knowing whether these transmission routes occur in the wild, and their relative importance, is critical for understanding maintenance, improving control measures and ultimately preventing human disease. The Norway rat (*Rattus norvegicus*) is the primary reservoir of leptospirosis in the urban slums of Salvador, Brazil. There is biological evidence for potentially three different transmission routes of leptospire infection occurring in the rodent population. Using newly obtained prevalence data from rodents trapped at an urban slum field site, we present changes in cumulative risk of infection in relation to age-dependent transmission routes to infer which intra-specific transmission routes occur in the wild. We found that a significant proportion of animals leave the nest with infection and that the risk of infection increases throughout the lifetime of Norway rats. We did not observe a significant effect of sexual maturity on the risk of infection. In conclusion, our results suggest that vertical and environmental transmission of leptospirosis both occur in wild populations of Norway rats.

1. Introduction

There are often multiple potential routes of intraspecific transmission of pathogens within wildlife and other populations. Elaborating the various intra- and inter-specific routes of transmission, and their relative importance, are essential for understanding the dynamics of the agent, with implications for interventions to reduce infection prevalence in the reservoir host and to control or prevent subsequent human disease [1]. Obtaining evidence of transmission routes by experimental infection in a laboratory setting is difficult and often does not represent transmission as it would occur in the wild. Inferring the relative importance of different potential transmission routes from field data may therefore be of both fundamental and practical interest. However, inferring routes of transmission from statistical associations is not straightforward. There is a need to consider multiple statistical models with different underlying assumptions to better understand associations between risk and reality.

Leptospirosis is a zoonosis caused by pathogenic bacteria of the genus *Leptospira* [2]. Many mammals serve as reservoir hosts, becoming chronically infected within their kidneys and shedding infectious leptospires in urine. Humans are incidentally infected and there are very few cases of human-to-human transmission [3,4]. The main routes of human infection are through contact with environmental sources contaminated with animal urine or through direct contact with animal reservoirs [2]. Available vaccines to prevent human leptospirosis are often not effective [5] as they do not protect against all *Leptospira* serovars, do not induce long-lasting immunity, and have side effects [6]. Control of transmission within and from the reservoir host may therefore be critical for disease control.

Urban slum dwellers, who account for one third of people living in urban settings, are at increased risk of water borne and zoonotic infectious disease, as a result of substandard housing and the lack of sanitary services [7]. Salvador, a coastal city in north-east Brazil, has experienced a recent human population increase, leading to the creation and expansion of urban slums [8]. Pau da Lima, an urban slum in Salvador, Brazil registers annual outbreaks of leptospirosis [8] where annual flooding events, associated with the rainy season, wash contaminated soil and water into areas of potential human use. Several studies have demonstrated an increased risk of *Leptospira* transmission associated with residence regions prone to flooding, open sewers, proximity to accumulated refuse, and sightings of rats at the home [9,12]. In addition, cases cluster at areas prone to flooding, where residents are mainly squatters living close to open sewers [9].The incidence of leptospirosis in the slums of Salvador is high. A recent community-based cross-sectional survey of 3,171 slum residents found an overall prevalence of Leptospira antibodies of 15.4% [9].

Residents in the slums live in close proximity to the primary animal reservoir, the Norway rat (*Rattus* *norvegicus*) [8,10,11] and environmental reservoirs contaminated with leptospires shed in rat urine. Isolates of leptospires infecting Norway rats in Salvador have been repeatedly identified *Leptospira interrogans* serovar Copenhagenii (serogroup Icterohaemorrhagiae) [13]. Paired results between qPCR of urine or kidney samples were previously shown to be 100% concordant [13]. The prevalence of *Leptospira* kidney carriage among rats in Salvador ranges between 60% and 80%, and age-stratified rates of shedding are known [11,13], but we do not currently understand the pathways of intra-specific transmission of leptospires in the rat reservoir. There are multiple potential routes: 1) vertical and pseudo-vertical transmission, where rats acquire infection in utero or acquire infection via suckling from infected dams (herein, for practical reasons, we make no attempt to distinguish these potential routes); 2) direct transmission, either by sexual contact or by some other direct mechanism such as bites; and 3) infection from exposure to environmental sources contaminated with bacteria. There is evidence that vertical and sexual transmission are biologically feasible, namely the presence of leptospires in the mammary gland, milk and semen of rats ([14] and Lin Zhan, unpublished observations), and high concentrations of leptospires are shed in the urine, so transmission through contact with the contaminated environment can be assumed to occur [13], but the epidemiological significance of the transmission routes is unknown.

