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Full Length Research Paper

A pilot study on isolation of *Asaia* and detecting its co-presence with *Plasmodium falciparum* in two major malaria vectors in Senegal

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The declining of malaria creates the opportunity to accelerate its elimination. However, the elimination program is threatened by the spread of insecticide resistance among *Anopheles* (*An.*) mosquitoes, stressing the urgent need for new vector control strategies. Recent evidence of stable infection of anopheles' symbionts that could affect the development of *Plasmodium* parasites within mosquitoes, has paved the way for the use of such organisms to target and control malaria vector populations. We have isolated a new *Asaia* strain from a wild population of *Anopheles gambiae* s.l. and, for the first time, from natural population of *Anopheles funestus*, and insectary colony of *Anopheles coluzzii*. The new anopheline strain has been named *Asaia (A.) aff. bogorensis* GD01 because of its close relationship with *A. aff. bogorensis* isolated from plant flowers. We highlighted an antagonistic effect of *A. aff. bogorensis* GD01 on the sporogonic development of *Plasmodium falciparum* within the two major malaria vectors (*An. gambiae* and *An. funestus*) in Senegal. The putative impact of *A. aff. bogorensis* GD01 infection on the *P. falciparum* sporogonic development offers an opportunity for new malaria vector control approaches that can be added to the limited arsenal required to fulfil the World Health Organization (WHO) recommendations for searching new tools to achieve malaria elimination goal. Although this is an innovative and promising malaria control tool, more investigations are required to better characterize *Asaia* vs. *Plasmodium* interactions, before any *Asaia*-based intervention. This is required to ensure their safe use as an alternative or complementary vector control strategy to achieve malaria elimination goal.

Key words: *Asaia*, *Plasmodium falciparum,* malaria, *Anopheles gambiae, Anopheles funestus*, Senegal.

INTRODUCTION

Among the arthropod-borne diseases affecting human, malaria is the primary global health problem with over than 600,000 annual deaths (World Health Organization [WHO], 2016). Developing countries of the WHO African

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region are the most affected, accounting for almost 90% of the global deaths (WHO, 2016). They also suffer a huge economic burden due to the disease (Sachs and Malaney, 2002). Nowadays, efforts to control malaria integrate preventive strategies, such as insecticide-based vector control interventions with rapid diagnostic testing and effective drug treatments. These efforts are enhanced by strengthening surveillance and healthcare systems and research infrastructure (Breman et al., 2004; WHO, 2017).

Despite the global progress in the fight against malaria recorded so far, recurrent problems, including the increase and the spread of resistance phenomena among vectors and parasites as well as the lack of effective vaccines, highlight the need for a new generation of malaria control tools. Genetically modifying mosquitoes that transmit malaria to become resistant to parasites has been successfully attempted (Grossman et al., 2001). However, genetic manipulation tends to reduce mosquito fitness and thus the chance to successfully spread genes of interest among natural populations (Favia et al., 2007).

Recent evidences of the stable natural infection by endosymbionts in human malaria vectors, which appears to affect the development of the *Plasmodium* parasites, has shown the potential for the use of such organisms to control population of malaria vectors (Baldini et al., 2014; Shaw et al., 2016).

Asaia species are facultative intracellular, Gram-negative, aerobic, rod-shaped and peritrichously flagellated *Alphaproteobacterium* from the *Acetobacteraceae* family, primarily isolated from the tropical flowers of *Bauhinia purpurea* and *Plumbago auriculata* (Katsura et al., 2001; Moore et al., 2002; Yamada et al., 2000; Yukphan et al., 2004). Later, several strains of the genus were described as extracellular endosymbiotic bacteria in the salivary glands, gut and the reproductive organs of both males and females of anopheline mosquitoes. Indeed, Favia et al. (2007) reported a laboratory reared *Anopheles stephensis* strain stably harbouring an acetic acid bacterium of the *Asaia* genus (Favia et al., 2007).

Furthermore, the location of these strains in the reproductive organs suggests their potential vertical and sexual transmissions to females' offspring and mating partners, respectively. Since some *Asaia* strains have been suspected to affect the development of *Plasmodium falciparum* within its invertebrate host (Favia et al., 2008; Pumpuni et al., 1996), they may be good candidates for use as novel malaria vector control tool in combination with, or as an alternative to the existing core interventions to increase the efficiency of malaria control and elimination programs. However, to successfully use such an alternative tool, better understanding the nature of interactions between *Anopheles* - *Asaia* - *P. falciparum* is critical.

