HOSTED BY ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Review http://dx.doi.org/10.1016/j.apjtm.2015.11.012

Wolbachia pipientis: A potential candidate for combating and eradicating dengue epidemics in Pakistan

Uruj Tahir[1*], Umair Hassan Khan[2], Muhammad Saad Zubair[3], Bahar-e-Mustafa[2]

[1]*Department of Environmental Sciences, Fatima Jinnah Women University, Rawalpindi, Pakistan*

[2]*Department of Microbiology, University of Agriculture Faisalabad, Sub-Campus Toba Tek Singh, Pakistan*

[3]*Department of Microbiology, Government College University Faisalabad, Pakistan*

ARTICLE INFO

Article history:
Received 15 Sep 2015
Received in revised form 20 Oct 2015
Accepted 3 Nov 2015
Available online 14 Nov 2015

Keywords:
Dengue
Pakistan
Antiviral agents
Chemical methods
Biological control
Wolbachia pipientis

ABSTRACT

Dengue virus syndrome is an emerging global health challenge which is endemic in tropical countries like Pakistan. In recent years dengue incidences have increased considerably in different areas of Pakistan with more sever impacts on urban and peri-urban populations. This review is an effort to highlight the changing epidemiology of dengue fever, role of Government of Pakistan in disease management and control using preventive and community based approaches in the region. Moreover, there is an emphasis on application of *Wolbachia* as novel, inexpensive and environmentally benign candidate for control and eradication of dengue transmitting vectors.

1. Introduction

Infectious diseases continue to plague the global community with high rates (26%) of morbidity and mortality. The main causes for transmittance of such emerging and reemerging disease outbreaks are unprecedented shifts in (a) environmental or climatic conditions either due to anthropogenic activities, (b) overlapping of geographical ranges, (c) change in land-use patterns or (d) emergence of drug and insecticidal resistances among vector populations [1,2]. Vector-borne diseases such as Congo hemorrhagic fever, Malaria, Dengue, Yellow fever, Chikungunya *etc*. account for a significant threat to human population in terms of health and economic losses all over the world. Such outbreaks lead to widespread epidemics which may result in deaths of many hundreds and millions of people annually [3,4]. These diseases have been reported to exert more devastating effects on developing or under transition countries. Because different variables *e.g*. water and sanitation facilities, population density, literacy rate *etc*. are the major players for spread of epidemics in such countries as compared to developed ones, where main contributors include ambient temperature, moisture or humidity along with rainfall patterns [1,2,5–7]. Most of these transmitted ailments are concentrated and prevalent with higher impacts in regions of South-East Asia, Southern Europe, Western Pacific and Eastern Mediterranean countries, Latin America, Australia and Sub-Saharan Africa [1,2,6,7]. As for instance, approximately 1.1 million deaths at global scale have been attributed to malaria alone [8]. Around 91% deaths involving 86% of children (<5 years of age) were recorded in Africa because of malaria [9].

Recently dengue fever (DF) has been recognized as one of emerging infectious diseases worldwide. It represents a considerable havoc to people living in urban and peri-urban localities of tropics and subtropics [10]. The viral epidemic has led to hospitalization of 20 million people with around 24 000 deaths as documented by Sulehri *et al* [7], which now has raised up to 50–100 million infected people with 2.5 billion at risk of contracting dengue [11,12]. Pakistan has experienced a number of dengue outbreaks with more server impacts in recent years. These outbreaks are affecting larger proportion of urban population and putting significant stress on health care facilities. Unfortunately the magnitude and severity index of the disease remains unreported due to deficiency and difficulty

*Corresponding author: Uruj Tahir, Department of Environmental Sciences, Fatima Jinnah Women University, Rawalpindi, Pakistan.
E-mail: urujtahirjavaid@gmail.com
Peer review under responsibility of Hainan Medical College.

in data collection [6,11,13]. This review attempts to describe the prevalence of dengue virus infection and its associated serotypes in Pakistan with importance of potential biological control agents in eradication of the transmitting vector/s.

2. Dengue fever, signs and symptoms

Dengue globally affects 40% of world's population and occurs in repetitive cycles (3–5 years interval). The ailment presents high fever accompanied by frequent headaches, nausea, skin rashes, enlargement of lymph nodes, pain behind eyes, severe muscular, bone and joint pains, epitaxis along with leukocytopenia *i.e.* reduction in white blood cells count. Thus render individuals more prone to viral infection. DF is also characterized as bone break fever or bone crush fever due to severe pain in joints and bones [6,7,12,14–18].

3. Virology and serotypes

Being a member of a medically important viral family, Flaviviridae, the virus possesses single stranded positive sense RNA (+ve ssRNA). Albert Sabin in 1944 classified dengue virus into four antigenically related but immunologically distinct serotypes namely DENV-1, DENV-2, DENV-3 and DENV-4 [19–25]. All DENVs comprise of three structural [Capsid (C), membrane (prM/M) and envelope (E)] and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). Each of these serotypes has been reported to exhibit extensive genetic variability due to distinct genotypes. Each of these genotypes is associated with severe dengue epidemics [17,22,23,26].

4. Principal transmission vector, breeding habitats and virus acquisition

The infection is usually transmitted through bite of infected *Aedes aegypti* (*Ae. aegypti*) (female mosquito). The mosquito is adapted to feed exclusively on human blood and is a day time feeder [6,11,15,17]. *Ae. aegypti* is well-known for being susceptible to all dengue viral serotypes and as an efficient vector with capability of transmitting dengue virus to many individuals in shorter time span [20,26,27]. This species of mosquito has spread extensively in tropical and subtropical regions of the world as a result of increased national and international trade, shipping and tourism [6,11,15,17,28]. *Aedes albopictus* (*Ae. albopictus*) has also been reported to transmit dengue viruses however to much lesser extent [29].

Ae. aegypti usually breeds in dark places, stagnant water kept in containers for (indoor and outdoor) storage, water coolers, drums, barrels, plant saucers, open buckets, in used tyres and places where rainwater collects. The mosquito unlike rural settings is specifically adapted to urban and peri-urban settings (*i.e.* cleaner environment). In such localities mosquito population density has been reported to be proportional to human population density. Moreover, breeding habitats of mosquitoes have strongly been associated with elevated temperatures, water supplies, sewage and sanitation facilities [6,15].

Virus is acquired by mosquito during blood meals of viremic (infected) patients. The acquisition is followed by incubation period (8–10 d) where viral genome is replicated within the mosquito, transferred to salivary glands via intestinal tract of mosquito. Mosquito then transmits virus to healthy persons upon subsequent biting [17,21,30,31] and also to offsprings via transovarian transmission during reproductive cycles [7,21]. Once inside the human host virus starts replicating within the target organs like white blood cells, lymphatic tissues *etc*. and gets released into blood following circulation [32].

5. Dengue viral syndromes

Depending upon age and immunological status dengue virus results in clinically different syndromes ranging from (a) classic DF characterized as a milder a typical flu-like form with high grade fever, head and body aches. It affects infants, young children and adults but is not fatal. Nonspecific febrile sickness with rashes has been reported in infants and young children. While mild febrile syndrome with high fever, head and joint-muscular pains along with skin rashes has been observed in older children and adults (b) dengue hemorrhagic fever (DHF) characterizes acute and continuous fever (40–41 °C) lasting for 2–7 d, accompanying hemorrhagic manifestations (ecchymosis and petechiae) with reduction in platelets. It may lead to liver enlargement and circulatory dysfunction in severe cases, and (c) dengue shock syndrome, a complicated and prolonged illness, in which DENV-1 viral infection is followed by DENV-2 and DENV-3. Thus exhibits symptoms of both DF and DHF. Such infections lead to hypervolemic shock (*i.e.* increased plasma leakage due to vascular permeability) and are associated with higher morbidity and mortality rates [6,7,14,31,33–37]. Furthermore, dengue infections have been reported to be life threatening upon coexistence in asthmatic and diabetic patients or in individuals with other chronic diseases [31]. According to Raza *et al* [12] and Mukhtar *et al* [38] approximately 250 000–500 000 people suffer from DHF and/or dengue shock syndrome and about 20 000–25 000 are succumbed to death every year.

