Biocontrol efficacy of selected mosquitocidal bacteria

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ABSTRACT

Insect species represent the largest percentage of the world’s known species. They are undoubtedly the most adaptible life forms existing on the Earth. Less than 0.5 percentage of the total number of the known insect species are considered as pests. Among these, mosquitoes pose a major threat to public health by transmitting diseases like malaria, dengue, chikungunya etc. Only four classes of chemical insecticides have been approved by WHO with less target sites. Indiscriminate use of these synthetic insecticides and resultant selection pressure on insect populations has caused many mosquito species remain resistant to widely used insecticides. Hence, alternative approach has been initiated to use biological agents. This biocontrol strategy is important in order to counter the evolution of resistance in target populations and possible effects on non target organisms. Unlike chemical insecticides, biocontrol agents are host specific, safer to the environment, find easy application in the field, long-lasting effect with single application and cost effective production. In this context, this paper reviews about the important mosquitocidal bacteria and their efficacy against different mosquito species.

Keywords: Mosquitocidal, Insecticides, resistance, biocontrol agents

INTRODUCTION

Mosquitoes are considered as large group of insects present throughout the temperate and tropical regions and even beyond the Arctic Circle of the world (Harbach, 2007). India is ranked fifth in terms of mosquito biodiversity after Brazil, Indonesia, Malaysia and Thailand (Foley et al., 2007). They belong to family Culicidae, order Diptera and are divided into two subfamilies and 112 genera. At present, a total of 3,540 recognized mosquito species are recorded in the world. Among this, the Indian mosquito fauna includes 393 species which is divided among 49 genera and 41 subgenera. Most of the important disease vectors are the members of Anopheliae and Culicidae. In India, 31 species are currently recognized for
Vector borne diseases-Global burden

Vector-borne diseases are responsible for 17% of the global burden of parasitic and infectious diseases (WHO, 2008). Within the past two decades, many important vector-borne diseases have re-emerged or spread to new parts of the world. Traditionally, it is regarded as a problem of tropical countries; now pose an increasingly wider threat to global public health, both in terms of the number of people affected and their geographical spread (WHO, 2014). For example, some of the vector borne diseases such as dengue, chikungunya and West Nile virus are emerging in the countries where they are previously unknown. This is mainly due to seasonal weather variation, socio-economic status, vector control programmes, environmental changes and drug resistance which are highly like to influence current vector-borne disease epidemiology. These effects are likely to express in many ways from short term epidemics to long-term gradual changes in disease trends (Githeko et al., 2000).

Vector control strategies:

The control of mosquito borne diseases remain a major problem due to the absence of effective vaccines or specific anti-viral drugs. Vector control is a powerful preventive tool that is not used to its full potential. It is defined as measures of any kind, directed against vectors of diseases and intended to limit their ability to transmit diseases (Karunamoorti, 2013). Mosquito control is carried out mainly with chemical insecticides such as organophosphates, carbamates and pyrethroids. Synthetic chemical insecticides such as DDT, delmethrin, malathion, chloropyriphos, etc are popularly used as first line of defense against pest populations particularly mosquito vectors. In 1955, WHO proposed the eradication of most of the prevalent vector-borne disease Malaria with the use of residual house-spraying of DDT (Hemingway and Ranson, 2000). The utilization of these products has been limited because they are non-specific, pollute the environment and their target insects have high rates of resistance.

Synthetic insecticides:

World Health Organization has approved a list of synthetic insecticides which are used commercially to treat adult mosquitoes to date. They have been categorized under four different classes which include organochlorines (now banned in most countries), organophosphates, carbamates and pyrethroids (Zaim and Guillet, 2002). Only a limited number of insecticide classes are available for adult mosquito control. No new malaria mosquito adulticide has been approved by the WHO in the last 15 years (Nauen, 2007). It is important to note that these four chemical classes of insecticides possess only two different modes of action indicating less target diversity when compared to agricultural pesticides (Nauen and Bretchneider, 2002). Moreover, the use of chemical insecticides results in undesirable effects such as increased physiological resistance in vectors, environmental pollution lead to bio-amplification in food chain and killing of non-target populations such as earthworms, birds etc.

Insecticide resistance:

Resistance is defined as the developed ability in a strain of insects to tolerate doses of toxicant which would prove lethal to majority of individuals in a normal population of the same species (WHO,1957). The resistance to insecticides is considered to be a recent evolutionary adaptation in insects which occurs in less than one century with response to sequential application of insecticides. The possible mechanisms to develop resistance is to enhance the ability of insects to detoxify the insecticide molecules and to alter the target sites so that insecticide molecules no longer bind with the action sites (Brattsten, 1986).

The emergence of resistance act as major hurdle in the line of current vector control programmes. More than 40 years of intensive synthetic insecticides use to control arthropod pests and disease vectors have resulted in pesticide resistance among over 450 species (Georghiou, 1986). Resistance is commonly monitored by bioassay either by determining LC50 value or by using uniform diagnostic doses (Feng Cui et al., 2006). Susceptibility studies of malaria vectors A.stephensi Liston and A.subpictus Grassi , collected from different locations in arid and semi-arid regions of India are conducted by adulticide bioassay of DDT, malathion, deltamethrin and larvicide bioassay of fenthion, temephos, chloropyriphos using diagnostic doses. Both the Anopheles sp. showed variable
resistance to DDT and malathion, larvae of *Anopheles sp.* showed resistance to chloropyrophos followed by fenthion (Tikar et al., 2011). In recent years, the knowledge of resistance status is essential to select a particular insecticide against target species in vector control programs. Hence, Worldwide Insecticide resistance Network (WIN, http://win-network.kird.fr) collaborates with internationally recognized institutions in vector research to track insecticide resistance at a global scale. The objective of WIN is to provide WHO and member states to evidence and expertise resistance management and deployment of alternative arbovirus vector control measures (Corbel et al., 2016). In order to control human disease vectors, there is a need for alternate, more effective and environment-friendly control agents which enable long term sustainable results.