In this study, we present the first data on a possible antagonistic effect of *Asaia aff. bogorensis* GD01 on the sporogonic cycle of *P. falciparum* within wild populations of two major malaria vectors (*Anopheles gambiae* and *Anopheles funestus*) in central and south-eastern Senegal and its high and stable presence in an *Anopheles coluzzii* insectary strain.

MATERIALS AND METHODS

Study sites

Wild populations of *An. funestus* and *An. gambiae* s.l. were collected using the pyrethroid spray method from four different Senegalese sites located in the two administrative regions of Kedougou and Fatick, about 500 km apart from each other. Dielmo and Ndiop, located in the Fatick region, which host one of the longest epidemiological cohort studies on malaria, are two Sudanese savannah villages in central Senegal, near the Gambian border (Trape et al., 2011). Kedougou, Bandafassi and Tomboronkoto were chosen close to the Malian and Guinean borders in the South-Eastern Senegal. The region belongs to the Sudano-Guinean savannah ecological zone and has the highest malaria incidence (PNLP, 2015).

Samples collection

A total of 1,184 females of wild anopheline populations were tested. Of these, 375 *An. funestus* and 362 *An. gambiae* s.l. were from Fatick. While Kedougou's sample included only 447 females of *An. gambiae* s.l. The same specimens were tested to detect both *P. falciparum* and/or *A. aff. bogorensis* GD01 infections. In addition to the wild anopheline populations, 23 males and 127 females of an *An. coluzzii* insectary colony, maintained in our laboratory in Dakar (Boudin et al., 2005) were included in the study to assess the frequency of the *A. aff. bogorensis* GD01 in the mass rearing conditions.

Isolation and cultivation of the new *Asaia* strain

An. gambiae mosquitoes collected during routine entomological surveillance in Dielmo during the summer of 2011 were used as isolation sources. Prior to isolation, 11 alive individual mosquitoes were washed in a 10% solution of a commercial disinfectant-detergent (Amphomousse, Hydenet S.A., Sainghin-en-Melantois, France), then rinsed with sterile water and placed in a 1% solution of sodium hypochlorite for 1 min. After the final rinse in sterile phosphate-buffered saline, mosquitoes were placed in a sterile 1.5 µl plastic tube and triturated with a sterile micro-pestle in 600 µl of MEM tissue culture medium. The shell-vial culture approach (Kelly et al., 1991) for intracellular bacteria was employed to isolate the new *Asaia* strain. Isolation material, consisting of 200 µl of the supernatant of crushed mosquitoes, was inoculated into three different cell line monolayers respectively made of *Rhipicephalus microplus* tick (BME/CTVM2), *Aedes albopictus* (C6/36) and *Xenopus laevis* (XTC2) cells, then incubated at 28°C and 5% CO_2 atmosphere and checked every day to assess microbial growth. The catalase and oxidase activities were determinate as previously (Holt et al., 1994). The supernatant of the C6/36 infected cells, the only cell line producing intracellular gram-negative bacteria growth at day 7 post-inoculation, was collected and streaked on a 5% sheep's blood-enriched Columbia agar (BioMérieux) at 28°C and 5% CO_2 atmosphere. Microbial growth of the isolate occurred the 3rd day post-cultivation. Regarding molecular characterization, the

grown bacteria DNA was extracted, amplified and the 16S rDNA gene sequenced.

Molecular analyses

DNA extraction

Microbial genomic DNA was extracted from the whole body of individual mosquitoes using the BioRobot EZ1 System with the EZ1 DNA tissue kit (Qiagen) following the manufacturer's instructions.

