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Malaria, tuberculosis and HIV: what's new? Contribution of the Institut Hospitalo-Universitaire Méditerranée Infection in updated data

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Abstract

The Institut Hospitalo-Universitaire Méditerranée Infection is positioned for the diagnosis, prevention and treatment of the 'big three' killer diseases: malaria, tuberculosis and HIV. We implemented the use of new diagnostic samples such as stools and new diagnostic tests such as mass spectrometry for the dual identification of vectors and pathogens. Furthermore, advances in the prevention and treatment of malaria and tuberculosis are reviewed, along with advances in the understanding of the role of microbiota in the resistance to HIV infection. These achievements represent a major step towards a better management of the 'big three' diseases worldwide.

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Introduction

In 2016, approximately 3.2 billion people—nearly half of the human population—were at risk of malaria [1]. According to the latest World Health Organization (WHO) estimates, there were 216 million cases of malaria in 2016, causing 445 000 deaths. Sub-Saharan Africa still carries a disproportionately high share of the global malaria burden, with 90% of malaria cases and 92% of malaria deaths, mostly in young children. *Plasmodium falciparum* resistance to most antimalarial drugs has emerged in Southeast Asia and spread to Africa [2,3]. Since 2005, the WHO has recommended artemisinin-based combination therapies (ACT) as first-line treatment for uncomplicated malaria and intravenous artesunate for severe malaria. However, artemisinin

derivative—resistant *P. falciparum* strains have emerged in western Cambodia, Myanmar and Thailand and throughout Southeast Asia [4–6]. Additionally, the vectors of malaria have adapted their behaviour after introduction of long-lasting insecticide-treated bed nets (LLINs) and have become resistant to insecticides [7,8]. Of 76 malaria-endemic countries that provided monitoring data for the 2010–2016 period, 61 countries reported resistance to at least one insecticide in one malaria vector from one collection site, and 50 countries reported resistance to two or more insecticide classes [1].

Here we report a number of research projects conducted by research teams of the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, Marseille, France, which contributed to the knowledge, clinical management and control of malaria in Africa. We limit this report to recent and representative new data.

Malaria

Identification of malaria vectors

To establish monitoring and control of malaria vectors, precise identification of mosquitoes at the species level is essential.

Currently mosquitoes are classically identified using morphologic criteria or molecular methods. Nevertheless, these methods, requiring entomologic expertise and training, are long, expensive and labourious, thus limiting large-scale surveys. To overcome these limitations, matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) profiling was explored in the past decade as an identification tool for insects [9]. This economic, rapid and accurate innovative strategy has proven to be a relevant tool for *Anopheles* identification at the species level, also distinguishing cryptic species such as *An. gambiae* s.s. and *An. coluzzii* (Culicidae) [10]. This proteomic tool was successfully applied to the identification of mosquitoes at the adult [11] and juvenile [12] stages. Consequently, it is now conceivable to perform accurate live monitoring of mosquitoes before their emergence and to adjust control measures according to the mosquito detected [13]. The standardization of protocols from sample homogenization method to MS spectra analysis, taking into account mode and duration of sample storage, has been performed [14]. These optimized protocols will facilitate the creation and sharing of a mosquito MS reference database.

The elucidation of *Anopheles* trophic preferences remains a key factor in the management of malaria transmission by incriminating vectors involved in pathogen transmission. Recently the proof-of-concept use of MALDI-TOF MS profiling for the determination of *Anopheles* blood meal source was reported [15,16]. These studies underlined that correct identification of blood meal origin could be relevant for specimens freshly engorged, up to 24 hours after feeding. Moreover, it was demonstrated that the conventional field practice, consisting of crushing on Whatman paper filter the abdomen of blood-engorged mosquitoes, was compatible with future MS identification of the blood source [17]. More recently, the detection of the *Plasmodium* infectious status of *Anopheles* was positively revealed by MALDI-TOF MS profiling [18].

Taken together, these works highlight the potential of MALDI-TOF MS for elucidating host, *Plasmodium* and *Anopheles* interactions. Guidelines in the field should facilitate the creation and sharing of an open-source reference MS spectra database in the near future. This emerging tool opening new opportunities may revolutionize medical entomology.

