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ANOPHELES GUT MICROBIOTA PROVIDE POSSIBILITIES FOR THE DEVELOPMENT OF NEW STRATEGIES TO PREVENT TRANSMISSION OF MALARIA

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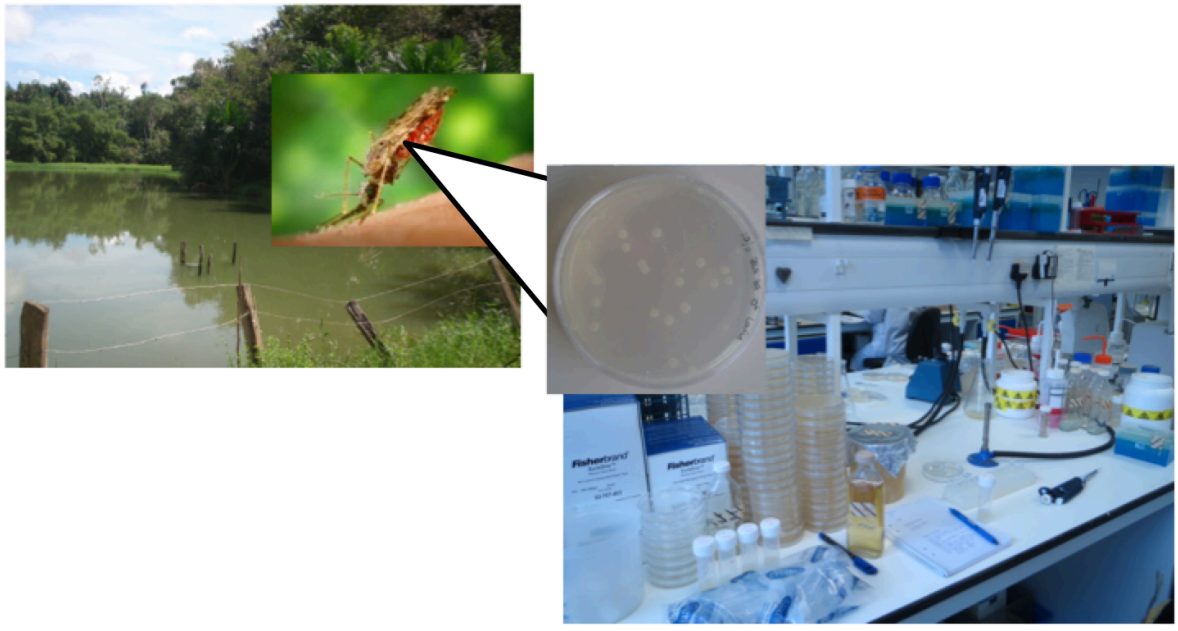

Introductory Research Essay

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Abstract

Every year, millions of people around the world are affected by malaria and many die from it. Attempts at eliminating and eradicating malaria have been made for over a hundred years but not succeeded, even though progress has been made. The most used prevention methods today are insecticides and drugs. However, the threat of resistance development in the mosquitoes and the parasites are forcing science to find new ways to prevent transmission and disease. One area of research involves the midgut bacteria of the vector mosquitoes. It has been shown that this has an effect on the parasite development. Studies have shown that there is a big diversity of bacteria found and different studies identify different numbers and different species of bacteria. The methods used for identifying the microbiota however has changed with technical development that might explain some of the differences observed. The identification of the midgut microbiota and what determines it, will hopefully lead to the identification of bacteria that can be used in one way or another to block the transmission of parasites in the malaria mosquitoes. A bacterium that has been suggested for disease reduction is *Wolbachia*, which modifies the host reproduction in order to spread. The identification of new malaria prevention methods is an opportunity to improve the lives of millions and the study of malaria-mosquito midgut bacteria might help towards this.

Introduction

Malaria is a vector-borne disease affecting millions of people around the world, most of them living in the poorest countries. It is a major public health problem and new strategies to deal with this and prevent people from being infected and getting malaria need to be developed. According to the latest World Malaria Report (WHO, 2013) there were 627,000 people who died from malaria in 2012. An estimated 207 million cases of malaria occurred and 3.4 billion people were at risk of getting malaria, 1.2 billion at high risk and 2.2 billion at low risk. Sub-Saharan Africa is most heavily affected where 80% of the estimated cases of malaria and 90% of the deaths due to malaria occurred. Of the dead, 77% were children younger than 5 years old. Malaria was in 2013 considered endemic in 104 countries and territories around the world (WHO, 2013). Here some basics of malaria will be described and the past, present and future control methods will be discussed. At the moment, studies looking at the midgut microbiota in the vector mosquitoes are being carried out and the role that the bacteria play in the transmission of malaria is investigated to develop new control strategies that may turn malaria into a disease of the past saving millions of lives.

Malaria

Causes and distribution

Malaria is caused by protozoan parasites of the genus *Plasmodium* and transmitted to humans by female mosquitoes of the genus *Anopheles*. There are five species of *Plasmodium* that cause malaria in humans, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Raghavendra et al. 2011). These parasites can be transmitted by about 70 out of 465 formally recognised species of *Anopheles*. A map of the 41 dominant vector species / species complexes for human malaria transmission has been created by Sinka et al. (2012) to show the world distribution (Fig. 1). Dominant is defined by Sinka et al. as “capable of transmitting malaria at a level of major concern to public health”. In different regions of the world, the number of dominant *Anopheles* species differs. E.g. in Africa there are a few dominant species of *Anopheles* while in South-East Asia and Pacific there are more. In many regions of the world, there are also more than one vector species present that occupy the same geographical area. (Sinka et al. 2012).