Endosymbionts and Plasmodium detection

Taqman® qPCR methods with custom designed primers and probe sets were used to screen samples for the presence of the *A. aff. bogorensis* GD01 targeting the *rpoB* gene (Fwd: 5'- GAC GCC AAG GAC CTG ATC TA -3'; Rev: 5'- ATA GGC CAG GAT TTC GTC CT -3'; Probe: 6-FAM- GGT CAC GAC CCT GCT CTA TG-TAMRA); and/or *P. falciparum* targeting the *pfEMP1* gene (Fwd: 5'- GGA CAT AAT AAA AGG TTT TTC TTC CA -3'; Rev: 5'-CAA AAT ACA CAA AAT ACA GAA CCA AA -3'; Probe: 6-FAM-CAT TAT GAT GTG ACG TGG TAG GAT GGG-TAMRA). All PCR reactions were performed in a total reaction volume of 20 µl, which contain 50 ng of genomic DNA, 10 µl of master mix Takyon® (Eurogentec, Liège, Belgium), 0.2 µM of each primers and probes, completed with nuclease free water. PCR conditions were set in the real time CFX96 Touch detection system BioRad thermal cyclers with one cycle of 5 min at 95°C, followed by 40 cycles of 10 s at 95°C and 45 s at 60°C. The sensitivity of both qPCR methods was checked using a serial of dilution cascade up to 10^{-3} of the positive controls of both microorganisms. The tests were performed with 3 replicates to ensure the consistency of the PCR sensitivity. Negative and non-template controls were included during all PCR reactions to ensure the absence of contamination. Therefore, DNA extracted from laboratory colony served as negative control during *P. falciparum* detection; while two strains, of respectively *Alcaligenes* species GD03 isolated from *An. gambiae* s.l. from Dielmo and the *Escherichia coli* No. ATCC 8739 isolate, were used as negative control for *Asaia* detection. All samples with Ct value ≤35 cycles were recorded as positive and all positive were confirmed by another PCR.

***A. aff. bogorensis* GD01 16S rDNA sequencing and phylogenetic analysis**

Genomic DNA extracted from pure *A. aff. bogorensis* GD01 isolate was amplified to generate a nearly complete 16S rDNA sequence (1366 bp) using universal 16S rDNA primers fD1 and rp2 (Weisburg et al., 1991). Sequencing of the amplified PCR products were performed as previously described (Drancourt et al., 1997). Sequences were assembled and corrected using the ChromasPro 2.0.0 software version, then compared with reference sequences available in the GenBank using the server BLASTN version to identify closely related species and/or strains. To infer taxonomic relationships with existing isolates, the 16S rDNA sequence of *A. aff. bogorensis* GD01 were aligned against reference sequences retrieved from the GenBank database using the ClustalW multisequence alignment program (Thompson et al., 1994) in the BioEdit software (Hall et al., 1999). The ML phylogenetic tree was reconstructed using Topali v2.5 according to the Hasegawa-Kishino-Yano (HKY85) substitution model (Hasegawa et al., 1985) which includes the proportion of invariable sites and the Gamma distribution. The robustness of individual branches was estimated by bootstrapping with 100 replicates (Felsenstein et al., 1985).

Data analysis

Genus and species assignment levels were set respectively at 99 and 97% of sequence similarity between the *A. aff. bogorensis* GD01 isolate and GenBank reference sequences. The prevalence of each microorganism in each vector species was estimated as the proportion of specimens positive for the considered parasites. Given the dichotomous nature of the results of the *Plasmodium* and/or *Asaia* infections, the measures of association of *Asaia* and *Plasmodium* infections were estimated using tetrachoric correlation in the psych package in R; after binarization of the response (Positive = 1 and Negative + 0). The tetrachoric correlation estimator was chosen since the Pearson correlation (φ) for dichotomous data is reported to underestimate when applied to latent variables (Revelle et al., 2017). Data were compared with the Pearson Chi^2 or Fisher exact tests where applicable with the statistical significant threshold set at P value ≤ 0.05. All statistical analyses were performed using R software 3.3.2 version (R Core Team, 2016).

RESULTS

A. aff. bogorensis GD01 strain isolation

The GD01 strain was isolated from wild *An. gambiae* mosquitoes collected in Dielmo (Senegal). The new isolate is an aerobic Gram-negative, catalase-positive, oxidase-negative rod-shaped (Figure 1) and peritrichously flagellated bacterium. BLASTN query, based on the 16S rDNA gene sequences, proved that the closest related bacterial species to the GD01 belong to the *Alphaproteobacterium* phylum and especially to the Acetobacteraceae family and *Asaia* genus. Phylogenetic analysis showed that the new strain clustered with *A. bogorensis* reference strain (GenBank # AB682073) (Figure 2) and was therefore provisionally named *A. aff. bogorensis* GD01.

Prevalence of *A. aff. bogorensis* GD01 versus *P. falciparum* in mosquitoes

All PCRs analyses were validated without amplification of negative controls and amplification of positive controls. Overall, the *A. aff. bogorensis* GD01 carriage was significantly higher among the insectary specimens (60.00%, 90/150) compared to the wild anopheline specimens (5.06%, 60/1184) (χ^2 = 397.06, df = 1, p < 0.001) (Table 1). The *A. aff. bogorensis* GD01 infection was found both in wild populations of *An. gambiae* s.l. (14.36%, 52/362) and *An. funestus* (1.60%, 6/375) from Dielmo and Ndiop, where the carriage was significantly higher in *An. gambiae* s.l. (χ^2 = 39.651, df = 1, p < 0.001) (Figure 3). In south-eastern Senegal, where only *An. gambiae* s.l. samples from Bandafassi and Tombrokoto were tested, *Asaia* infection was less important with only 2 infected (0.45%) out of the 447 specimens tested (Figure 3). Moreover, as shown in Figure 4, the insectary females were more significantly infected than males

