SHORT COMMUNICATION

Culex torrentium mosquitoes from Germany are negative for Wolbachia

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Abstract. Wolbachia (Rickettsiales: Anaplasmataceae) infects a wide range of arthropods, including several mosquito species. The bacterium is known to induce a plethora of phenotypes in its host, examples being the reproductive phenotype cytoplasmic incompatibility or resistance against infection with arboviruses. The latter is especially relevant when assessing the vector competence of mosquito species for emerging arboviruses. Thus, knowledge of Wolbachia infection status is important for the assessment of vector competence.

To facilitate Wolbachia screening in mosquito populations, a quantitative polymerase chain reaction (qPCR) assay was developed to enable high-throughput analysis of mosquito samples. Using this assay, the Wolbachia infection status of the two most common Culex mosquito species in Germany, Culex pipiens biotype pipiens Linnaeus (Diptera: Culicidae) and Culex torrentium Martini (Diptera: Culicidae), was assessed. About 93% of all tested C. pipiens biotype pipiens individuals were positive for Wolbachia, whereas none of the C. torrentium samples was found to be infected. Furthermore, other applications of the qPCR assay were explored by assessing a potential link between the levels of Wolbachia and West Nile virus (WNV) infections in German C. pipiens biotype pipiens mosquitoes. No relationship was found between the two variables, indicating that a Wolbachia-induced antiviral phenotype in this mosquito population is not exclusively attributable to the general level of bacterial infection.

Key words. Culex, Wolbachia, qPCR, West Nile virus.
fruit fly Drosophila melanogaster Meigen (Hedges et al., 2008; Teixeira et al., 2008), where persistent Wolbachia infections are present, both in nature and in laboratory stocks (Hoffmann et al., 1998; Clark et al., 2005). Subsequently, more and more studies have elucidated the phenomenon of antiviral and anti-protozoan protection of several mosquito species by either artificial Wolbachia infection with different strains (Walker et al., 2011) or natural infection (Glaser & Meola, 2010). These possible antiviral and anti-protozoan effects are especially interesting in mosquitoes that are potential vectors of arboviruses and protozoan parasites. These effects have, however, a broad range of phenotypes, including reduced viral proliferation or transmission, no effect on viral infection, reduced infection rates and even enhanced viral infection rates (reviewed in Johnson, 2015).

Mosquitoes of the Culex pipiens complex and Culex torrentium Martini (Diptera: Culicidae), are widespread and abundant in Central Europe (Rudolf et al., 2013; Hesson et al., 2014) and have been shown to transmit Sindbis virus in Sweden, West Nile virus (WNV) in Italy and Usutu virus in Austria and Germany (Lundstrom et al., 1990a, 1990b; Jost et al., 2011; Chiari et al., 2015; Hesson et al., 2015; Leggewie et al., 2016). To date, contradictory observations have been reported concerning the relationship between Wolbachia and Culex mosquitoes. Glaser and Meola reported that Wolbachia causes WNV resistance in naturally infected Culex quinquefasciatus Say (Glaser & Meola, 2010), whereas Dodson et al. reported that inoculation with the Wolbachia strain wAlbB resulted in an enhancement of WNV infection in Culex tarsalis Coquillett (Dodson et al., 2014). Furthermore, WNV resistance in C. quinquefasciatus as well as in C. pipiens seems to depend on Wolbachia density (Micieli & Glaser, 2014). Thus, not only knowledge about the presence or absence of Wolbachia, but also knowledge about its density might be required to assess the potential for antiviral protection (Moreira et al., 2009). What is more, knowledge about the Wolbachia infection status could contribute to the assessment of the vector potential of a given mosquito species.

As mentioned above, members of the Culex complex, namely C. pipiens biotype pipiens, C. pipiens biotype molestus and C. torrentium, are present in Germany (Rudolf et al., 2013) and are susceptible to WNV as well as Sindbis virus infection (Lundstrom et al., 1990a, 1990b; Chiari et al., 2015; Hesson et al., 2015; Leggewie et al., 2016). Earlier studies in which the presence of Wolbachia was determined showed that 80–100% of C. pipiens biotypes were infected (Rasgon & Scott, 2003; Duron et al., 2005; Fedorova & Shaikevich, 2007; Khrabrova et al., 2009; Zele et al., 2014; Raharimalala et al., 2016), but data on Wolbachia infection rates in C. torrentium are inconclusive. Studies conducted with Russian and Belgian populations of C. torrentium indicated that this species is not infected with Wolbachia (Fedorova & Shaikevich, 2007; Khrabrova et al., 2009; Raharimalala et al., 2016). However, Wolbachia DNA sequences were found in two C. torrentium samples from Italy (Ricci et al., 2002). Wolbachia infection rates for a larger panel of C. torrentium populations from Central Europe would be interesting given the high susceptibility of this species to WNV and Sindbis virus (Lundstrom et al., 1990a; Hesson et al., 2015; Leggewie et al., 2016). The question of the prevalence of Wolbachia in German mosquito species and the possible link to viral susceptibility led us to perform an analysis of Wolbachia infection rates in German Culex mosquito samples. To facilitate this study and future large-scale analysis of field-collected mosquito samples for the presence of Wolbachia, a quantitative real-time polymerase chain reaction (qPCR) based on the wsp (Wolbachia surface protein) gene sequence was designed. Using this method, a total of 505 individual mosquito samples from Germany were screened, which were previously identified as C. pipiens biotype pipiens or C. torrentium by a multiplex quantitative PCR (qPCR) assay (Rudolf et al., 2013). Moreover, any further application of the qPCR method was explored by screening adult C. pipiens biotype pipiens with known WNV infection status to investigate a potential link between WNV and Wolbachia infection.