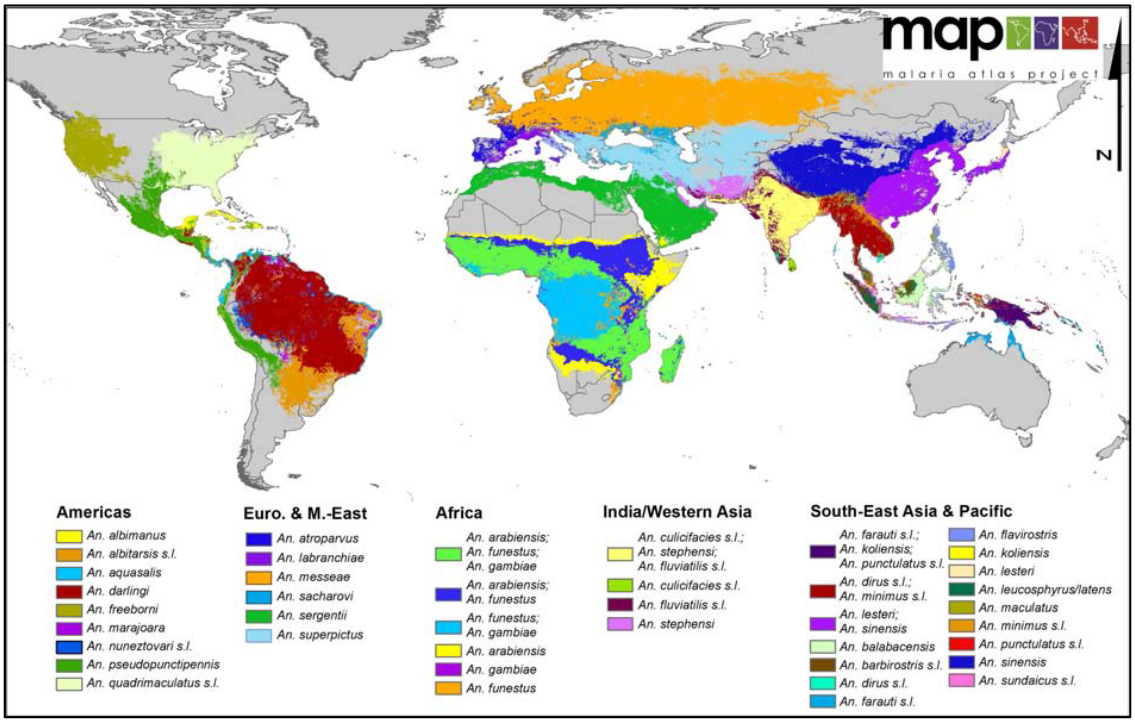

Figure 1. *World distribution of the dominant malaria vector species / species complexes (Sinka et al. 2012).*

Plasmodium life cycle

The malaria parasites have a complex life cycle that requires developmental stages in both mosquitoes and in humans. When a human is bitten by an infected mosquito, the parasite sporozoites are injected with the mosquito saliva and they travel through the blood to the liver. In the liver they invade the hepatocytes where they develop, multiply and differentiate to become merozoites (Tilley et al. 2011). Thousands of merozoites are then released into the blood again where they invade the erythrocytes. *Plasmodium* development within the erythrocytes occurs through a number of steps. The first step is the ring stage, the next step when they grow is the trophozoite stage and the last step when they divide until the erythrocyte ruptures is the schizont stage. About 20 merozoites are released from each schizont and these infect new red blood cells. For this intraerythrocytic cycle to occur, it takes about 48h (Tilley et al. 2011). In the erythrocytes some of the parasites, which at this stage are haploid and asexual, commit to sexual differentiation instead of continuing the asexual cycling. This produces micro- and macrogametocytes that are male and female gametocytes respectively. The gametocytes need to be taken up by a mosquito vector to be able to continue their development otherwise they will die (Baton and Ranford-Cartwright 2005). If they are taken up in a blood meal by a mosquito, they travel into the midgut lumen together with the rest of the blood meal. This quickly initiates gametogenesis, which is the transformation of the gametocytes into extracellular micro- and macrogametes, which fuse rapidly to form diploid zygotes. These zygotes undergo meiosis and transform into motile ookinetes that move to the midgut epithelium and cross it. When they arrive at the basal side of the epithelium, they transform into oocysts. The oocysts amplify through mitosis to increase the amount of parasites, which is called sporogony. Thousands of mature haploid sporozoites are packed in every oocyst before it ruptures. When this happens, the sporozoites then travel to

the end station in the mosquito, the salivary glands. Here they accumulate and wait for the mosquito to feed on a human again. During feeding, a small proportion, typically fewer than 100, of the sporozoites are injected (Baton and Ranford-Cartwright 2005) starting the cycle all over again.

History and interventions

The association between human hosts and malaria parasites has existed for thousands of years. *P. falciparum* originated in Africa and hypotheses supported to some extent by bioinformatics studies of genetic variation in the parasites (Hartl 2004) suggest that about 10,000 years ago populations expanded rapidly in Africa and spread worldwide together with increased human populations and migration (Enayati and Hemingway 2010; Hay et al. 2004). References to almost certainly cases of malaria occur through history, from Chinese documents from 2700BC to Mesopotamian clay tablets to Egyptian papyri and Hindu texts all from BC. The malaria parasites, however, were not discovered until the year 1880 when Alphonse Laveran found them in human blood of malaria patients. After this, in 1897, the transmission cycle involving mosquitoes in bird malaria was discovered by Ronald Ross. A year later Grassi, Bignami and Bastienelli demonstrated that human malaria parasites are also transmitted by mosquitoes (Cox 2010). The distribution of malaria before any interventions started, around 1900, reached latitudes of 64° north and 32° south (Fig. 2). Since then, human intervention methods to control malaria have decreased its distribution. The progress in the 20th century in reducing the area with risk of human malaria is seen in Fig. 2. The risk areas have decreased by about half from 1900-2002, from 53% to 27% of the land surface of the Earth.