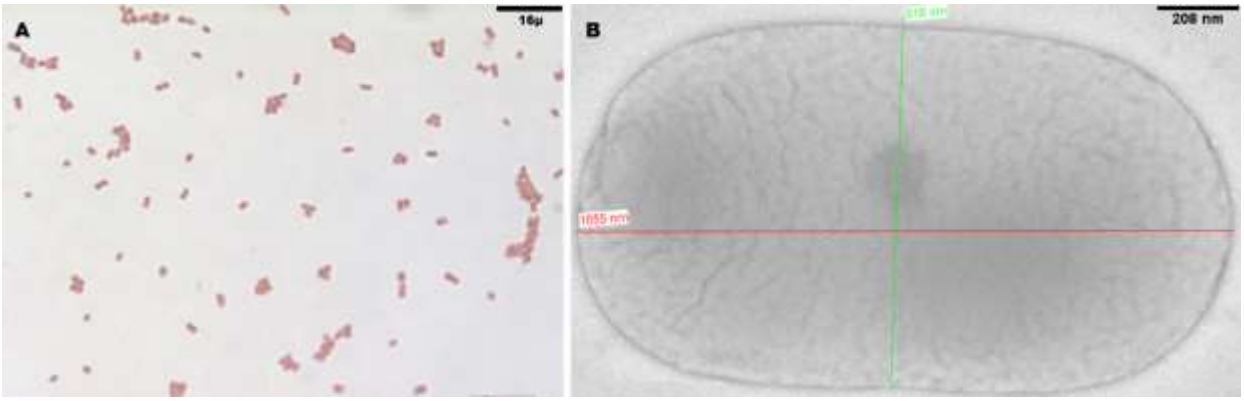

Figure 1. Gram-staining (A) and Transmission electron microscopy (B) of *Asaia aff. bogorensis* GD01.

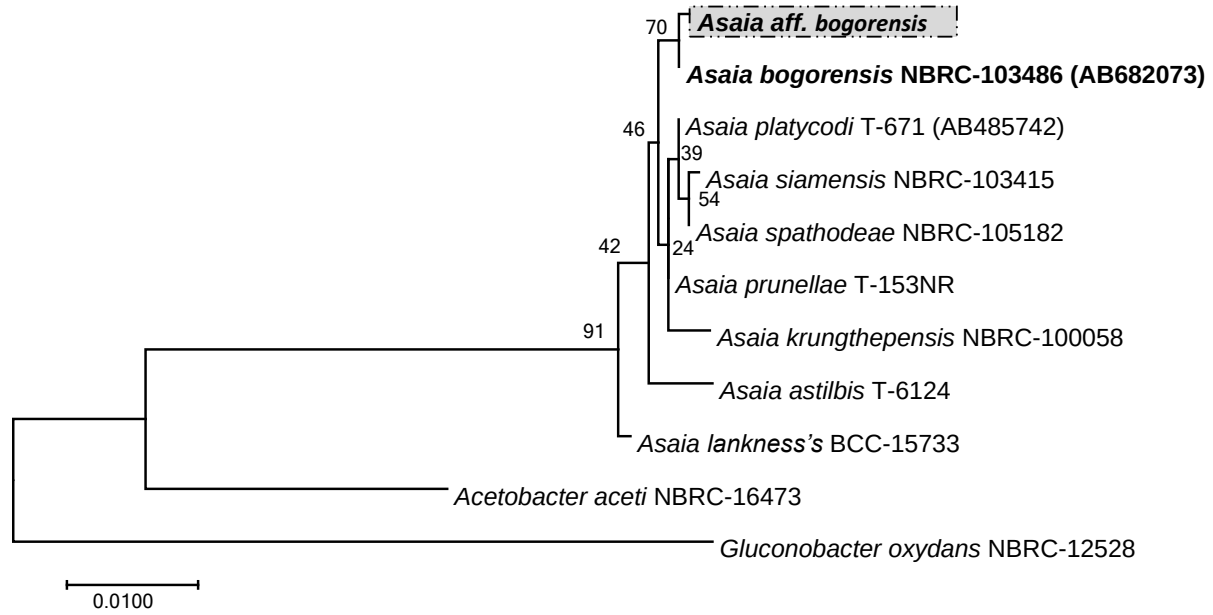

Figure 2. Phylogenetic tree highlighting (Gray box) the position of Asaia aff. bogorensis GD01 relative to others acetic acid bacteria of the Asaia genus, and Gluconobacter oxydans and Acetobacter aceti as out-groups. The accession numbers of the nucleotide sequences of the 16S rRNA gene retrieved from Genbank databases are presented in brackets