Entomologic investigation and control strategy in Africa

The efficiency of control strategies against malaria, such as indoor residual spraying, depends on the behaviour of mosquitoes, which varies by species. The implementation of LLINs has contributed to halving the mortality rate due to malaria since 2000 in Africa. Thus, to achieve the World Health Assembly's new target to reduce the burden of malaria over the next 15

years by 90%, it is necessary to understand how the spatio-temporal dynamics of malaria vectors and human exposure to bites are modified in the context of scaling up global efforts to control malaria transmission.

Parallel parasitologic and entomologic surveys from the Dielmo project, Senegal, were collated since June 1990; full descriptions of the site and data collection methods are reported by Wotodjo *et al.* [19]. A study was conducted in Dielmo, a Senegalese village, after the introduction of LLINs, and two rounds of LLIN renewal showed that implementation of LLINs correlated with a significant decrease in the biting densities of the main malaria vectors *An. gambiae* s.l. and *An. funestus*, thus reducing malaria transmission. The bulk of bites occurred during sleeping hours, but the residual vector populations of *An. gambiae* s.l. and *An. funestus* had increased propensity to bite outdoors, so the risk of infectious bites remained for LLIN users [20]. This study suggests that *An. funestus* has adapted its biting time to the new situation (use of impregnated nets). Behavioural change in response to insecticides may make control tools ineffective, thus negatively affecting malaria control strategies. This remarkable behavioural adaptation of mosquitoes to insecticide-based vector control interventions requires increased and close awareness in the context of preelimination of malaria [7]. These results highlight the need to amplify and correct the use of LLINs and to combine this intervention with complementary control measures against residual exposure, such as spatial repellents and larvae source management, to achieve the goal of eliminating malaria transmission.

The IHU teams also participated in the monitoring of entomologic status of French military bases in sub-Saharan Africa (Gabon, Ivory Coast, Republic of Central Africa, Senegal, Djibouti) [21–23]. This surveillance is based on identification of vectors, study of behaviour and evaluation of insecticide resistance. New tools for vector trapping and identification of pathogens in vectors have been developed to identify the best candidates for vector surveillance and pathogen transmission [24,25]. Additionally, we showed that the use of remotely sensed environmental and meteorologic data enables predicting the risk of malaria transmission in Africa [26].

Tools have been also developed to evaluate the risks of exposure of soldiers to vector bites through the identification of exposure biomarkers by analysing mosquito salivary antigenic proteins and serologic responses associated with the level of exposure [27,28]. These tools enabled evaluating the effectiveness of antivectorial strategies, estimating the risk of disease transmission and monitoring mosquito populations.

Diagnosis of malaria

PCR was been applied to detect *Plasmodium* spp. in ape faeces [29]. One IHU team demonstrated that malaria-infected

humans, like apes, shed detectable *P. falciparum* parasites in their stool, which, like in blood, may be detected [30]. The detection of *P. falciparum* in human faeces may facilitate the surveillance of malaria in Africa in large epidemiologic studies due to less invasive sampling.

WHO recommends a diagnosis confirmation by microscopy or rapid diagnostic tests (RDTs) in all patients with suspected malaria before treatment with antimalarial drugs. However, falsely negative RDT results were reported with the use of *P. falciparum* histidine-rich protein 2 (PfHRP2)-based RDTs. The performance of the PfHRP2-based RDTs may be affected by genetic variation of the *pfhrp2* gene with a new epitope that is not recognized or *P. falciparum* parasites that lack PfHRP2. We identified the first cases of parasites that lack PfHRP2 in Senegal [31]. The introduction of parasites lacking the PfHRP2 protein in an area will have an impact by increasing parasite prevalence, transmission, morbidity and ultimately malarial mortality.