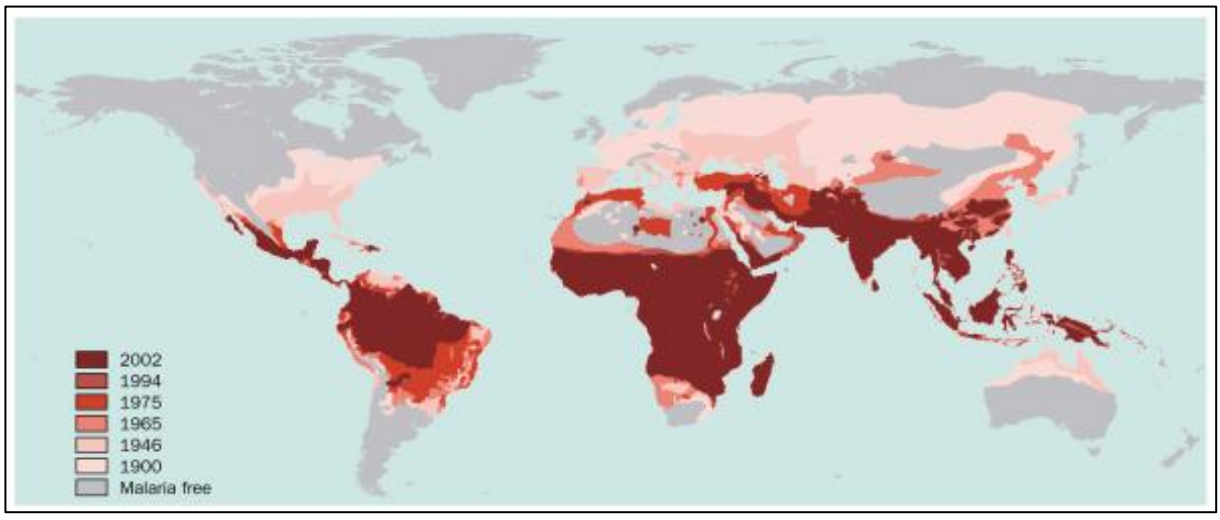

Figure 2. *World malaria distribution from 1900-2002 (Hay et al. 2004).*

Control efforts have been most successful in areas with low endemicity rates while it is increasingly difficult to control malaria in areas with high transmission (Hay et al. 2004)

Endemicity is defined by the proportion of a population that carries asexual parasites in their blood (Enayati and Hemingway 2010). During 1900-2002, the global population at risk has decreased from 77% to 48%. However, the population growth during the same period has been rapid and the number of people at risk has increased from about 0.9 to 3 billion (Hay et al. 2004). After the discovery of the transmission of malaria, interventions focused on environmental control of mosquito breeding-sites. This proved to be very successful in many places and environmental control was the main reason for the reduction in the global malaria distribution till 1946. In the 1940s the discovery of the insecticide properties of dichlorodiphenyltrichloroethane (DDT) enabled control on large scales (Hay et al. 2004). DDT was used (and is still being used in some places) to spray inside houses on the walls to kill indoor resting adult mosquitoes and in contrast to other insecticides only needed to be applied once every sixth month or year (Najera et al. 2011). The WHO Global Malaria Eradication Programme (1955-1969) supported the use of DDT and chemoprofylactic drugs to eradicate malaria. This was successful in many regions where malaria was eliminated, however sub-Saharan Africa was not included in the success (Hay et al. 2004). Also, the widespread use of DDT led to the first appearance of mosquito resistance in 1951 in Greece and in 1960 it was confirmed that parasites had developed resistance to the drug chloroquine. Fourteen years after the start of the Global Malaria Eradication Programme, it had to be recognised that eradication was not possible in all countries in the short term (Najera et al. 2011). During the 1970s and 1980s the funding for malaria control decreased and antimalarial drug resistance increased. However, more focus on research and development of malaria control also occurred at this time (Najera et al. 2011). In 1998, the Roll Back Malaria initiative was established with the aim of halving the global malaria risk, morbidity, and mortality by 2010 focusing on four main targets: 60% of children and pregnant women should have insecticide treated nets, 60% of malaria cases should get treatment within 24h of onset of symptoms, 60% of pregnant women should get an effective dose of an antimalarial drug at routine antenatal care visits weather or not infected with malaria (intermittent presumptive therapy), and 60% of epidemics should be detected within 2 weeks of onset and then within a further 2 weeks responded to appropriately (Hay et al. 2004). The aim of eradicating malaria was again brought up on the agenda when Bill and Melinda Gates called for it at a malaria forum in 2007 (Enayati and Hemingway 2010). Progress in the reduction of malaria incident and mortality rates has taken place in the 21st century. If the incidence and mortality rate had remained at the levels they were at in 2000, 500 million more cases and 3.3 million more deaths would have occurred between 2001 and 2012 (WHO 2013).

Control methods today and in the future

Current situation

Malaria control methods can be divided into two main strategies, prevention and treatment. Prevention includes approaches such as vector control, drug prophylaxis, and vaccines, while treatment includes drugs and blood transfusions for example. Different drugs are and have been successfully used through the years to treat malaria caused by the different parasites. However, the problem of drug resistance in the parasites is always an issue. At the moment the use of artemisinin-based combination therapies are supported by the WHO (Enayati and Hemingway 2010). Today there is no licensed malaria vaccine in commercial production, although there might soon be. The vaccine RTS,S/AS01 is at the end of phase 3 trial and has shown that in infants 6-12 weeks old and in children 5-17 months old in seven African

countries it provided protection against both clinical and severe malaria. After 12 months since the first vaccine dose the reduction in clinical malaria was about 50% for 5-17 months old children and about 30% for 6-12 weeks infants (Agnandji et al. 2011; Agnandji et al. 2012). The vaccine targets *P. falciparum* and is made up of a fusion protein (RTS) of a malaria antigen with hepatitis B surface antigen in a multi-component adjuvant (AS01) co-expressed with large amounts of unfused hepatitis B surface antigen (Hill 2011).