(χ^2 = 10.774, df = 1, p < 0.05). In Fatick, the *P. falciparum* carriage was significantly higher in *An. funestus* (5.87%) compared to *An. gambiae* s.l. (2.21%) (χ^2 = 5.4057, df = 1, p < 0.05), but was comparable (χ^2 = 0.1971, df = 1, p = 0.66) to the infection rate of *An. gambiae* s.l. (4.92%) in Kedougou (Table 1). Interestingly, none of the individuals tested was found to be co-infected with *A. aff. bogorensis* GD01 and *P. falciparum* in all malaria vector populations tested during the study period from both areas (Figure 5). Furthermore, the presence of *P. falciparum* was negatively correlated with *A. aff. bogorensis* GD01 (r_t = -0.42).

DISCUSSION

Despite unprecedented reduction of its incidence, malaria is still a major cause of human mortality and morbidity worldwide (WHO, 2017). One of the main control measures of the disease relies mainly on insecticide-based interventions (WHO, 2012). The two cores vector control tools to reduce malaria burden are the indoor residual spraying (IRS) and insecticide treated nets (ITNs) (WHO, 2017). The widespread expansion of these tools has reduced the global burden of malaria and renewed optimism about its elimination in some regions

Table 1. Prevalence of *Asaia aff. bogorensis* GD01 and *P. falciparum* in mosquitoes from the study sites and the insectary in Senegal.

Site	Mosquitoes species	Sex	Tested	P. falciparum (%)	*Asaia aff. bogorensis* GD01
Kedougou	*An. gambiae s.l.*	Female	447	22 (4.92)	2 (0.45)
Fatick	*An. funestus*	Female	375	22 (5.87)	6 (1.60)
	An. gambiae s.l.	Female	362	8 (2.21)	52 (14.36)
	Subtotal 1		737	30 (4.07)	58 (7.87)
Insectary	*An. coluzzii*	Male	23	-	8 (34.78)
		Female	127	-	82 (64.57)
	Subtotal 2	-	150	-	90 (60.00)
Total	-	-	1,334	-	150 (11.24)

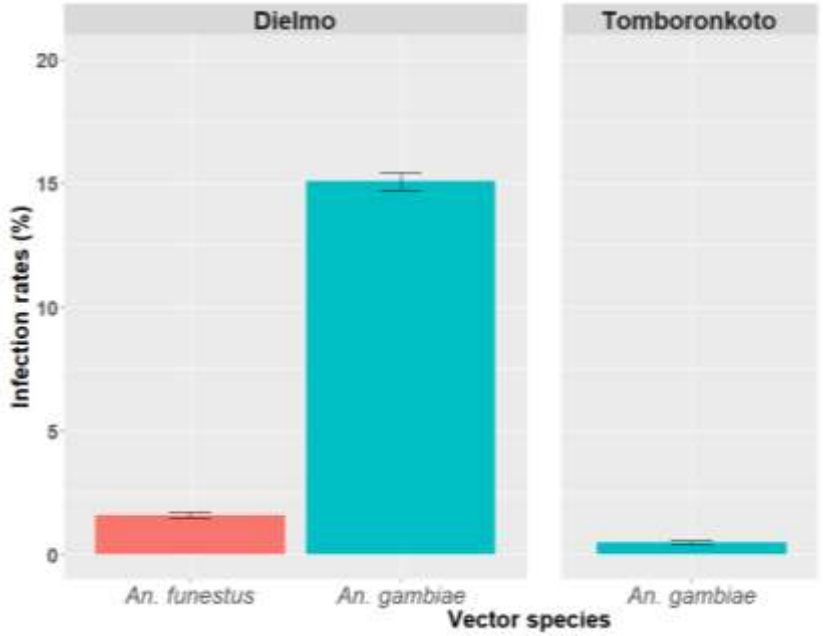

Figure 3. Prevalence of *Asaia aff. bogorensis* GD01 in wild populations of *An. gambiae s.l.* and *An. funestus* from Fatick and Kedougou (Senegal).