6. Dengue in Pakistan

Pakistan being a developing urban and agricultural economy is more likely to be at risk of vector-borne epidemics. Mosquito-borne diseases such as dengue have now become a public health concern. This is due to exertion of considerable burdens of morbidity, mortality and economic distresses in many parts of the region. Over populated cities, lack of access to proper sanitation facilities, unavailability and shortage of clean drinking water, population influxes at massive scale and lack of awareness about possible health effects posed by vector transmitted pathogens are some of the leading causes of spread of mosquito-borne diseases [1,2,39]. Moreover, raised ambient temperature, huge population, overcrowded cities with poor resource settings and lack of health facilities (in terms of vaccination access and coverage) also serve as important prerequisites for reproduction of mosquitoes. This situation further exacerbates transmission of pathogens dwelling within these host vectors causing long-term illnesses [31,40].

Dengue viral infection killing 365 individuals with 21 597 positive cases is becoming endemic in Pakistan. The viral epidemic persists all the year round with greater intensity in months of October–December (post-monsoon period). Additionally, recent floods have enhanced the prevalence of dengue in entire country through provision of large number of breeding sites for viral vectors, thereby made the situation more worst [13,15,17,24,41].

7. Prevalence of dengue viral serotypes in Pakistan

Outbreaks of DF recorded during 1994–2004 showed prevalence of DENV-1 and DENV-2 serotypes in different regions of Pakistan. Whereas DENV-3 was the commonest serotype in 2005 and led to severe dengue hemorrhagic infections in previously DENV-1 and DENV-2 sensitized populations. In recent years Pakistan is experiencing dengue outbreaks either major or minor mainly by DENV-2 and DENV-3 [42]. For instance, the predominance and co-circulation of DENV-2 and DENV-3 serotypes have been reported during 2006, 2007 and 2009 in Pakistan. Research studies revealed that DENV-2 (subtype IV), DENV-3 (subtype III) and DENV-4 prevailed in 2008. Likewise, serotype DENV-2 was reported to follow DENV-1 during dengue epidemics in 2010. However DENV-2 and DENV-3 serotypes caused most critical outbreaks in 2011 [12,18,21,41] suggesting that magnitude of dengue outbreaks is entirely dependent upon serotype, strain of virus, density of infected vector, number of susceptible individuals [25].

8. Demographical facts about DF in Pakistan

History of dengue outbreaks in Pakistan dates back to early nineties (1990) usually after the commencement of rainy season. The first ever case of dengue was reported in Karachi in 1994, followed by second dengue outbreak in Baluchistan in 1995 [6,7,18,43]. But now the infection is appearing on regular basis with greater impacts in densely populated urban areas including upper and central Punjab, Sindh (Hyderabad, Karachi) *etc*. during and soon after the rainy season [43]. In 2004, 25 cases of dengue were reported from Karachi and Islamabad. Around 500 positive cases (including 13 deaths) were recorded in Karachi by the year 2005, followed by 5400 cases (55 deaths) in Nawabshah, Karachi, Sukhar, Islamabad and Rawalpindi and a larger proportion of dengue cases in Azad Jammu and Kashmir in 2006 [6,44,45]. Hyderabad, Karachi, Mirpurkhas, Haripur, Lahore, Islamabad and Rawalpindi were the more affected areas due to dengue outbreak with 2700 positive cases and 24 deaths in 2007. Likewise 1800 cases were reported in Lahore in 2008, followed by 320 cases reported in Gujranwala, Lahore and Sheikhpura in 2010 [46,47]. The worst strike of dengue (DF and DHF) was witnessed in 2011, with >20000 cases (including 300 deaths), with major havoc in Lahore followed by Faisalabad, Rawalpindi and Sargodha [21,43].

9. Strategies for management and control of dengue viral outbreaks

Since the epidemiology of DF with reference to Pakistan has changed during the recent era with increased frequency of occurrence and more drastic effects. Therefore immediate actions need to be taken for controlling dengue transmitting vector in order to prevent widespread of epidemics. Government of Pakistan has taken some crucial and important steps in managing the disease by launching various health line projects and awareness campaigns *etc*.

10. Role of Government of Pakistan in reducing viral epidemics at administrative level

Government of Pakistan as well as government of Punjab has taken many initiatives at administrative level for controlling the spread of disease. For instance, Punjab Health Line Project for Dengue has been launched by government of Punjab for sharing information on disease recognition (signs and symptoms), identification of epidemically affected areas along with provision of help to suspected individuals. The government has also established 24/7 operating mobile teams for providing free treatment to affectees with particular emphasis in rural areas [6].

11. Environmental management and community based approaches for reduction of mosquito population

Government of Pakistan has also taken environmental management initiatives in an effort to control dengue transmitting vector. Specialized community based programs have been started for proper solid waste collection and disposal in addition to improved water storage practices. The local community has been actively engaged along with concerned (municipal and public health) authorities to ensure street cleaning, proper drainage systems and elimination of pools of stagnant water. Proper coverage of water storage containers is made mandatory for preventing access of female mosquitoes for egg laying. The water air coolers must be emptied or drained out when not in use. Water containing objects like plant saucers should be removed from house-gardens, old tyres must be recycled or disposed off properly [6,7,15,17,48]. Moreover latest GIS based systems and tools are now being used for identification and mapping of hotspots for dengue infection in order to develop and implement national preparedness plans such as disease early warning and response systems [6,12,18,21].

12. Educational initiatives

Numerous awareness campaigns have been launched in association with electronic and print media. Additionally, dengue related information has also been included in the syllabi of various educational institutes especially at school level [17]. The Health Services Academy has also launched (12 months) diploma courses in medical entomology and disease vector control in Pakistan at postgraduate level for training the manpower to effectively and efficiently deal with vector-borne diseases [49]. Such programs will be more effective in producing more specialists to address the challenges posed by vector-borne diseases like dengue.

13. Reducing contact with dengue mosquito through personal actions

One of the most important measures for reduction in dengue virus transmission is the interruption of host-mosquito contact. Individuals should wear full length pants, trousers and full sleeved clothes to cover the limbs. The conventional measures such as mosquito repellents or lotions must be used for protection and to avoid mosquito biting during day time hours. Moreover coils, electric vapor mats, trap lights, insecticide treated nets and curtains *etc*. should be used to prevent direct contact with mosquitoes [7,17,21]. Windows and doors should be kept closed. Moreover, use of indoor air conditioning systems for cooling can also be helpful in avoiding direct contact of humans with transmitting vector [17].

14. Chemical methods for controlling mosquito population

Spray teams have been assembled by governmental authorities. These teams are responsible for time to time spraying, fumigation or fogging in residential areas and streets of various cities [6]. Suitable and long-lasting insecticides such as larvicides or adulticides are periodically applied to breeding habitats for reducing and controlling vector population [7,17].

15. Discovering effective antiviral vaccinations

Since no effective treatment or medication is available for dengue virus and/or fever to date. Therefore discovery of effective antiviral drugs and/or vaccines capable of targeting multiple serotypes of dengue virus is exigently needed. The vaccination needs to be effective, must provide life time immunity against viral pathogens as well as lessen the risks of formation of cross reacting antibodies [18,50]. Live attenuated and inactive dengue viruses, cloned and chimeric viruses, subunit and DNA vaccines are some of the approaches that are recently being used for developing effective vaccination for treating dengue viral infections [31].