Vector control

The area of vector control has contributed to a lot of the control of malaria and successful eradication in the past as said above. Vector control includes chemical- and biological control and environmental management. The most common vector control methods at the moment are indoor residual spraying (IRS) and insecticide-treated nets (ITNs), and the development of long-lasting insecticidal nets (LLIN) that do not need to be retreated to keep its insecticide activity. IRS is the application of insecticides to the inside of walls and roofs to kill resting adult female mosquitoes reducing lifespan and thereby reducing vector density. ITNs decrease human-mosquito contact and kill mosquitoes and provide >70% protection compared to having no net and they are cost effective and simpler than IRS. For ITNs only pyrethroid insecticides are recommended due to their low mammalian toxicity together with residual activity (Enayati and Hemingway 2010; Raghavendra et al. 2011). Chemicals against mosquito larvae can also be used to eliminate or reduce the vector population. However, it requires a high coverage of breeding sites for a significant impact, which is operationally and logistically challenging (Enayati and Hemingway 2010; Raghavendra et al. 2011). Drug resistance in the malaria parasites and insecticide resistance in the mosquitoes pose major problems towards current malaria control. The insecticides used for IRS and LLINs have been in use for many years and pyrethroid- and DDT resistance is found in different places. Also, the first cases of artemisinin resistance have been reported from Cambodia in the same area as where resistance against chloroquine and pyrimethamine also emerged (Dondorp et al. 2010). Therefore new methods for controlling and eliminating malaria need to be and are being developed.

Many non-chemical control methods have also been proposed for larvae and adult mosquitoes. For example controlling larvae with predators like fish and using bacteria and fungi as larvicides and viruses against adults. These should all be species-specific, non-toxic to humans and easy to distribute. Environmental management refers to modifications to vector habitats to reduce larval development and/or human-mosquito contact. These techniques include for example changing flows of water and building houses with corrugated iron roofs rather than with plant material (Raghavendra et al. 2011).

Transgenesis

Other suggested approaches involve genetic modifications. One way of genetic modification is transgenesis. Here, transgenesis involves direct genetic transformation of mosquito vectors where a gene is inserted into the vector genome and subsequently expressed to inhibit pathogen development in the vector (Coutinho-Abreu et al. 2010). There are some basic requirements for this to be achieved. First, a method for introduction of foreign genes into the

germ line must be available. Second, there must be a suitable promotor to drive the transgene expression in the appropriate tissue and at the right time. Third, there must be appropriate gene products that can interfere with development of the parasite identified. Finally, there should be no fitness loss for the mosquito induced by the transgene (Jacobs-Lorena 2003). Some transgenic mosquitoes have been shown to prevent pathogen development by expression of molecules that impair the survival of the pathogens (Coutinho-Abreu et al. 2010). To begin with, proof-of-principle laboratory experiments showing anti-*Plasmodium* resistance in transgenic *Anopheles* were performed but only for non-human malaria parasites. Now there are also studies showing impaired transmission of *P. falciparum*. For example, Smith et al. (2013) have shown that transgenic *Anopheles stephensi* expressing a catalytically inactive mutant phospholipase A_2 can significantly impair the development of both rodent and human malaria parasites. These also had a fitness advantage when fed blood infected with *P. falciparum* (Smith et al. 2013). Also using *A. stephensi* and *P. falciparum*, Corby-Harris et al. (2010) showed that expression of a gene encoding a protein kinase (Akt) dramatically reduces parasite development and at the same time reduces the average mosquito lifespan (Corby-Harris et al. 2010). Studies show that it is possible to reduce transmission of *Plasmodium* by genetic modification of the mosquito. However, there are challenges to this approach. For example, transgenic mosquitoes in the wild should not experience any reduced fitness compared to natural populations (Coutinho-Abreu et al. 2010), few of the large number of *Anopheles* species able to transmit malaria have been shown possible to genetically manipulate and they exist as reproductively isolated populations preventing gene flow (Wang and Jacobs-Lorena 2013). Also, the issue of how transgenes should be introduced into wild populations (Jacobs-Lorena 2003) is something that needs to be solved.

Paratransgenesis

Another genetic approach to reduce transmission of malaria is to transform mosquito symbiotic bacteria or other microorganisms to deliver anti-*Plasmodium* molecules within them to interfere with development and transmission. Bacteria are potentially easier to introduce into mosquito populations than transgenes and they can bypass the problem of reproductively isolated mosquito populations that are common in endemic areas by being transmitted through contact and not through genes. In addition, genetically modified bacteria can be produced cheaply in large quantities even in low-income countries (Ricci et al. 2011). This strategy of transforming symbionts for the production of anti-pathogen effector molecules is called paratransgenesis and this has been demonstrated in other insects such as the bug that transmits Chagas disease (Beard et al. 2002). During parasite development in the mosquito, there are a number of bottlenecks to overcome (Abraham and Jacobs-Lorena 2004; Ghosh et al. 2000). From about 10,000 gametocytes taken up in a blood meal less than 10 oocysts will be formed (Riehle et al. 2007). The parasite development is at its most vulnerable stage in the mosquito midgut providing a good place for intervention. In the midgut, the parasites share the same location with the mosquito microbiota, which increases by 100-1000-fold when the parasites enter the midgut with a blood meal. This means that potentially a large number of effector molecules could be released at the time as the parasites are at their most vulnerable stage (Wang and Jacobs-Lorena 2013). Not only bacteria, but other microorganisms as well, fungi and viruses, have been suggested for use in paratransgenesis. For example, Fang et al. (2011) showed that an entomopathogenic fungus could be transformed to produce anti-*Plasmodium* molecules in *An. gambiae* that could block transmission of *P. falciparum* and Ren et al. (2008) showed that a densovirus could express

an exogenous gene in *An. gambiae* midgut, fat body and ovaries and was vertically transmitted.