We can gain insight into the contribution of various transmission routes by examining changes in the prevalence of leptospiral carriage related to increasing age and different life stages, such as sexual maturity. When rats are born, they are initially confined to the nest [15]. Once weaned, they leave the nest and begin to roam freely as sub-adults, eventually becoming sexually mature [15]. In urban settings, the time to sexual maturity is weight dependent, and so animals can become sexually mature over a wider range of ages. Vertical and pseudo-vertical transmission, in addition to exposure within the burrow nest, must occur prior to weaning. Together these determine the proportion of animals infected once they first appear in the free-roaming population. Once rats leave the natal burrow they are exposed to environmental contamination and (non-sexual) direct transmission, and after reaching sexual maturity they have the additional risk of direct transmission during sexual contact. For leptospirosis, wounding has been found to be associated in Norway rats with a higher *Leptospira* load in the urine and kidney [13,16] The level of wounding is also a risk factor for Hantavirus infection in wild rats [17], for which the primary route of infection is direct (via biting), but the presence of leptospires in saliva has not been tested for [13].

We wish to answer the question of which of the hypothesized transmission routes are biologically significant, but our approach differs from previous studies which made a priori assumptions about how risk of infection changes over time [20–22]. Such studies of wildlife disease have used age-prevalence data to infer evidence of transmission routes based on the force of infection (FOI), also known as the hazard of infection. The FOI is ‘the per capita rate at which susceptible hosts acquire infection’ [18] and can be represented algebraically based on a mathematical framework or, in the case of data analysis, modelled as a survival distribution [19]. In our application, we employ a FOI approach using a flexible survival distribution with demographic covariates to model the hazard of infection.

Here, therefore, we take two complementary approaches. First, we identify risk factors of infection from demographic factors (sex, age/mass and external indications of sexual maturity) and the presence of bites or healing wounds. Second, we use a survival analysis to estimate the changing risk of infection as the rats age and seek evidence for differential risk among different sub-populations of rats. Thus, we present an extension to the practice of analysing age-prevalence data by considering the changes in cumulative risk of infection based on demographic variables related to age-dependent transmission routes.

2. Materials and methods

* 1. Study site

The study was carried out in the Pau da Lima neighbourhood, an urban slum community comprised of three valleys in Salvador. Salvador is the third largest city in Brazil, with almost two thirds of its residents living in slums [7]. Human cases of leptospirosis occur annually, with higher incidence in the rainy season [8].

* 1. Data collection

Demographic information (sex, weight, body length, reproductive status (scrotal testes for males, and perforate vagina, enlarged breasts or evidence of lactation for females), pregnancy status for females) and the presence and severity of wounds/scars were recorded from external examinations of Norway rats trapped over five collection periods (June-July 2012, May-August 2013, October-December 2013, March-August 2014, September-December 2014). Wounding grade, previously identified as a risk factor for leptospiral infection among Norway rats, was recorded on a five point categorical scale 0 (absent), 1 (very light), 2 (light), 3 (moderate) and 5 (severe) following [23].

* 1. Statistical analyses
     1. Ageing field animals

Body weight has been the used as a surrogate variable for estimating the age of Norway rats in most field studies [24,25] but weight is non-linearly related to age and so may be misleading in analyses of transmission patterns [15]. To overcome this limitation, we impute the age of rats from their weight using the von Bertalanffy equation for growth.

where is the asymptote, is the constant growth rate and is the age at which maximum growth occurs [26]. We generated a standardized von Bertalanffy curve for wild Norway rats using weight and age data obtained from captive colonies kept in large outside enclosures [15]. In justification of this, the range of weights of male and female rats from Salvador were comparable to those obtained by [15], and weight distributions do not vary significantly by month or season of sampling, although distinct wet and dry seasons occur [27]. The fitted von Bertalanffy curve using the data from [15] had asymptote days and estimated values for growth rate (grams per day) and point of inflection days. All statistical analyses were performed in R [28]. Both male and female rats caught in the field had a similar range of weights, so we converted their weights to ages using one growth curve.