Phytochemicals (plants and plant-derived compounds) have always served as important source in discovery of many antiviral drugs. Therefore activity of various plant-derived phenolic compounds has been evaluated by the researchers. For instance, Zandi *et al* [51] assessed antiviral activity of bioflavonoids against DENV-2 serotype at different infectious stages and virus replication. They concluded that quercetin was most effective viral inhibitor amongst all the tested compounds. Similarly, *Carica papaya* leaf extracts exhibited potential activity against dengue on administration to dengue infected patient [52]. Moreover, significance of various phytochemicals against DF has been shared in details by Sohail *et al* [53].

Most recent advancements related to dengue specific antiviral agents have been reviewed and explained explicitly by Julander *et al* [50]. Idrees and Ashfaq [54] have also reviewed the potential of siRNA as next generation therapeutic agents against dengue viral infection. Reports have revealed that vaccination (comprised of proteins from all the 4 viral strains) against dengue virus is under trial and testing processes (on monkeys). It is likely to be proved effective in treating the disease caused by all serotypes [18]. Moreover, the trials of tetravalent vaccines exhibited 89% sero-conversion against four serotypes after administration of third dose. While the administration of this vaccine in two doses provided 80%–90% protection in children. Similar results for sero-conversion rates have been recorded in adult volunteers for other vaccines prepared by Walter Reed Army Institute of Research [7]. Furthermore, numerous academic and academy associated research centers are actively engaged in discovering and developing anti-dengue viral drugs with hopeful results [17]. Researches and fully funded projects on various aspects of dengue virus and transmitting vectors (mosquito) with reference to serotypic and genotypic analysis also need to be launched by government of Pakistan on large scale at national level for understanding disease development and complications. These researches will hopefully be leading to breakthroughs in discovering and developing ideal dengue vaccines [55,56].

16. Biological methods for controlling mosquito population

Due to absence of effective vaccination and/or antiviral agents, current dengue vector control methods heavily rely on usage of chemically-based insecticides. Though seem to be useful in reducing larval or adult populations of mosquitoes during dengue epidemics, these insecticides are effective against *Ae. aegypti* population only over a small geographical scale. Conversely, non-targeted populations of other insects are also at risk of being affected by insecticides. Moreover accumulation of these insecticides in various environmental compartments leads to health and pollution problems along with evolution of insecticidal resistance among mosquito populations over passage of time. Hence, failure of chemical insecticide-based programs in preventing disease incidences and their escalating costs, especially in developing countries, has spurred the exploration of novel, cost effective and environment friendly control measures. These strategies should be capable of efficiently eliminating reliance upon such chemical toxins [16,57,58].

Biological control usually refers to introduction and/or release of biological agents persisting in nature into particular environment for controlling pests either through predation, disease or parasitism. These techniques have been widely utilized for protection of agriculturally important crops and livestock against pest outbreaks, plants or animals causing damage to environment. However, application of such biological control agents in public health sector is relatively recent approach that is still at infancy. This strategy for combating and controlling mosquito-borne epidemics has attracted the attention of many researchers. Presently there exists numerous biological control methods and many are under development process for controlling dengue vector for instance copepods, pathogenic bacteria and fungi *etc*. [58–60].

17. Fish and copepods as biological control agents

Introduction of mosquito predators such as fish, spiders, geckos and copepods (small crustaceans) is somewhat beneficial in combating mosquito-borne diseases. They prey or feed on mosquito larvae thus minimizing the social distress [58]. Such copepods trails for eradication of dengue have been conducted in Honduras, Vietnam [60–63], where field trails proved to be successful together with government and community participation. However being expensive and requiring continuous intercessions these strategies are unlikely to be implemented in peri-urban and urban communities [16,58].

18. Bioinsecticides as biological control agents

Constant monitoring and community interventions for applications of natural predators along with adverse environmental impacts (including humans and nature) posed by chemically-based insecticide vector control approaches. Additionally the loss of sensitivity of these vectors against insecticides has necessitated the discovery of environment friendly alternatives *i.e.* biopesticides [64,65]. Microbial insecticides comprised of microorganisms and their byproducts seem to be more effective and valuable alternatives for vector reduction in terms of target specificity and lower toxicity to pesticide user [66]. The entomopathogenic bacterium *Bacillus thuringiensis* subsp. *israelensis* (BTi) was found to be a safer alternative for controlling mosquitoes. Larvicidal activities of BTi described explicitly by Boyce *et al* [67] and Ben-Dov [68] have proven to be very effective in eradication of mosquito larvae [69]. However, short residual activity of BTi formulations limits its applicability in controlling mosquito-borne diseases [70].

Use of fungi as biocontrol agents has also been proposed because of their natural tendency to produce infective spores (conidia). These spores can adhere and penetrate within insect's cuticle where they flourish on nutritional reserves of host along with release of toxins that eventually kill the host [71]. *Beauveria bassiana* has emerged as potential candidate for control of *Ae. aegypti* causing significant mortality at larval as well as adult phases [58]. *Metarhizium anisopliae* has also been reported to cause higher mortality in adult *Ae. aegypti* [72]. Luz *et al* [73] and Garcia-Munguia *et al* [74] documented that fungal infection not only kills *Ae. aegypti* larvae and adults but also reduces fertility and slay the eggs. Such applications offer effective control of vector population with slow evolution of fungal resistance in contrast to insecticides. However the application and implementation of fungal formulations requires lots of research about the effects exerted by fungi on mosquito vectors, as these evidences revealed success stories from laboratories instead of field settings [75].

19. *Wolbachia* as biological control agent

Microbes associated with numerous arthropod vectors *e.g.* symbiotic bacteria as potential biocontrol agents have now gained much of attraction due to their ability to interfere with transmission of pathogens. Use of endosymbionts such as *Wolbachia pipientis* (*W. pipientis*) for control of pathogen transmission through mosquito vectors has been reported by Bordenstein and Rosengaus [59] and Thomas *et al* [76]. *W. pipientis* has recently emerged as biological weapon for control of mosquito-borne diseases because of its natural capability of interfering with viruses persisting within mosquitoes, environmental benignity and cost effectiveness in contrast to chemical control methods [16,76,77].

W. pipientis is a Gram negative bacterium having intracellular lifestyle and infects somatic and germ line tissues of hosts [78]. The bacterium is known to infect up to 70–76% insect species existing on earth [16]. The bacterium usually resides in insects, filarial nematodes, arachnids, crustaceans and different arthropod species [79–82]. *Wolbachia* is known to have an essential role in embryogenesis, induction of alterations in reproductive cycles of hosts, as antibiotic treatment of infected individuals and in animals for inhibition of microfilariae [81,83].

Utilization of *W. pipientis* for controlling mosquito-borne diseases has been pursued as a recent strategy with more promising results to alter *Ae. aegypti* populations and reduce their capability to transmit dengue virus pathogens in humans [84,85]. A number of different *Wolbachia* strains including wMel and wMelPop from *Drosophila* and aAlbB from *Ae. albopictus* have been reported to be introduced into *Ae. aegypti* [86,87]. *Wolbachia* invades and sustains itself inside mosquitoes where it induces series of reproductive abnormalities within the host for increasing reproduction of female mosquitoes. Thereby enhancing their own reproduction and transmission within host populations [16,79,88,89]. These reproductive abnormalities include feminization (transformation of genotypic males into phenotypic females), modifications within male sperm and parthenogenic reproduction of females along with other behavioral effects plus altering the biting position *etc.* [16,89]. The bacterium has also been reported to affect nutritional status, development of their hosts, reduction of host's lifespan due to over-replication and occupation of brain tissues of the infected hosts and interference with pathogen replication [16,90–92].