Chemoprevention of malaria

In 2012, the WHO recommended that children younger than 5 living in areas of highly seasonal malaria transmission in the Sahel and sub-Sahel regions should receive seasonal malaria chemoprevention (SMC) for up to 4 months per year to prevent malaria [32]. Before this WHO recommendation, SMC was shown to be highly effective in Senegal [33,34]. However, it was questioned whether the intervention could be delivered on a large scale at reasonable cost; whether it would be accepted by families; and whether the drugs used for SMC would be safe when deployed on a large scale. SMC with sulfadoxine/pyrimethamine plus amodiaquine substantially reduced the incidence of outpatient cases of malaria and of severe malaria in children, but no difference in all-cause mortality was observed. However, introduction of SMC was associated with an overall decrease of malaria incidence in untreated age groups. Therefore, in many areas of Africa with seasonal malaria, there is a substantial burden in older children that could be prevented by SMC. SMC is well tolerated and efficient in older children, and it can contribute to reduce malaria transmission. In 2016, 15 million children younger than 5 received treatment by an SMC strategy.

Treatment of malaria

Clinical studies were extensively conducted between 1994 and 2011 in Cameroon, Republic of Congo and Ivory Coast, where first- and second-line drugs (chloroquine, amodiaquine and sulfadoxine/pyrimethamine monotherapies), novel drugs (pyronaridine, atovaquone/proguanil, chlorproguanil/dapsone) and novel drug combinations, including amodiaquine/

sulfadoxine/pyrimethamine and ACT, were evaluated [35–40]. On the basis of their earlier experience in Central and West Africa, IHU researchers have been evaluating since 2010 the therapeutic efficacy of different antimalarial drugs against *P. falciparum* and *P. vivax*, together with resistance markers, in Mauritania, where clinical data are scarce. These ongoing studies are being conducted in collaboration with the World Health Organization, University of Nouakchott Al-Aasriya and National Institute of Research in Public Health, and include local capacity building. Results suggest high levels of *P. falciparum* resistance to chloroquine and sulfadoxine/pyrimethamine, which is associated with a high prevalence of mutations in molecular resistance markers (*Pfcr*, *Pfdhps* and *Pfdhfr*) but which indicate high efficiency of ACT (>98% cure rate on day 28, excluding re-infections) [41–44]. In contrast, chloroquine monotherapy is highly effective in treating *P. vivax* malaria in the Saharan zone of Mauritania [45]. Fever and parasitaemia were cleared within 48 hours of initiating therapy, and there was no reappearance of fever, malaria-associated symptoms or parasitaemia during the 28-day follow-up period. Moreover, there was no molecular evidence for drug resistance in *P. vivax* isolates [46].

Malaria control and elimination strategies can be driven and monitored by analysing the genetic diversity and parasite population dynamics of *P. falciparum* in humans using multiple loci variable number of tandem repeats analysis performed on DNA extracted from dried blood spots on filter papers or malaria rapid diagnostic test nitrocellulose strips [47]. The high genetic diversity of *P. falciparum* populations assessed in Mali strongly advocates for the use of global malaria control strategies that are based on diversified and complementary control measures. Otherwise, polymorphic and panmictic parasite populations that are particularly prone to escaping single or uniform control interventions in addition to their resistance qualities might easily spread across regions [48].

Resistance of *Plasmodium*

A IHU-Méditerranée Infection team has been involved as part of the national reference centre for malaria-associated laboratory, and the team members have conducted epidemiologic and clinical analyses of imported malaria cases as well as assessments of malaria-endemic areas where French soldiers are staying or could stay in the future, including Ivory Coast, Republic of Central Africa, Senegal, Gabon, Congo, Djibouti, Mauritania, Congo, Cameroon and Mali. In addition, evaluation of the *in vitro* susceptibility of *P. falciparum* parasites (ex vivo and molecular markers) and clinical efficacy in patients were conducted to adapt chemoprophylaxis and malaria treatment and control strategies in French armed forces and in civilian recommendations [49–57]. Additionally, a traveller database that can be used as a surveillance system to assess and monitor the

emergence of drug resistance in endemic African areas where information is limited has been developed [58].

Malaria resistance to most antimalarial drugs has developed in Southeast Asia and has spread to Africa. In this context, the identification of molecular resistance markers to these antimalarial drugs is urgently needed for monitoring the emergence and spread of resistance to antimalarial drugs. The IHU-Méditerranée Infection participates in identification, development and/or validation of new molecular resistance markers to doxycycline (*pfmdt* and *pftetQ*) [59–62], quinine (*pfmhe-1*) [63], artemisinin (*pfK13*) [64–68] and quinolones (*pfmdr1*, *pfmdr2*, *pfmdr5*, *pfmdr6*, etc.) [69–72].