An additional complication to aging female rats by weight is the potential for age misclassification associated with pregnancy. To address this concern, prior to imputing age among female rats we compared weights between visibly pregnant rats (N=68 animals) and non-pregnant mature females (perforate vagina, placental scars indicating past pregnancy, or lactating, but not pregnant; N=72 animals). The was no difference in the weights of pregnant and non-pregnant females (two sample t-test, mean difference=20.81, t = -1.41, df = 132.22, p=0.159). This lack of effect may be due to an averaging out of the weights of animals at different stages of pregnancy or and inflation of error variance due to using the unmeasured age covariate. We nonetheless took the conservative step of adjusting the weights of pregnant females by the estimated mean difference as an average measure of pregnancy.

* + 1. Prevalence analysis

Infection was modelled as a binary response variable. Infection status was determined by qPCR analysis of urine or kidney samples for a gene specific to pathogenic leptospires (*Lip32*), as described in detail elsewhere [13]. For 3.3% of the animals, urine could not be collected and infection was determined by presence of leptospires on kidney qPCR.

There were 486 animals for which the presence of kidney carriage was established and complete records of all explanatory variables recorded: age, sex (male/female), weight, sexual maturity, and level of wounding. The prevalence of leptospiral carriage was independent of collection time ( = 6.02, degrees of freedom = 4, p = 0.20) and so this was not included as an explanatory variable in the model selection process.

Generalized linear models of leptospiral infection were fitted the bias reduction method developed by [29, 30] was used as there was complete or quasi-complete separation present during the generalized linear model fitting. For ease of statistical computation, we collapsed the level of wounding [23] into three grades: 0 (absent), 1 (very light and light combined) and 2 (moderate and severe combined). Age was adjusted so that the intercept was at our lowest recorded imputed age (27 days). A male was classified as sexually mature by the presence of scrotal testes and a female by pregnancy, lactation or presence of placental scars

We chose to use a backward elimination approach with a 5% significance threshold to obtain the final prevalence model. The final model was then used to identify risk factors for acquiring infection and to estimate the risk of infection for rats at different developmental stages and with different classes of wounding.

* + 1. Survival analysis

Our analysis of risk-factors for infection does not differentiate between recent and more long-standing infections. However, carriage in the urine and/or kidney suggests that exposure occurred at least 10 days prior to detection [31], so rats were infected at an unknown time prior to their capture. Additionally, uninfected animals at capture would have been at risk of subsequent infection at a later date. If is the time of first infection, is the observed time (age at capture) and is coded 0 and 1 to denote absence or presence of infection at capture, respectively, it follows that

In other words, positive animals were infected at the age of capture or before, and negative animals may be infected in the future. The probability of not yet being infected (according to being urine or kidney positive) at age *t* is the cumulative distribution function of , which we model as a Weibull distribution, wherewith scale parameter and shape parameter . To investigate the effect of explanatory variables on risk of infection, we specify a log-linear model for the scale parameter, Using the fact that we can use the binary data to estimate the parmeters of the survival distribution (for details, see Supplementary Materials).

To investigate the effect of multiple variables on the scale parameter () and whether risk varied by age, we fitted a model with sex, maturity status and a binary wounding variable (absent/present) as factors and then tested for significant interactions between the variables. The shape parameter determines how the risk of infection changes over a rat’s lifetime. For and the risk of infection decreases and increases, respectively, with age, whilst if the risk of infection is constant.

1. Results
   1. Ageing field animals

Weight and age distributions are shown in Figure 1. Although males (mean weight 309.24) were on average heavier than females (mean= 281.23) (two sample t-test, t = 2.76, df = 460.7, p <0.01), the weight distributions of males and females had similar ranges and shapes, which resulted in similar distributions of estimated ages (Figure 1). Most animals had an estimated age of less than 100 days; few animals were over 200 days old (10 in total).