Before migration into salivary glands of mosquito and transmission into humans, dengue virus requires sufficient incubation period. Hence lifespan of mosquitoes is very critical in disease transmission. Consequently wMelPop strain has been proposed to alter and shorten the lifespan of *Ae. aegypti* and favors elimination of older mosquitoes to lessen the risks of dengue transmission [90,93]. Similarly, wMel strain has also been reported to reduce (adult) mosquito's lifespan around 10% [94].

Wolbachia transfections can also induce cytoplasmic incompatibilities a type of embryonic lethality that occurs during mating of *Wolbachia* infected males with uninfected females. Thereby causes reduction in egg hatching and producing unviable offsprings [58,95]. Cook *et al* [96] and Helinski *et al* [97] reported transfection of *Ae. aegypti* with two *Wolbachia* strains namely wMel (benign) and wMelPop (virulent) and observed induction of cytoplasmic incompatibilities within their hosts. Such transfections offer a reproductive advantage to *Wolbachia* infected females over uninfected populations due to reduction in fecundity of uninfected females and impacting dengue virus transmission [98]. The cytoplasmic incapability is basically meant for *Wolbachia* for enhancing number of infected individuals within host population in order to boost their own maternal transmission [85].

To date many stable *Wolbachia* transfected cell lines have been established and maintained for controlling disease transmitting vector (*Ae. Aegypti*). McMeniman et al. [92] transferred wMelPop into *Ae. aegypti* after 4 years of maintenance of *Wolbachia* within *Ae. albopictus* and generated stable wMelPop-CLA (cell-line-adapted) lines with 100% maternal transmission rates. These transfected mosquitoes have exhibited 50% reduction in adult lifespan in contrast to uninfected counterparts [92,99,100]. Similarly wAlbB strain of *Wolbachia* (from *Ae. albopictus*) has also been transfected successfully into *Ae. aegypti* [101].

Additionally, *Wolbachia* transinfections have also been reported to reduce the susceptibility of *Ae. aegypti* to dengue virus [102]. As depicted by Pan *et al* [103] that wAlbB transfected *Ae. aegypti* exhibited inhibition of dengue virus due to activation of ROS of Toll pathway. Such *Wolbachia* mediated pathogen interferences have caused significant reduction in competency of vector for dengue virus. These transfections also offer some sort of protection against DENV within mosquito cell lines despite lack of whole organism or tissue specific immunity within these cells [94,101,104,105].

Moreover, considerably higher bacterium transmission frequencies to next progeny of *Wolbachia* transfected mosquitoes (females) have been documented by McMeniman *et al* [92], where the bacterium invades uninfected populations by inducing reproductive abnormalities. Thus favors propagation of infected females [92,106]. For example, 100% transmission efficiency of wMelPop-CLA strains has been reported in offsprings of transinfected *Ae. aegypti* [92]. wMelPop-CLA transfection in *Ae. aegypti* are known to induce higher metabolic rates in mosquitoes [107], feeding impairments in older mosquitoes [108], reduced hatching and egg survival during phases of dormancy [109]. Such transfections suggest *Wolbachia* as best control strategies against dengue vector by suppressing egg hatching in wet seasons [16]. Furthermore, modifications in larval nutritional levels, duration of larval development as well as wing size of male *Ae. aegypti* has also been observed by Barrett *et al* [110] and Yeap *et al* [111]. wMelPop-CLA infected mosquitoes also showed significant reduction in replication of DENV-2 along with 56% reduction in fertility of infected females as compared to uninfected ones [94,104]. Additionally, reduction in blood meal ingestion by wMelPop-CLA transfected mosquitoes has been observed by Turley *et al* [108] and Moreira *et al* [112]. All the three types of *Wolbachia i.e.* wMel, wMelPop and wAlbB have been reported to inhibit dengue virus replication at significant levels along with dissemination within *Ae. aegytpi* population, thereby causing either partial or complete blockage of virus transfer in humans [94,101,104]. Analysis of pooled saliva extracts revealed complete blockage of viral transmission by wAlbB and wMelPop-CLA infected mosquitoes [94].

Wolbachia transfections within host tissues have been reported to interfere with DENV replication where the bacterium competes for cellular resources and sequesters fatty acids of host cells that are required for DENV replication [104,113,114]. The immunofluorescence studies of *Wolbachia* transfected *Ae. aegypti* revealed no co-localization of bacterium with dengue virus and suggested metabolic competition between the two [104]. This phenomenon has been illustrated in detail by Bourtzis *et al* (2014). This metabolic competition is further supported by the fact that significant differences were observed in density as well as tissue distribution between wMel and wMelPop strains (though closely related to each other) in infected *Ae. aegypti*. This wMelPop strain is known for boosting phenotypic interferences in mosquitoes against dengue virus due to higher concentration [94,104]. Studies have also revealed a strong negative correlation between genome copy of dengue virus and *Wolbachia*, where dengue infection has been inhibited completely at *Wolbachia* density of up to 961.8 wsp/actin [115]. This has suggested dependency of *Wolbachia* mediated viral interferences on bacterial density within the transfected hosts [16].

Many researchers have reported that *Wolbachia* strains utilize host microRNAs (miRNAs) for altering and manipulating gene expression of host and displaying antiviral response by establishing infections in *Ae. aegypti* [116–120]. The upregulation of host aae-miR-2940 in cell lines of *Wolbachia* transfected mosquitoes has been reported by Hussain *et al* [116] where it targets metalloprotease genes and facilitates colonization of bacterium within the host. Similarly *Wolbachia* exploits this MiRNA of *Ae. aegypti* for targeting methyltransferase gene *DnmmtA2* and alters it expression which eventually interferes with dengue virus replication. This interference inhibits virus establishment within the host thus providing a pathway for controlling disease transmission. Moreover, induction of aae-miR12 by *Wolbachia* down regulates DNA replication (*MCM6*) and monocarboxylate transporter (*MCT1*) genes. Therefore, plays an important role in maintaining *Wolbachia* infection within cell lines of host [117].

Field trials for release of *Wolbachia* infected mosquitoes have been performed in different countries. For instance, wMel infected *Ae. aegypti* were introduced in two towns in North Queensland, Australia during wet season where wMel strains successfully invaded and transformed the local populations of *Ae. aegypti* within few months of being released [118–122]. While persistence of virus blocking property of *Wolbachia* in field released infected mosquitoes was confirmed by Frentiu *et al* [123]. These trials suggested that *Wolbachia* strains can efficiently be deployed as biocontrol agents against dengue vectors. No further evidences of transfer of bacteria into humans via mosquito bites, or the lateral transfer to nontarget species as well as to mosquito predators has been observed [16]. But the likelihood for horizontal transfer of *Wolbachia* DNA within mosquito genomes has been reported by McNaughton *et al* [124] and Popovici *et al* [125].