New antimalarial drugs

The IHU-Méditerranée Infection also participates in the identification of new antimalarial drugs by *in vitro* screening in collaboration with several international departments of medical chemistry and international pharmaceutical laboratories [73–77] and in the development of potential antimalarial drugs like cepharanthine [78,79], atorvastatin [80,81] and methylene blue. Methylene blue is active *in vitro* against *P. falciparum* and *P. vivax* in the nanomolar range without cross-resistance with standard antimalarial drugs; it shows synergistic effects in combination with artemisinin derivatives and prevents cerebral malaria in an experimental cerebral malaria murine model [82–86]. The development of these antimalarial drugs is based on the evaluation of *in vitro* activity against *P. falciparum* clones, evaluation of *ex vivo* activity against *P. falciparum* field isolates from several areas, evaluation of *in vivo* activity in experimental models (uncomplicated malaria and cerebral malaria) and identification of the mode of action and potential resistance mechanisms.

HIV

The IHU-Méditerranée Infection is clearly an important place for the diagnosis and treatment of patients carrying the HIV virus. Because of the specificity of our institute, which is dedicated to the study and understanding of the microbiome, research strategies are currently focused on analysing the microbiota of HIV-infected patients. Ongoing studies suggest that HIV-infected patients and naive controls do not carry the same bacterial gut microbiota composition, suggesting that the virus and/or the treatment significantly influences the microbiota. In addition, studies are carried out on patients who have been positive for HIV and who seem to have totally eliminated the virus from their body. Preliminary results of the microbiota analysis of a patient who apparently eliminated the virus indicate a particular gut microbiota composition with species that are not found in negative controls. This corroborates the published results from

Vesterbacka et al. [87], who demonstrated that genus diversity and richness of faecal microbiota are significantly higher in elite controllers versus naive controls. Additional analyses are underway to confirm the link between the microbiota composition and the virus elimination. We have further studied the bacterial species present exclusively in the microbiota of the 'self-cured' patient and found that they produce bioactive compounds that are able to block the *in vitro* replication of HIV. Interestingly, Wang et al. [88] had reported that a heat-stable molecule derived from *Streptococcus cristatus* induces APOBEC3 expression and inhibits HIV-1 replication. In order to understand whether the bacterial species that we have isolated from self-cured patients use this mode of action or another, we are currently studying the molecular mechanisms by which biologically active compounds could block *in vitro* the virus replication in target cells exposed to HIV. This may open new ways to treat patients with derivatives of these bioactive molecules or to consider treating patients by introducing the protective bacteria into their microbiota in order to increase its richness.

Tuberculosis

In 2015, the number of tuberculosis incident/prevalent cases was estimated to be 10.2 per million/10.1 per million, respectively, including 8.8 per million/8.9 per million in HIV, with an estimated number of deaths of 1.3 million (1.1 million in HIV-infected persons). Mortality, incidence and prevalence have declined since 2005, but the Global burden of disease Tuberculosis group concluded that despite the observed reduction of tuberculosis cases, it still represents a large disease burden globally [89]. Our group has been working on mycobacteria and mycobacterial diseases since 1995, and has been focusing essentially on trying to shorten *Mycobacterium tuberculosis* (MTB) diagnosis; more recently we have been focusing on new therapeutic approaches to fight resistance but also to reduce the treatment length and MTB clearance and contagion.

MTB is known as an airborne organism transmitted between humans through close contact. However, we demonstrated that MTB was able to survive in soil, where it may remain virulent outside its host for an extended period of time, suggesting that other transmission modes might exist [90]. The diagnosis of tuberculosis is sometimes fastidious as a result of poor sputum quality sampling, in particular in children and women, or in cases of comatose patients. In such situations, alternative specimens are most often obtained by invasive procedures such as aspirates or bronchoscopy. We reported that stools might also be valuable and alternative specimens for culture and PCR [91]. This was confirmed by Nicol et al. [92] using Xpert MTB/Rif testing in children with pulmonary tuberculosis and was successfully used in patient with laryngeal tuberculosis [93]. Samples from which MTB culture is