Larval samples from C. pipiens biotype pipiens and C. torrentium mosquitoes were obtained from egg raft collections carried out in the Hamburg area of Germany (Altes Land, 53°35′N, 9°32′E; Langenlehsten, 53°30′N, 10°44′E, and Hamburg City, 53°32′N, 9°57′E) and Lake Constance in Germany (Radolffzell-Böhringen, 47°44′N, 8°58′E, and Mettnau, 47°43′N, 8°59′E). The egg collection was carried out using gravid traps filled with hay infusion placed in proximity to natural breeding sites of Culex mosquitoes. Traps were checked twice a day for freshly deposited egg rafts, which were retrieved from the water surface using a wooden spatula and placed in individual plastic cups for transportation to the laboratory. Subsequently, egg rafts were reared separately in dechlorinated water and four to five hatched larvae from each raft were used for DNA extraction (DNasey Blood and Tissue kit; Qiagen, Hilden, Germany). The egg rafts were then identified as either C. pipiens biotype pipiens or C. torrentium by a multiplex qPCR assay as described previously (Rudolf et al., 2013). The DNA samples from larvae pools were then analysed for the presence of Wolbachia using a TaqMan-based quantitative real-time PCR based on the wsp gene sequence. Primers for the Wolbachia qPCR were chosen based on a consensus sequence derived from available wsp gene sequences. All qPCR reactions were set up using the HotStar Taq Master Mix kit (Qiagen) including 0.6 µM of the forward and reverse primers OSM_324 (5′-TAGGCGATTGAAGAT ATGC-3′) and OSM_324 (5′-CTAGCTTCTGAAGATTG-3′), 0.2 µM of the probe OSM_324 (5′-FAM-CACCAACACCAACA CCAAGC-BHQ1-3′) and 1 µL of DNA. The PCR was subsequently optimized for MgCl2 concentration, annealing temperature and reaction mixture. The resulting qPCR protocol contained a supplementation of the PCR reaction with 4.5 mM MgCl2 and used the following thermal profile: 95°C for 15 min and cycling at 95°C (5 s), 55°C (1 min) and 72°C (30 s) for 45 cycles. A PCR product based on previously published primers (Zhou et al., 1998) was used to evaluate our qPCR protocol according to Minimum Information or Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for qPCRs (Bustin et al., 2009). The reaction efficiencies were between 85 and 100% (Fig. 1A) and the coefficient of determination (R2) was 0.99 (Fig. 1B) using this qPCR protocol. A linear correlation between the measured concentration and input concentration was found at each dilution step (Fig. 1C). Thus, all parameters of the wsp qPCR lay within generally accepted ranges and indicated a highly efficient and reliable qPCR assay (Bustin et al., 2009) which can be used as an alternative to the
SYBR Green qPCR developed by de Oliveira et al. (2015). Furthermore, this TaqMan assay could be used in a multiplex setting for simultaneous measurement of Wolbachia DNA, host genes or DNA of pathogens of interest.

Using the new qPCR assay, a total of 317 C. pipiens biotype pipientis and 188 C. torrentium samples were screened and remained grouped according to species, irrespective of geographical origin. In this way, an average picture of the infection status of these species could be obtained. The chi-square test was applied to assess differences in the proportion of Wolbachia-positive samples between the species groups. All statistical analysis was performed using Graphpad Prism 6 software (GraphPad Software, Inc., San Diego, CA, U.S.A.). The qPCR revealed that 93% of C. pipientis biotype pipientis samples were Wolbachia positive (Fig. 2A). Previous studies have investigated the infection status of C. pipiens mosquitoes from various countries, including Russia, Kazakhstan, Kyrgyzstan, Belarus, Portugal, Spain, France, Italy, Switzerland, Belgium, U.K., the Netherlands, Greece, Turkey, Cyprus, Tunisia and Australia. They all confirmed a Wolbachia infection status of up to 100% (Vinogradova et al., 2003; Duron et al., 2006; Khrabrova et al., 2009; Raharimalala et al., 2016). However, one study, which included two C. torrentium individuals from Italy, reported both of them to be positive for arthropod-specific Wolbachia (Ricci et al., 2002). In any case, the prevalence of Wolbachia in German C. torrentium populations seems to be low or zero, illustrating an obvious significant difference in the Wolbachia infection load between the two mosquito species (Fig. 2A; \( P < 0.001 \)). This observation could help to explain other interesting phenomena such as the difference in genetic variability between C. torrentium and C. pipientis reported by others (Werblow et al., 2014). Werblow et al. hypothesize that the decreased mitochondrial diversity in C. pipientis, with respect to C. torrentium populations, is the result of CI and subsequent mitochondrial sweep. A Wolbachia-free C. torrentium population in Germany supports this hypothesis.