(Dieme et al., 2017). However, the spread of insecticide resistance is likely to threaten the effectiveness of current vector control interventions against malaria (WHO, 2014).

In addition to the physiological resistance, main vectors species have undergone behavioural avoidance of treated surfaces preventing them from being impacted by core vector control interventions, which target indoor resting/feeding populations, thus maintaining residual transmission (Killeen et al., 2013). Therefore, there is an increasing need to develop innovative control measures to tackle the issue of outdoor biting/resting strains as well as anopheline populations harbouring insecticide resistance gene and/or mechanisms (WHO, 2012). A promising control approach against malaria could be the use of symbiotic organisms, naturally and stably present in mosquito, to deliver anti-pathogen effector molecules (Mancini et al., 2016). Previous studies have demonstrated the potential of the acetic acid bacterium *Asaia* as one of the most promising mosquito symbionts for such an approach (Mancini et al., 2016). Bacteria of the genus *Asaia* are stably associated with and can quickly colonize numerous mosquito tissues, including malaria vectors such as *An. gambiae* and *An. stephensi* (Wilke and Marrelli, 2015).

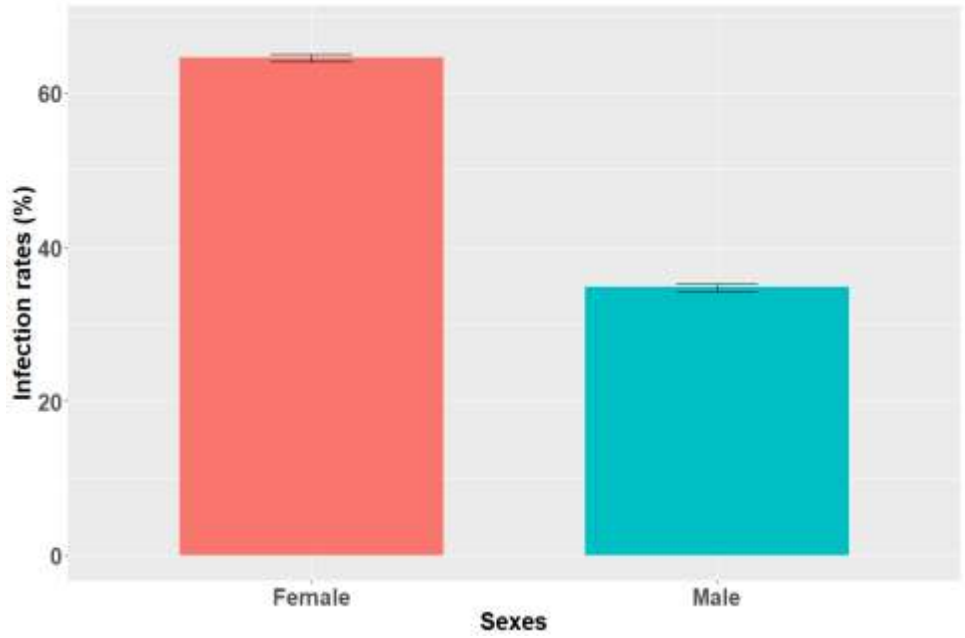

Figure 4. Prevalence of *Asaia aff. bogorensis* GD01 in female (red) and male (green) specimens of *An. coluzzii* insectary colony.

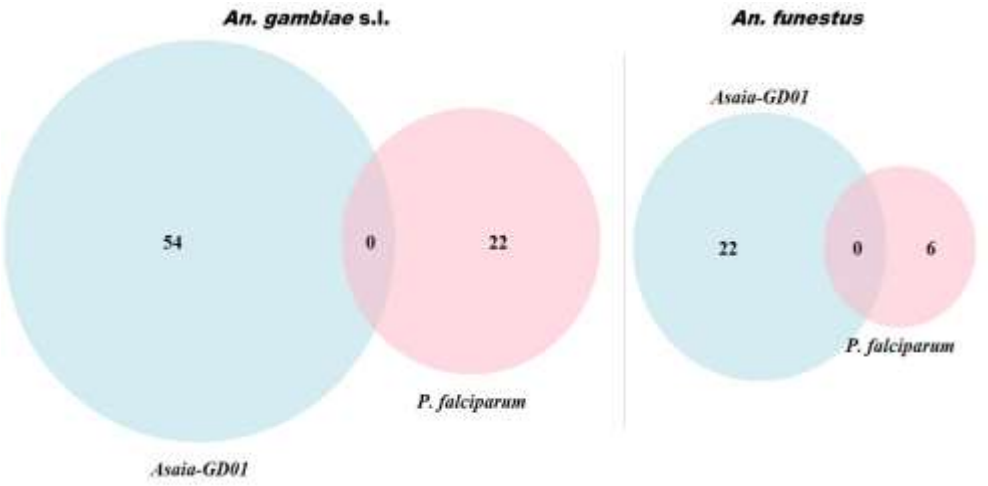

Figure 5. Impact of the presence of *Asaia aff. bogorensis* GD01 on *P. falciparum* sporogonic development within wild malaria vectors in Senegal. The presence of *Asaia aff. bogorensis* GD01 in mosquitoes is negatively correlated with *P. falciparum* carriage.