20. *Wolbachia* based biocontrol research being carried out in Pakistan

The *Wolbachia* strains that have already been explored and successfully exploited in other parts of the world can also be evaluated for stopping dengue virus transmission through eradication of vector populations in Pakistan. However implementation and application of such control strategies is largely dependent upon geographical and climatic conditions of particular settings, scientific evidences as well as region specific appropriateness [126]. Therefore, scientific community needs to explore the potentials of native or indigenous endosymobiotic strains and their transfer within *Ae. aegypti*. Many research projects in collaboration with international institutes are currently being conducted in Pakistan for reduction in transmission of dengue virus. For example, a project aimed at making *Ae. aegypti* incapable of carrying and transmitting dengue virus in humans through genetic modifications using *Wolbachia* has been launched in collaboration with Michigan State University, USA at Government College University, Lahore [127]. Moreover, interagency partnerships and collaborations of research organizations for *Wolbachia* based control technologies in Pakistan can be helpful in providing new means and tools for precise assessment of *Wolbachia* transfections in mosquitoes, their impact on vector population as well as role in blocking virus transmission *etc*. These collaborations among research organizations at international level may facilitate and strengthen the research networks by encouraging capacity building. They can also play crucial role in filling the funding gaps that hinder focused research efforts [128,129]. Resource mobilization, political participation and support are also needed to promote the efforts being made to prevent and control dengue using biological control agents in the region. Government of Pakistan and higher education commission needs to setup and release dengue virus research funds for reproducing researches going on in other parts of the world like Australia, USA and British biotechnology company Oxitec *etc*. [129,130]. Such initiatives will encourage the researchers to explore and evaluate the capability of indigenous *Wolbachia* species to eliminate dengue epidemics through high-tech facilities and equipment. Various institutes of Pakistan intend to collaborate with international institutions to figure out the potentials and implementation of *W. pipientis* for minimizing and eradicating dengue vector populations form the region.

Though *W. pipientis* seems to be very efficient in reduction and eradication of dengue vector, still the interactions and molecular mechanisms which affect the performance of mosquitoes are largely unknown. Moreover, effects exerted by *Wolbachia* on dengue virus transmission and epidemiology in endemic areas are yet ambiguous and require large scale clinical trials before being released into field [131]. These untold stories have opened new portals for research activities to understand biological and evolutionary relations between *Wolbachia* and their symbionts. The knowledge obtained can potentially be applied for control of mosquito-borne diseases.

21. Conclusions

Prevalence of dengue epidemics throughout Pakistan is a leading cause of morbidity and mortality of thousands of individuals every year. This situation has spurred the development of effective control measures for controlling and eradicating the transmission vectors. Though many efforts at governmental and community levels have been made to effectively deal with *Ae. aegypti*. Unfortunately the failure of chemical based strategies, development of resistance against insecticides with subsequent threats to humans and environment, hurdles in implementation of other biological control measures due to effectiveness over small geographical scales has forced the scientific community to discover economically feasible and environment friendly alternatives. *Wolbachia* mediated vector control strategies as described above offer promising results for control of dengue virus. Though still at infancy this strategy seems to be very effective without posing any threat to human health as well as the environment. So there is a dire need to explore the potentials of native and/or indigenous endosymobiotic *Wolbachia* strains and their transfer within *Ae. aegypti* for ensuring our region to be free of dengue virus. All this seems to be possible only with support of government and private funding agencies.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Nieto NC, Khan K, Uhllah G, Teglas MB. The emergence and maintenance of vector-borne diseases in the Khyber Pakhtunkhwa province, and the federally administered tribal areas of Pakistan. *Front Physiol* 2012; **3**: 250.

[2] Bhutta ZA, Sommerfeld J, Lassi ZS, Salam RA, Das JK. Global burden, distribution and interventions for infectious diseases of poverty. *Infect Dis Poverty* 2014; **3**: 21-27.

[3] Hill CA, Kafatos FC, Stansfield SK, Collins FH. Arthropod-borne diseases: vector control in the genomics era. *Nat Rev Microbiol* 2005; **3**: 262-268.

[4] Burt A. Heritable strategies for controlling insect vectors of disease. *Philos Trans R Soc B* 2014; **369**: 1-6.

[5] World Health Organization. *Dengue hemorrhagic fever, diagnosis, treatment, prevention and control*. 2nd ed. Geneva: World Health Organization; 2010, p. 4-45.

[6] Khan E, Hasan R. Dengue infection in Asia: a regional concern. *J Postgrad Med Inst* 2011; **26**(1): 1-6.

[7] Sulehri MA, Hussain R, Gill NI. Dengue fever, its diagnosis, treatment, prevention and control. *A P M C* 2012; **6**(1): 22-27.

[8] Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 cases of death for 20 age groups in 1990 and 2010: a systematic analysis for the global burden of disease study 2010. *Lancet* 2012; **380**(9859): 2095-2128.

[9] Nsungwa-Sabiiti J, Peterson S, Pariyo G, Ogwal-Okeng J, Petzold MG, Tomson G. Home-based management of fever and malaria treatment practices in Uganda. *Trans R Soc Trop Med Hyg* 2007; **101**: 1199-1207.

[10] Focks DA, Brenner RJ, Hayes J, Daniels E. Transmission thresholds for dengue in terms of *Aedes aegypti* pupae per person with discussion of their utility in source reduction efforts. *Am J Trop Med Hyg* 2000; **62**: 11-18.

[11] Khalil MAM, Tan J, Khalil MAU, Awan S, Rangasami M. Predictors of hospital stay and mortality in dengue virus infection-experience from Aga Khan University hospital Pakistan. *BMC Res Notes* 2014; **7**: 473-480.

[12] Raza FA, Rehman S, Khalid R, Ahmad J, Ashraf S, Iqbal M, et al. Demographic and clinic-epidemiological features of dengue fever in Faisalabad, Pakistan. *PLoS One* 2014; **9**(3): e89868; http://dx.doi.org/10.1371/journal.pone.0089868.

[13] Shakoor MT, Ayub S, Ayub Z. Dengue fever: Pakistan's nightmare. *WHO South-East Asia J Pub Health* 2012; **1**(3): 229-231.

[14] Naseem S, Farheen A, Muhammad A, Fauzia R. Dengue fever outbreak in Karachi, 2005 – a clinical experience. *Infect Dis J* 2005; **14**(4): 115-117.

[15] Jahan F. Dengue fever in Pakistan. *Asia Pac Fam Med* 2011; **10**: 1-4.

[16] Iturbe-Ormaetxe I, Walker T, O'Neill SL. *Wolbachia* and the biological control of mosquito-borne disease. *EMBO Rep* 2011; **12**(6): 508-518.

[17] Sherin A. Dengue fever: a major public health concern in Pakistan. *KUST Med J* 2011; **3**(1): 1-3.

[18] Zafar H, Bukhari KT, Lodhi GM. Global prevalence of dengue viral infection, its pathogenesis, diagnostic and preventive approaches. *Asian J Agri Biol* 2013; **1**(1): 38-42.

[19] Halstead SB. Dengue virus-mosquito interactions. *Annu Rev Entomol* 2008; **53**: 273-291.

[20] Raja NS, Singh NN, Mehmood T, Sethi H, Raja NH, Janjua KA. Dengue viral infections: a major public health issue. *Int J Pathol* 2009; **7**: 4-12.

[21] Khanani MR, Arif A, Shaikh R. Dengue in Pakistan: journey from disease free to a hyper endemic nation. *J Dow Univ Health Sci Karachi* 2011; **5**(3): 81-84.

[22] Costa RL, Voloch CM, Schrago CG. Comparative evolutionary epidemiology of dengue virus serotypes. *Infect Gene Evol* 2012; **12**: 309-314.

[23] Drumond BP, Mondini A, Schmidt DJ, Bronzoni RVD, Bosch I, Nogueira ML. Circulation of different lineages of dengue virus 2, genotype American/Asian in Brazil: dynamics and molecular and phylogenetic characterization. *PLoS One* 2013; **8**(3): 1-10.

[24] Koo C, Nasir A, Hapuarachchi HC, Lee K, Hasan Z, Ng L, et al. Evolution and heterogeneity of multiple serotypes of dengue virus in Pakistan, 2006–2011. *Virol J* 2013; **10**: 275-285.