Paratransgenesis in *Anopheles*

In *Anopheles*, paratransgenesis was first described by Yoshida et al. (2001) who showed that a genetically transformed *Escherichia coli* expressing a single-chain immunotoxin could block *P. berghei* oocyst development in *An. stephensi* by 58% and significantly reduce the oocyst number from a mean of 45.4 to 8.6 oocysts per gut (Yoshida et al. 2001). Riehle et al. (2007) also genetically engineered *E. coli* to express molecules (SM1 and PLA2) in *An. stephensi* that inhibited *P. berghei*, leading to a reduction in oocyst numbers by 41% and 23%, respectively. However, *E. coli* survived poorly in mosquitoes and instead *Pantoea agglomerans* was selected for survival in *An. gambiae* (Riehle et al. 2007). Wang et al. (2012) used *P. agglomerans* to deliver different kinds of effector molecules in *An. stephensi* and *An. gambiae* midguts and looked at the effect on *P. berghei* and *P. falciparum*. This paratransgenesis inhibited parasite development by up to 98% and the prevalence decreased by up to 84% for two of the effector molecules expressed, scorpine and $(EPIP)_4$. Also, the number of bacteria increased more than 200 times during the first two days after a blood meal and there was no negative effect on the longevity of the mosquitoes in the laboratory. However, an important issue to address is the development of parasite resistance to the anti-*Plasmodium* effector molecule similar to that of drugs against the blood-stage parasites. A solution could be a combination of multiple effector molecules with different modes of action, which will also increase the effectiveness of the interference (Wang and Jacobs-Lorena 2013).

Thorsellia

A bacterium with potential for use in paratransgenesis is *Thorsellia*. These bacteria are so far only found in vector mosquitoes and their breeding waters, which is a positive characteristic for paratransgenesis. It was first isolated in *An. arabiensis* from Kenya (Lindh et al. 2005). This bacterium was phylogenetically placed within the *γ-Proteobacteria* class but outside all families and orders within. It was later given its name *Thorsellia anophelis* and suggested to be a new species belonging to the *γ-Proteobacteria* (Kampfer et al. 2006). *Thorsellia* has since then been shown to be the dominant species in adult *An. gambiae* s.l and also found in the surface microlayer water from breeding sites in rice paddies in Kenya. It was shown that it can utilize blood to enhance growth and it can tolerate alkaline environments (pH 9.1) and displays α-hemolysis (Briones et al. 2008). Also, in another study *T. anophelis* was the dominant bacterium in 1-day old none-fed adult *An. gambiae* in Kenya (Wang et al. 2011). *T. anophelis* has also been found in *An. culicifacies* larvae from Iran (Chavshin et al. 2014), in *An. darlingi* in Brazil (unpublished) and in *An. stephensi* from India (Rani et al. 2009). In another recent study *Thorsellia* was found in *Culex tarsalis* (Duguma et al. 2013). This was the first study to show that *Thorsellia* is found in other species than *Anopheles*. Since then *Thorsellia* sequences have also been found in *Aedes* mosquitoes (unpublished results). Together these findings suggest that *Thorsellia* could be well established in mosquito guts and in particular in vectors of human disease and a possible candidate for paratransgenesis.

Anopheles midgut microbiota

In order to find suitable bacteria for use in paratransgenesis the microbial communities in mosquito midguts need to be studied. Since the pioneering study by Jadin et al. (1966) the microbiota of mosquitoes was not studied much until about 15-20 years ago (Ricci et al. 2011).

Culture-dependent methods

Two early studies on *Anopheles* midgut bacteria and the effects on *Plasmodium* were carried out in the mid-1960s (Jadin et al. 1966; Jadin 1967). After that, studies investigating the *Anopheles* microbiota appear in the 1990s. These studies used culture-dependent methods to look at the midgut microbiota in laboratory-reared *An. stephensi* (Pumpuni et al. 1993), in laboratory reared *An. stephensi*, *An. gambiae* and *An. albimanus* (Pumpuni et al. 1996), in *An. funestus* and *An. gambiae* s.l. from Kenya and *An. gambiae* s.l. from Mali (Straif et al. 1998) and in insectary and field-caught *An. albimanus* from Mexico (Gonzalez-Ceron et al. 2003). Pumpuni et al. (1993) identified three genera, Straif et al (1998) identified twenty genera and Gonzales-Ceron et al. (2003) identified two genera in field-collected but no bacteria in insectary mosquitoes. Pumpuni et al. (1996) showed that the proportion of newly emerged adults harbouring bacteria differed between the *Anopheles* species, from 17-90%. Three bacterial species were common to all the *Anopheles* species. Two studies also looked at the effect of Gram-negative bacteria on *Plasmodium*. Pumpuni et al. (1993) showed that all four strains tested inhibited oocyst formation while Straif et al. (1998) found no association between Gram-negative midgut bacteria and *P. falciparum* sporozoites in *An. gambiae* or *An. funestus*.

Culture-dependent methods and DNA-sequencing methods

After these reports, came studies that also used culture-independent methods using only DNA sequences to look at the *Anopheles* midgut bacteria and using 16S rRNA gene sequences to identify bacterial colonies. The first was by Lindh et al. (2005) that investigated *An. gambiae* s.l. and *An. funestus* from Kenya by both culture-dependent- and culture-independent methods and identified seven genera by each method (Lindh et al. 2005). In a study on three *An. stephensi* from an insectary in Italy 16S rRNA gene libraries from total DNA of the abdomens showed that 90% of the clones belonged to the genus *Asaia* and thus was the dominant genus out of four genera. In the same study, 16S rRNA gene libraries were also obtained from three *An. gambiae* field-collected in Burkina Faso and three *An. maculipennis* field-collected in Italy. In *An. gambiae* seven genera were identified with *Sphingomonas* as the most common with 30% of the sequences and in *An. maculipennis* three genera were identified with *Serratia* as the most common with 50% of the sequences (Favia et al. 2007). In Brazil, nine *An. darlingi* females were captured and DNA extracted yielding 56 16S rRNA sequences with the majority belonging to the family *Enterobacteriaceae* (Terenius et al. 2008). In Kenya, *An. gambiae* s.l. and their breeding water in rice paddies were DNA extracted to look at the bacterial content. The result as mentioned above showed that *Thorsellia anophelis* was the dominant bacterium in adult mosquitoes and it was also found in the breeding waters suggesting acquisition from the water and persistence through metamorphosis into adult