* 1. Prevalence analysis

The prevalence of leptospiral carriage in the population was 80% (N=486). Prevalence was independent of collection time ( = 6.02, degrees of freedom = 4, p = 0.20) and so was not included as an explanatory variable in the model selection process (Table 1).

The final model of leptospiral infection among rats included age, wounding, sexual maturity and an interaction between wounding and age. Risk of being infected increased with age, level of wounding and being sexually mature (Table 2), but the risk of infection no longer increased for older animals when they had higher levels of wounding.

An estimate of the probability of infection at weaning is given by the intercept term in the final model. Thus, the youngest animal trapped (27 days old), and so likely to have just left the nest, without wounding and sexually immature, had an estimated probability of infection of 0.21 (95% confidence interval: 0.13-0.33).

* 1. Survival analysis

The final survival model included wounding, sexual maturity, sex and an interaction term between wounding and sexual maturity (Table 3). Having wounds, being sexually mature and being female increased the risk of infection, but beyond these individual effects, being both wounded and sexually mature decreased the risk of infection. The estimate of the shape parameter was 0.81 (95% confidence interval 0.52-1.28). Hence, there was no significant change in the risk of infection as the rats aged.

These effects are more clearly seen in Figure 2, which shows the cumulative distribution function of the Weibull distribution with parameters estimated from the survival model and standard errors calculated using the delta method (see Supplementary Materials for more detail). As well as females having a consistently higher risk of infection than males, wounding clearly increased the risk of infection among immature animals (Figure 2a); sexual maturity increased the risk of infection among those without wounds (Figure 2b); but for those with wounds, there was no significant difference in risk between mature and immature animals (Figure 2c).

1. Discussion

Direct evidence of different transmission routes occurring successfully and significantly in the field is more informative than experimental approaches, which, at best, can only represent the potential for transmission. Additionally, laboratory studies of rats cannot simultaneously investigate all the variables and interaction effects examined in the present study. For leptospirosis, as for other zoonoses, control of the primary reservoir can reduce the risk of zoonotic pathogen transmission to humans [32]. Key to controlling infection in a reservoir host is an understanding the dynamics of that infection. An understanding of transmission is key to understanding the dynamics of any infection.

Previous studies have used the force of infection to understand how transmission occurs in wild populations [21,22]. The notable difference in our study is that changes in risk were identified based on demographic variables instead of specified functions of hazard (piece-wise or step functions, for example). In wildlife systems, there is not a distinct time threshold for when animals reach different phases of their life cycle. For example, rats can become sexually mature over a range of ages, and so it would be inappropriate to use a step function to model change in risk for sexually maturity. Non-linear functions of hazard can be specified based on a priori assumptions about changes in risks, but by allowing covariates to create this change in risk the variation of demographic processes in wild animals can be accounted for. Methods utilising age-prevalence data are in continuing development. For example, recently developments include a framework to combine age-prevalence data with individual level antibody response data [33] and a framework to estimate individual time since infection using multiple data sources [34].

One hypothesized transmission route of leptospire infection is vertical transmission. As rats confined to the nest were never sampled, it is impossible to distinguish true vertical (in utero) from pseudo-vertical transmission (e.g. suckling), or from transmission from mother to pups in the burrow. Juvenile rats have been documented to leave the natal burrow at 25 days of age, although initial movements are limited to those close to the natal burrow space [15]. Milk transfer/production by mothers has been estimated to cease by 27 and 30 days postpartum, respectively, corresponding to the onset of free-roaming behaviour [35,36]. Hence a 27 day old animal can be taken to be one that has not been exposed to any of the other transmission routes, and its calculated probability of infection of 0.21, and relatively narrow confidence interval, suggest strongly that a significant proportion of animals leave the nest infected. Among the possible mechanisms behind this, pathogenic leptospires are present in the milk and breast tissue of chronically infected lactating females [14], but this does not necessarily indicate that transmission to suckling animals occurs at epidemiologically significant rates. Other studies have reported isolation of leptospires from faetal Norway rats, so this potential transmission route requires additional study [37].