[25] Khan J, Khan A. Incidence of dengue in 2013: dengue outbreak in District Swat, Khyber Pakhtunkhwa, Pakistan. *Int J Fauna Biol Stud* 2015; **2**(1): 1-7.

[26] Murray NEA, Quam MB, Wilder-Smith A. Epidemiology of dengue: past, present and future prospects. *Clin Epidemiol* 2013; **5**: 299-309.

[27] Rodenhuis-Zybert IA, Wilschut J, Smit JM. Dengue virus life cycle: viral and host factors modulating infectivity. *Cell Mol Life Sci* 2010; **67**: 2773-2786.

[28] Ali A, Rehman H, Nisar M, Rafique S, Ali S, Hussain A, et al. Seroepidemiology of dengue fever in Khyber Pakhtunkhawa, Pakistan. *Int J Infect Dis* 2013; **17**: 518-523.

[29] Lambrechts L, Scott TW, Gubler DJ. Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. *PLoS Negl Trop Dis* 2010; **4**(5): 646-654.

[30] Bosio CF, Fulton RE, Salasek ML, Beaty BJ, Black WC. Quantitative trait loci that control vector competence for degue-

2 virus in the mosquito *Aedes aegypti*. *Genetics* 2000; **156**: 687-689.
[31] Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, et al. Dengue: a continuing global threat. *Nat Rev Microbiol* 2010; **8**(12): 7-16.
[32] Kunst RL. Vector control for dengue and other mosquito-borne diseases. In: Robinson WH, Bajomi D, editors. *Proceedings of the sixth international conference on urban pests*. Hungary: OOK-Press Kft; 2008, p. 315-317.
[33] Shu PY, Hang JH. Current advances in dengue diagnosis. *Clin Diagn Lab Immunol* 2004; **11**(4): 642-650.
[34] Siqueira JJ, Martelli CM, Coelho GE, Simplicio AC, Hatch DL. Dengue and dengue hemorrhagic fever, Brazil, 1981–2002. *Emerg Infect Dis* 2005; **11**(1): 48-53.
[35] Rajapakse S. Dengue shock. *J Emerg Trauma Shock* 2011; **4**(1): 120-127.
[36] Usman M, Jamal S, Tabassum S, Zafran M. Outcome of dengue fever in adults. *Gomal J Med Sci* 2011; **9**(11): 70-73.
[37] Sellahewa KH. Pathogenesis of dengue hemorrhagic fever and its impact on case management. *ISRN Infect Dis* 2012; **2013**: 6571646; http://dx.doi.org/10.5402/2013/571646.
[38] Mukhtar F, Salim M, Farooq A. Outbreak of dengue in Lahore: study of risk factors. *J Ayub Med Coll Abbottabad* 2012; **24**(2): 99-101.
[39] Manderson L, Aagaard-Hansen J, Allotey P, Gyapong M, Sommerfeld J. Social research on neglected diseases of poverty: continuing and emerging themes. *PLoS Negl Trop Dis* 2009; **3**(2): 322-327.
[40] Liese BH, Schubert L. Official development assistance for health-how neglected are neglected tropical diseases? An analysis of health financing. *Int Health* 2009; **1**(2): 141-147.
[41] Rasheed SB, Butlin RK, Boots M. A review of dengue as an emerging disease in Pakistan. *Public Health* 2013; **127**: 11-17.
[42] Khan MA, Ellis EM, Tissera HA, Alvi MY, Rahman FF, Masud F, et al. Emergence and diversification of dengue 2 cosmopolitan genotype in Pakistan, 2011. *PLoS One* 2013; **8**(3): 1-5.
[43] Ahmed S, Mohammad WW, Hamid F, Akhter A, Afzal RK, Mahmood A. The 2011 dengue hemorrhagic fever outbreak in Lahore: an account of clinical parameters and pattern of hemorrhagic complications. *J Coll Physicians Surg Pak* 2013; **23**(7): 463-467.
[44] Khan E, Siddiqui J, Shakoor S, Mehraj V, Jamil B, Hasan R. Dengue outbreak in Pakistan 2006: experience at tertiary care center. *Trans R Soc Trop Med Hyg* 2007; **101**: 1114-1119.
[45] Tang JW, Khanani MR, Zubairi AM, Lam WY, Lai F, Hashmi K, et al. A wide spectrum of dengue IgM and PCR positivity post-onset of illness in a large dengue 3 outbreak in Pakistan. *J Med Virol* 2008; **80**: 2113-2121.
[46] Humayoun MA, Waseem T, Jawa AA, Hashmi SS, Akram J. Multiple dengue serotypes and high frequency of dengue hemorrhagic fever at two tertiary care hospitals in Lahore during the 2008 dengue virus outbreak in Punjab, Pakistan. *Int J Infect Dis* 2010; **14**(3): 54-59.
[47] Mahmood N, Rana MY, Qureshi Z, Mujtaba G, Shaukat U. Prevalence and molecular characterization of dengue viruses serotypes in 2010 epidemic. *Am J Med Sci* 2012; **343**: 61-64.
[48] Kay BH, Nam VS, Tien TV, Yen NT, Phong TV, Diep VTB, et al. Control of Aedes vectors of dengue in three provinces of Vietnam by use of *Mesocyclops* (Copepoda) and community-based methods validated by entomologic, clinical, and serological surveillance. *Am J Trop Med Hyg* 2002; **66**: 43-48.
[49] Rathor HR, Mnzava A, Bile KM, Hafeez A, Zaman S. Launching the first postgraduate diploma in medical entomology and disease vector control in Pakistan. *East Mediterr Health J* 2010; **16**: 76-81.
[50] Julander JG, Perry ST, Shresta S. Important advances in the field of anti-dengue virus research. *Antivir Chem Chemother* 2011; **21**: 105-116.
[51] Zandi K, Teoh B, Sam S, Wong P, Mustafa MR, AbuBakar S. Antiviral activity of four types of bioflavonoid against dengue virus type-2. *Virol J* 2011; **8**: 560-571.
[52] Ahmad N, Fazal H, Ayaz M, Abbasi BH, Mohammad I, Fazal L. Dengue fever treatment with *Carica papaya* leaves extracts. *Asian Pac J Trop Biomed* 2011; **1**(4): 330-333.
[53] Sohail F, Alam GM, Khan RR, Naeem R. How to cope with dengue in developing countries like Pakistan. *Asian J Animal Vet Adv* 2011; **6**(12): 1094-1124.
[54] Idrees S, Ashfaq UA. RNAi: antiviral therapy against dengue virus. *Asian Pac J Trop Biomed* 2013; **3**(3): 232-236.
[55] Arunachalam N, Tana S, Espino F, Kittayapong P, Abeyewickreme W, Wai KT, et al. Eco-bio-social determinants of dengue vector breeding: a multicountry study in urban and peri-urban Asia. *Bull World Health Organ* 2010; **88**(3): 173-184.
[56] Whitehorn J, Farrar J. Dengue. *Br Med Bull* 2010; **95**: 161-173.
[57] McGraw EA, O'Neill SL. Beyond insecticides: new thinking on an ancient problem. *Nat Rev Microbiol* 2013; **11**: 181-193.
[58] Frentiu FD, Walker T, O'Neill SL. Biological control of dengue and *Wolbachia*-based strategies. In: Gubler DJ, Ooi EE, Vasudevan S, Farrar J, editors. *Dengue and dengue hemorrhagic fever*. 2nd ed. London: CAB International; 2014a, p. 537-547.
[59] Bordenstein S, Rosengaus RB. Discovery of a novel *Wolbachia* super group in Isoptera. *Curr Microbiol* 2005; **51**: 393-398.
[60] Kay BH, Vu SN. New strategy against *Aedes aegypti* in Vietnam. *Lancet* 2005; **365**: 613-617.
[61] Marten GG, Borjas G, Cush M, Fernández E, Reid JW. Control of larval *Aedes aegypti* (Diptera: *Culicidae*) by cyclopoid copepods in peridomestic breeding containers. *J Med Entomol* 1994; **31**: 36-44.
[62] Nam VS, Yen NT, Phong TV, Ninh TU, Mai LQ, Lo LV, et al. Elimination of dengue by community programs using *Mesocyclops* (copepoda) against *Aedes aegypti* in central Vietnam. *Am J Trop Med Hyg* 2005; **72**: 67-73.
[63] Kay BH, Hanh TTT, Le NH, Quy TM, Nam VS, Hang PVD, et al. Sustainability and cost of a community-based strategy against *Aedes aegypti* in northern and central Vietnam. *Am J Trop Med Hyg* 2010; **82**: 822-830.
[64] Weill M, Lutfalla G, Mogensen K, Chandre F, Berthomieu A, Berticat C, et al. Insecticide resistance in mosquito vectors. *Nature* 2003; **423**: 136-137.
[65] Federici BA, Park HW, Bideshi DK. Overview of the basic biology of *Bacillus thuringiensis* with emphasis on genetic engineering of bacterial larvicides for mosquito control. *Open Toxinol J* 2010; **3**: 83-100.
[66] Ramírez-Lepe M, Ramírez-Suero M. Biological control of mosquito larvae by *Bacillus thuringiensis* subsp. *israelensis* insecticides-pest engineering. In: Perveen F, editor. *Insecticidespest engineering*. Rijeka: InTech; 2012, p. 239-264.
[67] Boyce R, Lenhart A, Kroeger A, Velayudhan R, Roberts B, Horstick O. *Bacillus thuringiensis israelensis* (Bti) for the control of dengue vectors: systematic literature review. *Trop Med Int Health* 2013; **18**(5): 564-577.
[68] Ben-Dov E. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins* 2014; **6**: 1222-1243.
[69] Lacey LA. *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *J Am Mosq Control Assoc* 2007; **23**: 133-163.
[70] Margalith Y, Ben-Dov E. Biological control by *Bacillus thuringiensis* subsp. *israelensis*. In: Rechcigl JE, Rechcigl NA, editors. *Insect pest management: techniques for environmental protection*. Boca Ratonv: CRC Press; 2000, p. 243-301.
[71] Scholte EJ, Knols BG, Samson RA, Takken W. Entomopathogenic fungi for mosquito control: a review. *J Insect Sci* 2004; **4**: 19.
[72] Scholte EJ, Takken W, Knols BGJ. Infection of adult *Aedes aegypti* and *A. Albopictus* mosquitoes with the entomopathogenic fungus *Metarhizium anisopliae*. *Acta Trop* 2007; **102**: 151-158.
[73] Luz C, Tai MHH, Santos AH, Silva HH. Impact of moisture on survival of *Aedes aegypti* eggs and ovicidal activity of