mosquitoes (Briones et al. 2008). In India, lab and field collected *An. stephensi* were analysed by both culture and culture-independent methods. This showed that bacterial diversity was higher in the field (53 genera) than in the laboratory (15 genera). It was also higher in females than in males and in larvae than in adults. A total of 215 operational taxonomic units (OTUs) were identified at 97%. This means that the 16S rRNA gene sequences were grouped into clone clusters with its nearest neighbours shown by BLASTn and if the sequences were more than 97% identical they were said to belong to the same OTU (Rani et al. 2009). In a study on only laboratory-reared adult female *An. gambiae* by culture assay, it was shown that there is a great variation in both load and composition of the microbiota between individuals and generations of the same laboratory colony. Most abundant was *Chryseobacterium meningosepticum* (Dong et al. 2009). Another study, from Iran, looked at both *An. stephensi* and *An. maculipennis* from the laboratory and the field plus breeding water. Using culture methods they isolated 13 bacterial species from all samples (42 adults, 22 larvae and breeding water). Bacteria were only detected in a low percentage of the mosquitoes and no significant correlation was seen between the bacterial species and the *Anopheles* species or the breeding site (Dinparast Djadid et al. 2011). In a study with 100 wild larvae and 96 wild female adult *An. stephensi* from two locations in Iran, 40 bacterial species belonging to 12 genera were isolated from larvae and 25 species in 5 genera were isolated from adults. The larvae contained almost twice the number of species as adults. Five of twelve genera were found in both regions, coastal and hilly. *Pseudomonas* species were the most common in both larvae and adults (Chavshin et al. 2012). The microbial diversity during development and with different food sources was investigated in laboratory-reared *An. stephensi* by Ngwa et al. (2013). They showed that the eggs, larvae and water had the highest diversity and sugar, uninfected blood, *P. falciparum* infected blood and *P. berghei* infected blood gave similar denaturing gradient gel electrophoresis pattern. 36 16S rRNA gene sequences were identified in total and nine were identified as *Elizabethkingia meningoseptica* that was found in all developmental stages and all dietary conditions (Ngwa et al. 2013). In another culture-dependent study midgut bacteria in 227 field-collected *An. gambiae* from four locations in Cameroon were investigated (Tchioffo et al. 2013). The bacteria were isolated on an agar particularly good for *Enterobacteriaceae*. The number of good quality 16S rRNA gene sequences obtained was 464. The most common were the families *Enterobacteriaceae*, *Pseudomonas* and *Aeromonas*. The 16S rRNA sequences were aligned with sequences from other insect vectors showing that some genera were more related to strains isolated from Cameroon than from other *Anopheles*, while other genera were more related to strains in other *Anopheles* from more distant areas. Also the abundance of the different bacterial genera in the mosquitoes differed between the four locations (Tchioffo et al. 2013). The first study of the microbiota in wild *An. culicifacies* looked at 68 larvae and 34 females in three biotopes in Iran. Culture methods resulted in 57 isolates belonging to 12 genera and 19 species with diversity varying with mosquito stage and area. *Pseudomonas* was most common and identical *Pseudomonas* 16S rRNA gene sequences were found in mosquitoes from areas 45 km apart (Chavshin et al. 2014).

Next-generation sequencing methods

The development of next-generation sequencing is now further increasing our knowledge by making it possible to get a larger picture of the microbial communities within the mosquitoes with the generation of thousands of DNA sequences from one individual. Three studies have looked at the *Anopheles* midgut bacteria using pyrosequencing, Wang et al. (2011), Boissiere

et al. (2012) and Osei-Poku et al. (2012). Wang et al. (2011) looked at the gut microbiota across the life history of *An. gambiae* in semi-natural habitats in Kenya. More than 650,000 sequences were obtained from 27 samples and 426,324 sequences were analysed to give 138 families and 337 genera. The OTU diversity was highest in the breeding water and then decreased in the order larvae, pupae, sugar-fed females and blood-fed females. A bloodmeal decreased the diversity but increased the bacterial load and favoured the family *Enterobacteriaceae* that possess large genetic redox capacity that helps to cope with the blood meal catabolism. Also, laboratory and field adult mosquitoes showed similar gut communities (Wang et al. 2011). Boissiere et al. (2012) investigated midgut microbiota in *An. gambiae* collected in two locations in Cameroon and reared the wild larvae to adults in an insectary. Gut bacteria in *P. falciparum*-positive and -negative mosquitoes resulted in 3,000-10,000 reads per gut. It was shown that 20 genera were shared by 80% of the individuals but the majority of species were found only in a few mosquitoes, a great richness was found. However, only a few dominant species were identified as only 28 genera were found to be present in at least one gut by more than 1%. The bacterial content of adult mosquitoes differed according to breeding site suggesting that the native aquatic breeding source determines the composition. Field and laboratory mosquitoes were also compared and in this study they differed drastically with a loss of diversity in the laboratory mosquitoes which contained 96% *Flavobacteria* while field collected contained 94% *Proteobacteria*. This study also indicated that *Enterobacteriaceae* correlated with *Plasmodium* infection (Boissiere et al. 2012), however this was later shown to be the opposite in another study by the same group (Tchioffo et al. 2013). In a study by Osei-Poku et al. (2012) eight different mosquito species of non-bloodfed females in Kenya including *Anopheles*, *Aedes*, *Culex* and *Mansonia* were investigated for gut microbiota by pyrosequencing. Almost 50,000 sequences were obtained and 33,757 sequences were analysed giving 789 OTUs with 53 OTUs comprising more than 1% in any gut. The median was 42 OTUs/gut though 1 OTU typically made up two-thirds, the gut microbial diversity was low. 144 genera were identified with *Aeromonas* as the most common in all species. 22 genera made up more than 1% in at least one individual and *Asaia* was found in all species. Different mosquito species had similar gut bacteria but individuals in a population had very variable bacteria. The sampling location showed no significant correlation with gut composition (Osei-Poku et al. 2012).