Our parsing of the risk of rat infection by age, sexual maturity, sex and wounding provides clues as to the relative roles of direct and environmental transmission. Our finding that rats with wounds have a significantly increased risk of infection confirms previous studies carried out in Salvador and Vancouver, Canada [11,16]. This may be true direct transmission (via biting for example), or an increased risk created by a different behaviour of those animals most likely to be wounded, or an increased risk of transmission from exposure, directly through those wounds, to leptospires in the environment. With regards to biting (mechanical transmission of leptospires contaminating the buccal cavity) Norway rats spend a considerable proportion of their time grooming (~40% for males [38]) and as the average infected rat sheds 5.9 x 106 leptospires per mL of urine [13] there is potential for contamination of saliva with leptospires during urogenital grooming that can subsequently be transmitted by bite. To support this theory there have been reports of humans acquiring leptospirosis following rat bite [39]. However, our attempts to detect leptospires in the saliva of infected Norway rats found only a minority were positive (7/18) and those that were had concentrations two orders of magnitude less than an infective dose (Zhan et al, unpublished observations). Thus, given this low concentration, and the short period of contact between saliva and an open wound in the act of biting, we consider epidemiologically significant transmission by this route very unlikely. It is far more plausible that wounds are a favourable entry port for environmental contamination, where contact with water and soil (where concentrations are similar to that in saliva [13]) is repeated and longer-lasting than biting. We did not see an effect of wounding on mature rats. This may be because most animals are infected by adulthood, and so wounding is no longer a risk factor. However, we would expect a difference in risk by wounding level at sexual maturity if older higher ranking animals were less likely to be wounded and hence infected.

In both analyses, sexually mature animals were, overall, more likely to be infected. This could reflect an increased risk of having acquired infection by non-sexual routes, such as a higher cumulative exposure to the environment, or a separate transmission route such as sexual transmission. However, we did not observe a significant change in risk with the onset of sexual maturity in the plots of cumulative risk of infection, after accounting for age and wounding, which would have been an indication of sexual transmission. Among unwounded animals, those that were sexually mature were more likely to be infected but they were also older. Sexual transmission may occur, therefore, and unpublished evidence of leptospires in semen (Zhan et al, unpublished observations) supports this, but, we propose, not at an epidemiologically significant rate.

Finally, the cumulative distribution plots all showed that females had a higher risk of infection than males, but only earlier in life. A previous study of leptospiral infection in Salvadoran rats also found evidence that female rats were infected at higher rates as juveniles than males [11]. Given that there were no interactions between sex and the other variables in the model, there is no evidence to suggest that the additional risk to females is from a one-way sexual transmission route or a differential effect of wounding on the sexes. Instead, the additional risk for females may come from some behavioural or physiological difference between the sexes that is apparent from the early life stages.

It should be noted that our observations in both the prevalence and survival model are strongly dependent on the imputed age. We obtained a growth curve on captive reared wild Norway rats from [15]. However, those animals were reared in a rural setting and it has been found than Norway rats grow quicker in urban settings [40]. Eye lens weight is a good predictor of age for Norway rats regardless of location differences [40]. Our inferences are subject to change with different growth rate parameters, but without eye lens samples we had to rely on published growth curves.

This study has illustrated novel methods of identifying evidence of multiple transmission routes of leptospirosis from prevalence data obtained from the field. The survival model extends the standard practice of analysing prevalence data in an epidemiological setting by considering risk over time. The combination of potential vertical, direct and environmental routes is shared with other species and applicable to other zoonotic pathogens. In the present case, we found support for significant risk of infection within the natal burrow – in effect, vertical transmission – and environmental transmission. Beyond that, while direct transmission, either sexually or in the act of biting, is biologically possible, evidence for this being epidemiologically significant was weak. These observations, obtained using data of transmission in the wild, are significant to inform a tractable mathematical model of Norway rat leptospirosis.

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Figure 1: Histograms of the weights and estimated ages of male and female animals from the field.

Figure 2: The cumulative distribution function of the Weibull distribution with parameters estimated from the survival model and 95% confidence intervals with standard errors calculated using the delta method illustrating the risk of infection with increasing age when (a) all animals are immature, (b) all animals are without wounds and (c) all animals are wounded.

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