Metarhizium anisopliae under laboratory conditions. *Mem Inst Oswaldo Cruz* 2008; **103**: 214-215.
[74] Garcia-Munguia AM, Garza-Hernández JA, Rebollar-Tellez EA, Rodríguez-Pérez MA, Reyes-Villanueva F. Transmission of *Beauveria bassiana* from male to female *Aedes aegypti* mosquitoes. *Parasites Vec* 2011; **4**: 24.
[75] Knols BGJ, Bukhari T, Farenhorst M. Entomopathogenic fungi as the next-generation control agents against malaria mosquitoes. *Future Microbiol* 2010; **5**: 339.
[76] Thomas P, Kenny N, Eyles D, Moreira LA, O'Neill SL, Asgari S. Infection with the wMel and wMelPop strains of *Wolbachia* leads to higher levels of melanization in the hemolymph of *Drosophila melanogaster*, *Drosophila simulans* and *Aedes aegypti*. *Dev Comp Immunol* 2010; **35**: 360-365.
[77] Kambris Z, Cook PE, Phuc HK, Sinkins SP. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* 2009; **326**: 134-136.
[78] Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, et al. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochem Mol Biol* 1999; **29**: 153-160.
[79] Jeyaprakash A, Hoy MA. Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* 2000; **9**: 393-405.
[80] Bandi C, Trees AJ, Brattig NW. *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. *Vet Parasitol* 2001; **98**: 215-238.
[81] Foster WA, Takken W. Nectar-related vs. human-related volatiles: behavioral response and choice by female and male *Anopheles gambiae* (Diptera: *Culicidae*) between emergence and first feeding. *Bull Entomol Res* 2004; **94**(2): 145-157.
[82] Lauren R, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL. Functional test of the influence of *Wolbachia* genes on cytoplasmic incompatibility expression in *Drosophila melanogaster*. *Insect Mol Biol* 2009; **20**(4): 75-85.
[83] Hoerauf A, Volkmann L, Nissen-Pàehle K, Schmetz C, Autenrieth I, Büttner DW. Targetting of *Wolbachia* endobacteria in *Litomosoides sigmodontis*: comparison of tetracyclines with chloramphenicol, macrolides and ciprofloxin. *Trop Med Int Health* 2000; **5**: 275-279.
[84] Hoffmann S, Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. *Wolbachia* and virus protection in insects. *Science* 2009; **322**(5902): 702-709.
[85] Ricci I, Valzano M, Ulissi U, Epis S, Cappelli A, Favia G. Symbiotic control of mosquito borne disease. *Pathog Glob Health* 2012; **106**(7): 380-385.
[86] Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc Natl Acad Sci U S A* 2010; **107**: 769-774.
[87] Kumar S, Blaxter ML. Simultaneous genome sequencing of symbionts and their hosts. *Symbiosis* 2011; **55**: 119-126.
[88] Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. How many species are infected with *Wolbachia*? A statistical analysis of current data. *FEMS Microbiol Lett* 2008; **281**: 215-220.
[89] Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol* 2008; **6**: 741-751.
[90] Sinkins SP, O'Neill SL. *Wolbachia* as a vehicle to modify insect populations. In: Handler AM, James AA, editors. *Insect transgenesis: methods and applications*. Boca Raton: CRC Press; 2000, p. 271-288.
[91] Brownstein JS, Hett E, O'Neill SL. The potential of virulent *Wolbachia* to modulate disease transmission by insects. *J Invertebr Pathol* 2003; **84**: 24-29.
[92] McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang YF, et al. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* 2009; **323**: 141-144.
[93] Texeira L, Ferreira A, Ashburner M. The bacterial symbiont *Wolbachia* induces resistance to RNA virus infections in *Drosophila melanogaster*. *PLoS Biol* 2008; **6**(12): 2753-2763.
[94] Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, et al. The wMel Wolbachia strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* 2011; **476**(7361): 450-455.
[95] Iturbe-Ormaetxe I, Burke GR, Riegler M, O'Neill SL. Distribution, expression and motif variability of ankyrin domain genes in *Wolbachia pipientis*. *J Bacteriol* 2005; **187**: 5136-5145.
[96] Cook PE, McMeniman CJ, O'Neill SL. Modifying insect population age structure to control vector-borne disease. *Adv Exp Med Biol* 2008; **627**: 126-140.
[97] Helinski ME, Valerio L, Facchinelli L, Scott TW, Ramsey J, Harrington LC. Evidence of polyandry for *Aedes aegypti* in semifield enclosures. *Am J Trop Med Hyg* 2012; **86**(4): 635-641.
[98] Turelli M. Cytoplasmic incompatibility in populations with overlapping generations. *Evolution* 2010; **64**(1): 232-241.
[99] Williams CR, Johnson PH, Long SA, Rapley LP, Ritchie SA. Rapid estimation of *Aedes aegypti* population size using simulation modeling, with a novel approach to calibration and field validation. *J Med Entomol* 2008; **45**: 1173-1179.
[100] McMeniman CJ, Lane AM, Fong AW, Voronin DA, Iturbe-Ormaetxe I, Yamada R, et al. Host adaptation of a *Wolbachia* strain after long-term serial passage in mosquito cell lines. *Appl Environ Microbiol* 2008; **74**: 6963-6969.
[101] Bian G, Xu Y, Lu P, Xie Y, Xi Z. The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS Pathog* 2010; **6**(4): 1-10.
[102] Bourtzis K, Dobson SL, Xi Z, Rasgon JL, Calvitti M, Moreira LA, et al. Harnessing mosquito-*Wolbachia* symbiosis for vector and disease control. *Acta Trop* 2014; **132**: 150-163.
[103] Pan X, Zhou G, Wu J, Bian G, Lu P, Raikhel AS, et al. *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. *PNAS* 2012; **109**: 23-31.
[104] Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya and plasmodium. *Cell* 2009a; **139**(7): 1268-1278.
[105] Frentiu FD, Robinson J, Young PR, McGraw EA, O'Neill SL. *Wolbachia*-mediated resistance to dengue virus infection and death at the cellular level. *PLoS One* 2010; **5**: 1-8.
[106] Ritchie SA, Johnson PH, Freeman AJ, Odell RG, Graham N, Dejong PA, et al. A secure semi-field system for the study of *Aedes aegypti*. *PLoS Negl Trop Dis* 2011; **5**: e988; http://dx.doi.org/10.1371/journal.pntd.0000988.
[107] Evans O, Caragata EP, McMeniman CJ, Woolfit M, Green DC, Williams CR, et al. Increased locomotor activity and metabolism of *Aedes aegypti* infected with a life-shortening strain of *Wolbachia pipientis*. *J Exp Biol* 2009; **212**: 1436-1441.
[108] Turley AP, Moreira LA, O'Neill SL, McGraw EA. *Wolbachia* infection reduces blood-feeding success in the dengue fever mosquito, *Aedes aegypti*. *PLoS Negl Trop Dis* 2009; **3**: e516; http://dx.doi.org/10.1371/journal.pntd.0000516.
[109] McMeniman CJ, O'Neill SL. A virulent *Wolbachia* infection decreases the viability of the dengue vector *Aedes aegypti* during periods of embryonic quiescence. *PLoS Negl Trop Dis* 2010; **4**(7): e748; http://dx.doi.org/10.1371/journal.pntd.0000748.
[110] Barrett T, Troup DB, Wilhite SE, Ledoux P, Rudnev D. NCBI GEO: archive for high-throughput functional genomic data. *Nucleic Acids Res* 2009; **37**: 885-890.
[111] Yeap HL, Mee P, Walker T, Weeks AR, O'Neill SL, Johnson P, et al. Dynamics of the popcorn *Wolbachia* infection in outbred *Aedes aegypti* informs prospects for mosquito vector control. *Genetics* 2011; **187**(2): 583-595.
[112] Moreira LA, Saig E, Turley AP, Ribeiro JMC, O'Neill SL, McGraw EA. Human probing behavior of *Aedes aegypti* when infected with a life-shortening strain of *Wolbachia*. *PLoS Negl Trop Dis* 2009b; **3**(12): e568; http://dx.doi.org/10.1371/journal.pntd.0000568.
[113] Heaton NS, Perera R, Berger KL, Khadka S, Lacount DJ, Kuhn RJ, et al. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases

cellular fatty acid synthesis. *Proc Natl Acad Sci U S A* 2010; **107**: 17345-17350.

[114] Heaton NS, Randall G. Dengue virus-induced autophagy regulates lipid metabolism. *Cell Host Microbe* 2010; **8**: 422-432.

[115] Lu P, Bian G, Pan X, Xi Z. *Wolbachia* induces density-dependent inhibition to dengue virus in mosquito cells. *PLoS Negl Trop Dis* 2012; **6**: e1754; http://dx.doi.org/10.1371/journal.pntd.0001754.

[116] Hussain M, Frentiu FD, Moreira LA, O'Neill SL, Asgari S. *Wolbachia* uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector *Aedes aegypti*. *PNAS* 2011; **108**: 9250-9255.

[117] Osei-Amo S, Hussain M, O'Neill SL, Asgari S. *Wolbachia*-induced aae-miR-12 miRNA negatively regulates the expression of *MCT1* and *MCM6* genes in *Wolbachia*-infected mosquito cell line. *PLoS One* 2012; **7**: e50049; http://dx.doi.org/10.1371/journal.pone.0050049.

[118] Raquin V. *Innate immunity and multi-infections in the mosquito (Diptera: Culicidae): functional study of the interactions Wolbachia-arbovirus-Aedes albopictus* [PhD thesis]. France: University of Lyon; 2012.

[119] Schnettler E, Donald C, Human S, Watson M, Siu RWC, McFarlane M, et al. Knockdown of piRNA pathway proteins results in enhanced Semliki Frest virus production in mosquito cell. *J Gen Virol* 2013; http://dx.doi.org/10.1099/vir.0.053850-0.

[120] Zhang G, Hussain M, O'Neill SL, Asgari S. *Wolbachia* uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti*. *PNAS* 2013; http://dx.doi.org/10.1073/pnas.1300052110.

[121] Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaexte I, Johnson PH, Muzzi F, et al. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* 2011; **476**: 454-457.

[122] Bull JJ, Turelli M. *Wolbachia* versus dengue: evolutionary forecasts. *Evol Med Pub Health* 2013; **1**: 197-207.

[123] Frentiu FD, Zakir T, Walker T, Popovici J, Pyke AT, van-den-Hurk A, et al. Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with Wolbachia. *PLoS Negl Trop Dis* 2014b; **8**(2): 1-10.

[124] McNaughton D, Clough A, Johnson PH, Ritchie SA, O'Neill SL. Beyond the back yard: lay knowledge about *Aedes aegypti* in northern Australia and its implications for policy and practice. *Acta Trop* 2010; **116**: 74-80.

[125] Popovici J, Moreira LA, Poinsignon A, Iturbe-Ormaexte I, McNaughton D, O'Neill SL. Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by *Aedes* mosquitoes. *Mem Inst Oswaldo Cruz* 2010; **105**: 957-964.

[126] Liaqat I, Jahan N, Ahmad SI. Challenges and future prospects for dengue vector control. *Afr J Microbiol Res* 2013; **7**(33): 4220-4227.

[127] DAWN. GCU research on mosquito's genetic modification. [Online] Available at: http://www.dawn.com/news/1036840 [Accessed on 27th June, 2015].

[128] Guruprasad NM, Jalali SK, Puttaraju HP. *Wolbachia*-a foe for mosquitoes. *Asian Pac J Trop Dis* 2014; **4**(1): 78-81.

[129] World Health Organization. Global strategy for dengue prevention and control; 2012–2020. [Online] Available at: http://www.who.int/immunization/sage/meetings/2013/april/5_Dengue_SAGE_Apr2013_Global_Strategy.pdf [Accessed on 27th June, 2015].

[130] Khan S. Dengue virus: latest research and Pakistan case. [Online] Available at: http://ideaskb.blogspot.com/2012/08/dengue-virus-latest-research-and.html [Accessed on 27th June, 2015].

[131] James S, Simmons CP, James AA. Ecology: mosquito trials. *Science* 2011; **334**: 771-772.