Moreover, these bacteria are easily cultivable and can be genetically manipulated for subsequent reinsertion into the insect host. Indeed, Bongio and Lampe (2015) have successfully created one of the first *Asaia* para-transgenetic strains harbouring a scorpine antimicrobial peptide and an anti-Pbs21 scFv-Shiva1 immunotoxin. The two proteins are known to be effective anti-plasmodial effectors as shown by the observed significant inhibition of parasite development.

In addition to being easily transformable and transmissible, horizontally and vertically, this bacterium has intrinsic biological characteristics that make it a good candidate. The acetic acid bacterial genus of *Asaia* has been first discovered and isolated from *B. purpurea* and *P. auriculate* flowers (Yamada et al., 2000). Here, we have isolated a strain of *Asaia* spp. from wild population

of *An. gambiae* s.l. The genus of *Asaia* belongs to the Phylum of Proteobacteria, the Class of Alphaproteobacteria, the Order of Rhodospirillales, to the family of Acetobacteraceae (Yamada et al., 2000).

Isolation of certain bacterial strains can sometimes be difficult, especially for those, like *Asaia*, that require specific culture conditions for optimal growth. During this study, an optimal growth was obtained after 72 h of incubation at 28°C in atmospheric condition supplemented with 5% of CO_2. Furthermore, the growth of *Asaia* colonies from a crushed mosquito could be inhibited by fast-growing bacteria such as *Burkholderia* species, which produces bactericidal or fungicidal substance (Andreolli et al., 2011) or *Proteus* (invasive bacterial species). Another limiting factor that makes *Asaia* difficult to isolate, could be the tiny colonies, on blood agar plate 72 h post-incubation; as observed on the cystine-lactose electrolyte-deficient and MacConkey agars (Tuuminen et al., 2006).

Currently, only 8 species of *Asaia* have been described so far: *Asaia astilbis* (Suzuki et al., 2010), *A. bogorensis* (Yamada et al., 2000), *Asaia krungthepensis* (Yukphan et al., 2004), *Asaia lannensis* (Malimas et al., 2008), *Asaia platycodi* (Suzuki et al., 2010), *Asaia prunellae* (Suzuki et al., 2010), *Asaia siamensis* (Katsura et al., 2001) and *Asaia spathodeae* (Kommanee et al., 2010). Phylogenetic analysis places the new strain in the *A. bogorensis* cluster and has been therefore provisionally named *A. aff. bogorensis* GD01. The close relatedness of the latter with strains isolated from plant flowers suggests that mosquitoes are potentially infected when feeding on plants. More investigations are needed to describe the complete epidemiological cycle of *A. aff. bogorensis* GD01 in Senegal. Previous studies have reported the *Asaia* genus from several arthropods including malaria vectors, such as *An. stephensis* (Favia et al., 2007) and *An. gambiae* (Chouaia et al., 2010; Damiani et al., 2010) but also from *Aedes aegypti*, a vector of arbovirus (Chouaia et al., 2010; Crotti et al., 2009).

In its culicid hosts, *Asaia* is found as an extracellular symbiont in the midgut, salivary glands and reproductive organs of female and male (Favia et al., 2007). Putative infection routes facilitating the spreading *Asaia* spp. in mosquito populations could be made by co-feeding, sexual mating, and maternal or paternal transmission (Damiani et al., 2008).

We have developed and checked two real time PCR systems for routine screening of *Asaia* spp. and *P. falciparum* infections, respectively. Both systems were cross-checked to ensure their specificity and sensitivity and also to exclude any risk of environmental contamination. Screening of the wild population of two malaria vectors from two Senegalese areas and one insect colony showed widespread infection with *Asaia* in wild and colonized malaria vectors in Senegal. Indeed, *A. aff. bogorensis* GD01 has been found in natural populations of *An. gambiae* s.l. as previously reported (Chouaia et al., 2010; Damiani et al., 2010) and for the first time, in wild population of *An. funestus*, the second major vector of malaria in Senegal (Samb et al., 2016).