Effect on *Plasmodium*

That the microbiota of *Anopheles* plays a part in the transmission of malaria has been shown by studies that used antibiotics to sterilize the guts of the mosquitoes and then feeding them with *Plasmodium*-infected blood with or without bacteria. Gonzales-Ceron et al. (2003) investigated *P. vivax* in *An. albimanus* and saw that oocysts decreased with addition of bacteria and that *Serratia marcescens* was the most effective (Gonzalez-Ceron et al. 2003). Dong et al. (2009) also showed that aseptic mosquitoes of *An. gambiae* were more susceptible to *P. falciparum* infection through basal immune activity (Dong et al. 2009). The effect of the microbe community on *An. stephensi* fitness in the form of longevity, blood-meal digestion, egg laying, body size and mating was investigated by Sharma et al. (2013). Gut sterilization reduced the lifespan by 5 days for males and by 2 days for females, blood-meal digestion was reduced as was egg laying. Body size and mating was not affected. Also, the *P. vinckei petteri* oocyst prevalence and intensity were measured and both decreased when fed bacteria (Sharma et al. 2013). Tchioffo et al. (2013) compared the role of seven different bacteria (*E. coli, S. marcescens, Pseudomonas stutzeri, Enterobacter spp. Acinetobacter septicus, Comamonas*

spp. and *Bacillus pumilis*) on *P. falciparum* transmission in *An. gambiae*. All but *A. septicus* significantly reduced the prevalence and the intensity of oocysts. *E. coli, S. marcescens,* and *Pseudomonas* had the most impact (Tchioffo et al. 2013). Bahia et al. (2014) also studied the effect of midgut microbiota on *P. falciparum* development *in vitro* and *in vivo* and *An. gambiae* survival. Seven bacterial species were individually introduced into sterile guts in mosquitoes that were later fed with *P. falciparum* infected blood. All seven bacteria species reduced the oocyst load but to different extents. *S. marcescens* had the strongest anti-*Plasmodium* activity both *in vivo* and *in vitro* and it secreted a soluble factor against *Plasmodium* (Bahia et al. 2014).

Endosymbiotic *Wolbachia*

Another approach that has been proposed using bacteria in *Anopheles* to prevent the transmission of malaria involves the use of the bacteria *Wolbachia*. *Wolbachia* are endosymbiotic, intracellular, Gram-negative bacteria that were first described within the reproductive tissues of the mosquito *Culex pipiens* in 1924 (Walker and Moreira 2011). These bacteria cannot be cultured outside of host cells and belong to the *α-Proteobacteria* and are members of the family *Rickettsiales*. Together all *Wolbachia* make up a monophyletic group (Bourtzis et al. 2014). *Wolbachia* is clustered into supergroups based on genetic similarity and tend to associate with particular host classes. The supergroups are named A, B, C, D, E, F and H and they can be further divided into strains with names based on their host species (LePage and Bordenstein 2013). Supergroups A and B are the only supergroups known to infect mosquitoes. *Wolbachia* infect many species of arthropods and filarial nematodes and due to the very large number of species belonging to arthropods it makes *Wolbachia* very widespread from a biodiversity perspective (LePage and Bordenstein 2013). The bacteria are vertically and maternally transmitted through the egg cytoplasm and manipulate the host reproduction in order to enhance their spread by, for example, cytoplasmic incompatibility (CI), feminization, killing of male embryos and parthenogenesis (meaning asexual reproduction when embryos develop from unfertilized eggs) (Walker and Moreira 2011). These phenotypes increase the proportion of infected females in the host population, which increases transmission to the next generation. CI is the most studied host-manipulation and works by causing death in embryos formed by eggs from uninfected females and sperm from infected males while the opposite cross and crosses between females and males with the same *Wolbachia* strain infection are viable. This creates a fitness advantage for infected females compared to non-infected females (LePage and Bordenstein 2013). The discovery that some *Wolbachia* strains increase the resistance against infectious agents causing diseases like dengue, yellow fever, Chikungunya and malaria and also shorten the adult lifespan of the host, which means that the virus/parasite does not have time to develop, makes it an interesting field of study (LePage and Bordenstein 2013; Walker and Moreira 2011).

Reduction of disease transmission

Two strategies based on *Wolbachia* for the reduction of disease transmission are an incompatible insect technique and a population replacement strategy. The incompatible insect technique involves releasing *Wolbachia*-infected males that induce CI. This will lead to sterilization of a large proportion of the females reducing the overall number of vectors

(LePage and Bordenstein 2013). By releasing only infected males, infected *Anopheles* will not feed on humans, as it is only females who do that. Compared to other methods that reduce insect populations, such as insecticides, this method has the potential to affect parts of the vector population otherwise not reached that can cause quick population recovery when the treatment is stopped. This "self–delivering" system could reach mosquitoes that might not come in contact with insecticides (Slatko et al. 2014). However, infected males need to be repeatedly introduced like insecticides to prevent recovery of mosquito populations. The population replacement strategy involves releasing *Wolbachia*-infected male and female mosquitoes that have increased resistance to the pathogen and will through CI replace the local uninfected population (LePage and Bordenstein 2013). This transmission works like a rapid self-spreading method of *Wolbachia* into natural populations from a relatively small number of released infected mosquitoes (Walker and Moreira 2011). This strategy was tested for the prevention of dengue in 2011 at two locations in Australia through the Elimination Dengue Program by the release of *Wolbachia*-infected *Aedes* mosquitoes, 10,000-22,000 per week for ten weeks. This proved to replace the native population with one that was dengue-free (Hoffmann et al. 2011; LePage and Bordenstein 2013). Another release of *Wolbachia*-infected mosquitoes was carried out through the same Elimination Dengue Program in Brazil in September 2014 (http://www.eliminatedengue.com/brazil/progress/article/343). This progress with *Aedes* mosquitoes provides hope for the use in *Anopheles* mosquitoes as well.