attempted are most often sputum or bronchoalveolar lavage fluids, which are usually contaminated by oropharyngeal flora. Decontamination and concentration of sputum to enhance MTB recovery is usually done by centrifugation, which exposes the laboratory staff to infectious aerosols. We developed an innovative technique that uses magnetic beads that bind specifically to MTB, and we showed that this technique enhances sensitivity of MTB isolation by culture in the sputum specimen [94]. MTB was long considered to be a slow-growing bacterium. Traditionally, the culture media were agar- and egg-based media incorporating green malachite and Middlebrook broth. We managed to grow MTB on blood agar in 1 to 2 weeks [95], confirming a previous publication [96]. In the search of reducing MTB culture time, which takes more than 2 weeks on conventional media, we set up a step-by-step protocol with a new MOD4 media (blood and egg lecithin) in microaerophilic atmosphere or ascorbic acid supplement and autofluorescent detection, allowing a marked reduction of culture time, with, in some occasion, rifampin susceptibility testing available after 72 hours of growth [90]. On the basis of this knowledge, we developed a novel solid medium for culturing MTB isolates from clinical specimens that we named MOD9, a blood-free derivative of MOD4; we showed that the detection time was significantly shorter compared to conventional Lowenstein medium [97] and standard culture broth [98].

Apart from shortening the culture time, another goal of our team was to shorten the time of colony identification. This was obtained in our group by using real-time high-resolution imaging. In a proof-of-concept study using an imaging assay, we succeeded in reducing the colony identification time by 4.4-fold [99]. Once isolated, identification of MTB complex (*M. tuberculosis*, *M. bovis* and the bacillus Calmette-Guérin-derived clones of *M. africanum*, *M. canetti*, *M. microti*, *M. caprae* and *M. pinnipedii*) colonies at the species level is important for therapeutic and epidemiologic applications. In order to achieve this goal, we used pyrosequencing, which proved to be a robust, specific, rapid and easy-to-perform assay to identify MTB complex isolates at the species level [100]. Although pyrosequencing has been extensively used for MTB isolate identification and antibiotic resistance evaluation because it is advantageous in terms of time, its cost is significantly higher than traditional methods. In order to reduce the cost of the assay, we developed direct MALDI-TOF MS for identification of mycobacteria. Using one colony cultivated on agar, we identified mycobacteria with a score of 1.22, 98.3% of isolates. MALDI-TOF MS with a score of ≥ 1.3 has a predictive value of 100% and might be a cheaper way for appropriate identification of mycobacterial colonies [101].

Another insight into tuberculosis by our institute is the treatment of resistant MTB infection. The first extensively drug-resistant tuberculosis patient in France was identified in

Marseille in 2011 with a Beijing genotype, and we suggested, on the basis of the literature, that trimethoprim/sulfamethoxazole might be an effective alternative for this patient [102]. Thus, we evaluated the *in vitro* susceptibility of MTB to trimethoprim/sulfonamide and concluded that sulfonamides could be considered as antituberculous antibiotics [103]. Later, we reported our first totally resistant MTB patient, for whom, and based on our previous data, we successfully used antileprosy treatment (sulfonamides, minocycline, clofazimine) [104]. The role of second-line injectable antituberculous drugs has been questioned as a result of adverse events [105].

Optimizing the treatment of MTB relies on finding an efficient and safe treatment with few adverse events that may be given orally to increase patient compliance. The treatment should avoid intravenously delivered drugs, should be as short as possible and should be available at low cost in low-income countries. Antileprosy drugs might meet these criteria, but a deep mining of the enormous collection of undeveloped or forgotten antimicrobial compounds is still needed [106].

Conclusions

The IHU-Méditerranée Infection is in position for the diagnosis, prevention and treatment of the 'big three' killer diseases: malaria, tuberculosis and HIV. Research teams contribute to the knowledge, clinical management and control of these 'big three' diseases. The IHU-Méditerranée Infection participates in advances in the prevention and treatment of malaria and tuberculosis, and in the understanding of the role of microbiota in the resistance to HIV infection. These achievements represent a major step towards a better management of the 'big three' diseases worldwide.

Conflict of interest

None declared.

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