Differences in the prevalence of Wolbachia might play an important role in WNV risk assessments in Germany, as Wolbachia can have significant effects on WNV replication in Culex mosquitoes (Glaser & Meola, 2010; Micieli & Glaser, 2014). Thus, a higher Wolbachia prevalence might be associated with increased resistance to WNV or lower transmission rates. To investigate a potential link between WNV and Wolbachia infection, a qPCR assay to assess the Wolbachia status of 106 adult C. pipientis biotype pipientis individuals with known WNV status from a previous study was used (Leggewie et al., 2016). To normalize for differences in DNA concentrations, a qPCR targeting the microsatellite locus CQ11 previously used for taxonomic
identification was used (Rudolf et al., 2013). Spearman's rank correlation and linear regression analysis were used to assess the relationship between levels of Wolbachia and WNV infections. Finally, the Mann–Whitney $U$-test was applied to analyse the relative amounts of Wolbachia in WNV-positive and -negative mosquitoes. A $P$-value of $<0.05$ was deemed statistically significant for all tests. Of the tested samples, only six matched the criteria of Wolbachia-negative and WNV-positive. Therefore, it was not possible to compare relative WNV CT (threshold cycle) values with respect to Wolbachia infection status (i.e. Wolbachia-positive and Wolbachia-negative). However, it was possible to look for variation in the relative WNV CTs with respect to the level of Wolbachia infection. There was a positive correlation between the relative Wolbachia and WNV infection levels in individuals harbouring both pathogens. This indicates that, in C. pipiens biotype pipiens, a high Wolbachia infection level is associated with a high WNV infection level and vice versa. However, the low Spearman's correlation coefficient ($r$) of 0.46 indicates little direct interaction between the two variables ($P = 0.001$). This is undermined by the linear regression analysis, which showed that a linear model is unsuitable to explain a possible relationship between Wolbachia and WNV infection levels in C. pipiens biotype pipiens individuals ($R^2 = 0.18$; slope $= 0.85 \pm 0.26$; $P = 0.002$). In addition, the fact that the relative amount of Wolbachia did not significantly differ between WNV-positive and -negative mosquitoes further suggests that the general level of Wolbachia infection does not have a causative effect on the WNV infection level (Fig. 2B; WNV positive: $n = 48$; WNV negative: $n = 52$; $N = 106$; $P = 0.93$). This observation is in contrast to the findings of experimental studies which have reported an enhanced WNV transmission rate and 2–3log higher viral loads in a Wolbachia-free C. quinquefasciatus strain compared with a Wolbachia-positive reference strain (Glaser & Meola, 2010). Other groups have, however, shown that the opposite effect is also possible. For example, Wolbachia-free C. tarsalis mosquitoes are more susceptible to WNV infection when infected with Wolbachia from Aedes albopictus Skuse (Diptera: Culicidae) (Dodson et al., 2014). Thus, the role of Wolbachia infection in WNV infections in mosquitoes is far from being clear on the basis of the available data. Our correlative study does not reveal a potential link of Wolbachia infection status and the susceptibility to WNV in Culex mosquitoes from Germany. However, it is possible that the location of Wolbachia within the mosquitoes might play a role in such a link and potentially explain the diversity of findings made in this area of research. Previous studies have indicated that not only the general or cellular density but also the specific tissue location of Wolbachia is an important factor contributing to antiviral protection (Frentiu et al., 2010). The wPip strain of Wolbachia has been found in different somatic and reproductive tissues in C. pipiens mosquitoes. However, the density of wPip in different C. pipiens colonies and natural populations might vary and thus antiviral protection might also be variable among different mosquito strains (Dobson et al., 1999; Micieli & Glaser, 2014; Pietri et al., 2016). Future studies applying the qPCR method to screen individual organs for Wolbachia will contribute to understanding the interspecies variation of antiviral protection.

Acknowledgements

This work was financially supported by the Leibniz Association, grant numbers SAW-2011-BNI-3-29 and SAW-2014-SGN-3. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.
Conflict of interest

The authors declare no conflicts of interest.

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Accepted 29 July 2017
First published online 14 September 2017