Infection of *Anopheles* and effect on *Plasmodium*

An issue with the use of *Wolbachia* in malaria control has been that *Anopheles* species have not been shown to harbour natural *Wolbachia* infections. However, recently a study reported evidence of this in *An. gambiae* (M and S molecular forms) in field populations in Burkina Faso (Baldini et al. 2014). They identified *Wolbachia* in female- and male reproductive organs and in larvae in two seasons by 16S rRNA gene sequencing and determined that they were maternally transmitted in the laboratory. The frequency of infection was 10.8% in all samples, 12.8% in M samples and 4.2% in S samples. They also concluded that the strain identified belongs to a phylogenetic supergroup different from the arthropod-associated A and B supergroups, potentially *Anopheles* specific (Baldini et al. 2014). However, we have found that this is not correct and that their strains belong to supergroup A and B, not a new group (unpublished). Earlier studies have shown that somatic *Wolbachia* infections in *An. gambiae* can significantly reduce *P. falciparum* oocyst development (Hughes et al. 2011) and *P. berghei* oocyst numbers (Kambris et al. 2010) and a recent study showed that *Anopheles* mosquitoes could be stably infected with *Wolbachia* microinjected into eggs (Bian et al. 2013). The outcome from that study was an *An. stephensi* with few defects that caused CI and resistance to *P. falciparum* infection. The *Wolbachia* was also maternally transmitted to the next generation (Bian et al. 2013; LePage and Bordenstein 2013). However, there are studies that show the opposite, that *Wolbachia*-infected *Anopheles* can enhance *Plasmodium* development, for example *P. berghei* in *An. gambiae* (Hughes et al. 2014). This highlights the need for answering questions like what is the effect of *Wolbachia* on all the five *Plasmodium* species that infect humans and what kind of mechanisms cause interference and enhancement of *Plasmodium* by *Wolbachia*? Since many human malaria parasite species are transmitted by the same vectors and occur in the same place, the effects on all parasites need to be investigated (Hughes et al. 2014).

Conclusion

The need for new strategies to fight malaria is evident as the current methods of insecticides and drugs face the problem of resistance-development in both the malaria mosquitoes and the malaria parasites. One thing that has been shown is that the mosquito gut microbiota plays a role in the success of parasite transmission. This has the potential to be used in strategies to block transmission of malaria. The most vulnerable stage of *Plasmodium* development occurs in the midgut when the ookinetes try to cross the midgut epithelium after ingestion of a blood meal. At this point the bacterial load in the midgut also increases. The investigation of the microbiota of different malaria mosquitoes using both culture-dependent and culture-independent methods in the same study and in different studies have shown that different genera were identified by the two methods and in the different studies. This highlights that the two methods can yield different results and factors like the type of agar used for isolation of bacteria and the primers used for amplification of bacterial DNA, which have differed between studies, might influence which bacteria were identified. When the mosquito microbiota was first started to be investigated, studies showed detection of bacteria in only some of the mosquitoes and only a few species were identified. Now however, with developments in DNA-sequencing techniques this is no longer the case. The investigation of the microbiota of different malaria mosquitoes by culture independent methods using next-generation-sequencing has now made it possible to investigate and compare large numbers of bacterial DNA-sequences to get a more comprehensive picture of what there is. This information will enable comparisons between the microbiota in different *Anopheles* and other mosquito species and their breeding sites and in different geographical locations to find differences and similarities in diversity and composition. So far, studies using next-generation-sequencing have been carried out with some of the *Anopheles* species transmitting human malaria and in some countries, for example Kenya, Cameroon, Mexico, Brazil, India and Iran. Many different bacteria have been identified in these studies and the bacteria have differed in composition and load dependent on life stage, gender, food source, geographic location and laboratory or field collections. By using next generation sequencing thousands of DNA sequences from every individual sample analysed can rather quickly and cheaply be obtained, which identifies more species and shows an increased bacterial richness in the samples than with other methods. However, this new technique requires more knowledge in the field of bioinformatics to deal with the large quantities of data output. This information on midgut bacteria could then contribute to identification of a suitable candidate for paratransgenesis to prevent the transmission of malaria.

The approach of paratransgenesis has potential as bacteria can be introduced into mosquito populations rather easily and they can work around the problem of reproductively isolated mosquito populations, which is common in endemic areas. A bacterium could infect all the different mosquito species present while, for example, if transgenic mosquitoes were to be used many different transgenic mosquito species would be needed in order to target all the vectors. Genetically-modified bacteria can be produced at a low cost and in large amounts even in low-income countries and there are molecules that have been expressed showing inhibition of human malaria parasite development and decreased prevalence in vector mosquitoes. A potential bacterial-paratransgenesis candidate should be present in malaria mosquitoes and not be harmful to other organisms. Of specific interest is *Thorsellia* that has been found in different *Anopheles* species and in different places in the world. They have also only been found in vector mosquitoes and their breeding waters.

Another interesting bacterium is *Wolbachia* that has been proposed for malaria prevention by strategies of population replacement, incompatible insect technique and lifespan shortening of the vector mosquitoes. The success with *Aedes* using this technique, the recent stable infection of *Anopheles* with *Wolbachia* resistant to *P. falciparum* and the discovery of natural infection of *Anopheles* with *Wolbachia* are all positive developments in this field.

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