Asaia carriage was higher in the insectary colony and was found in both females and males. The higher infection rates in colonized population compared natural populations could be explained by the confined environment which favours higher horizontal and vertical transmission rates. Indeed, Mancini et al. (2016), using confined semi-field conditions to evaluate the ability of a modified *Asaia* strain to diffuse targeted vectors populations, have demonstrated its ability to spread at a high rate in the dominant malaria vectors by exploring both horizontal and vertical transmission, and sometimes even transstadial diffusion mechanisms. In this regard, the infection in males, which are non-hematophagous, provide a potential for their use to spread *Asaia* strains interfering with malaria transmission in African vectors, as suggested previously for *An. stephensi* (Damiani et al., 2008).

Indeed, transmission from males to females during mating can be exploited to colonize mosquitoes in the wild (Wilke and Marrelli, 2015). This may also explain the observed difference of rates infection between sexes among our insectary population; with females being more infected than males. Indeed, males performing mainly a reproductive role can mate with several females and thus sexually infect their mating partners.

Higher impact of *Asaia* infection on females has been previously reported involving paternal transmission of a recombinant strain during the mating (Mancini et al., 2016). The different infection rates observed between natural populations can be explained by different levels of environmental transmission risk involving flower nectar. In fact, bacteria of the genus *Asaia* have been originally isolated from tropical plant species of *B. purpurea* and *P. auriculata* (Katsura et al., 2001; Moore et al., 2002; Yamada et al., 2000; Yukphan et al., 2004). Acetic-acid bacteria (Acetobacteraceae) are acquired naturally in nectars, fruit sugars, and phloem sap. Their transmission to oral nectar-feeding species has been extensively documented for several insect species, such as *Ae. aegypti*, *Apis mellifera*, and *Drosophila melanogaster* (Chouaia et al., 2010; Crotti et al., 2009), and may occurs with different transmission level according to plants species present in an area.

It is interesting to note that the results obtained here suggest that mosquitoes carrying *A. aff. bogorensis* GD01 are likely to be resistant to *Plasmodium* infection. Indeed, the presence of the endosymbionts was negatively correlated with *Plasmodium* parasite carriage, suggesting a potential antagonist effect on malaria parasite development within the vector. Although Capone et al. (2013) did not attribute the decrease in *Plasmodium* infection intensity to active replication of *Asaia*, they have already reported a decrease in average *Plasmodium* infection rates, and meanwhile the number of *Asaia*

has increased.

But, the amount of *Asaia* and the number of parasites in single guts was surprisingly positively correlated due to suitability of the mosquito midgut environment for the development of both *Plasmodium* oocysts and *Asaia*. However, the co-localisation of both parasite in the mosquito midgut and salivary glands could lead to a competition for the location and nutriments, or secreted putative antiplasmodial proteins. Indeed, Capone et al. (2013) have shown '*in vitro'* that *Asaia* induces the expression of antimicrobial peptides affecting the development of plasmodium species. This corroborates our observation and therefore offers a new field of investigation for the use of *Asaia* spp. as malaria control tools in addition to the existing tools and fulfil WHO recommendations for the search for new tools to faster malaria elimination (WHO, 2012).

Conclusions

To the best of our knowledge, this study is the first to investigate the occurrence of *Asaia* in wild anopheline vector populations and its impact on *P. falciparum* development. It is also the first reporting *Asaia* infection among wild populations of *An. funestus*, one of the major malaria vectors in tropical Africa, especially in Senegal. We also observed a rapid spread of *Asaia* infections in adult males and females in a colony of insects, paving the way for potential massive production of anopheles-*Asaia*$^{+}$ line for the malaria control purpose. However, further studies are needed to better characterize the potential impact of *Asaia* infection on the development of *Plasmodium* and to ensure their safe use as an alternative or complementary control strategy to achieve elimination status.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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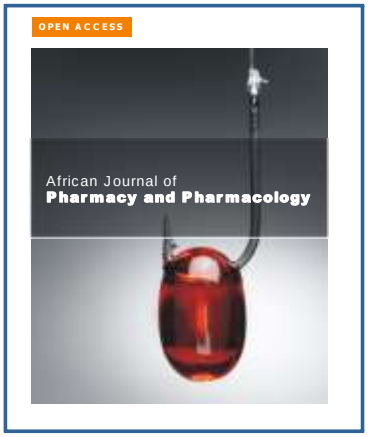

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