**Biological agents:**
The balance of nature depends to a large extent on the regulation of population densities by parasitoids, predators, competitors, parasites and pathogens. These natural enemies play an important role in checking the proliferation of vectors in nature. In this aspect various biological control agents have been thoroughly investigated with the support of World Health Organization Special Programme for Research and Training in Tropical Diseases(WHO/TDR) (Mulla, 1990). A large number of mosquito pathogens and parasites have been isolated and studied for the biocontrol of mosquitoes. The term biological control is defined as the control of pests, including the vectors of human disease by the direct or indirect use of natural enemies with or without their metabolites (WHO, 1982). The first biocontrol was *Bacillus popilliae*, entomopathogenic bacteria which was used against larvae of Japanese beetle. It was the first bacterium registered as insecticide in United States (Zhang et al., 1997). Processed formulations were applied into soil and the pest population remains suppressed for more than 10 years after one application.

**Entomopathogenic Bacteria:**
Insect pathogenic bacteria are present in the families Pseudomonadaceae, Enterobacteriaceae, Lactobacillaceae, Micrococcaceae and Bacillaceae. In the past few decades, several bacterial isolates and strains of spore forming bacteria have been isolated that produce parasporal proteins which show high toxicity against insects (Katara et al., 2012). Based on the safety to non-target organisms, only members of Bacillaceae (Order: Eubacteriales) were the most studied, commercialized, and successfully used in microbial control of lepidopteron, dipteran and coleopteran insect pests (Lacey et al., 1986). Among these, two bacteria such as *Bacillus thuringiensis serovar israelensis (Bti)* and *Bacillus sphaericus (Bs)* have been successfully tested against mosquito larvae. There are certain guidelines for laboratory and field testing of mosquito larvae and it remains as universal method to test any biocontrol agent (WHO, 2005).

**Bacillus thuringiensis israelensis (Bti):**
*Bacillus thuringiensis* is a gram positive, rod shaped, spore forming bacterium characterized by its ability to exhibit insecticidal properties. This bacillus crystalline inclusions dissolve in the larval midgut, releasing one or more insecticidal crystal proteins (also called delta endotoxins) of 27 to 140 kilodaltons (kDa) (Hofte and Whitely, 1989). This appears to be a synergistic interaction between four proteins resulting in a highly complex mode of action which lead to the toxicity of mosquito larvae and with no resistance development. The *Bti* spores and parasporal crystals must be ingested by the larval (feeding) stage of target organism to cause mortality. The toxin binds to a receptor on the midgut cell wall resulting in pore formation in the cell and leading to death of the larva. *B. thuringiensis* was found to induce cellular and oxidative stress prior to mosquito death (Ahmed, 2013).

*Bti* is highly pathogenic against mosquitoes (*Culicidae*) and black flies (*Simuliidae*) and has some virulence against certain other Diptera especially *Chironomidae* (midges). Previously, formulations of *Bacillus thuringiensis* have been used successfully as biocontrol agent to control agricultural pests, but their role in control of dipteran species was recognized only after the discovery of *B. thuringiensis* serovar *israelensis* (*Bti*). In 1975-76 under a World Health Organization sponsored project, a new *Bt* strain was discovered in Israel by Goldberg and Margalit (1977). This strain was isolated from *Culex* sp. dead larvae mosquito. Later, it was identified as *Bt israelensis*, serotype H14 according to its flagellar antigenicity. As a result of extensive research on the efficacy and evaluation of agents, *B. thuringiensis* (H-14) was effective in the field and registered for mosquito control in 1980 (Mulla et al., 1984).
Table 1: List of *Bacillus thuringiensis israelensis* strains reported as mosquitocidal bacterial strains

<table>
<thead>
<tr>
<th>Bacillus thuringiensis israelensis</th>
<th>Selected references</th>
<th>Source of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B.t. jegathesan</em></td>
<td>Seleena and Lee, 1990; Seleena et al., 1995</td>
<td>Malaysia (soil)</td>
</tr>
<tr>
<td><em>B.t. medellin</em></td>
<td>Orduz et al., 1992; 1996; Thiery et al., 1996</td>
<td>Colombia (soil)</td>
</tr>
<tr>
<td><em>B.t. jegathesan</em></td>
<td>Seleena and Lee, 1990; Seleena et al., 1995</td>
<td>Malaysia (soil)</td>
</tr>
<tr>
<td><em>B.t. sotto</em></td>
<td>Ohba et al., 2000; Ohugushi et al., 2003</td>
<td>Okinawa, Japan (soil sample)</td>
</tr>
<tr>
<td><em>B.t. fukuokaensis</em></td>
<td>Ohba and Aizawa, 1990; Lee and Gill, 1997; Guerchicoff et al., 1997</td>
<td>Japan</td>
</tr>
<tr>
<td><em>B.t. kyushaensis</em></td>
<td>Ohba and Aizawa, 1979; Held et al., 1990</td>
<td>Japan (B.morri breeding site)</td>
</tr>
<tr>
<td><em>B.t. israelensis</em></td>
<td>Goldberg and Margalit, 1977</td>
<td>Israel (sewage pond)</td>
</tr>
<tr>
<td><em>B.t. morrisoni</em></td>
<td>Padua et al., 1984</td>
<td>Phillipines (soil sample)</td>
</tr>
<tr>
<td><em>B.t. darmastadensis</em></td>
<td>Padua et al., 1980</td>
<td>Japan</td>
</tr>
<tr>
<td><em>B.t. canadensis</em></td>
<td>Ishi and Ohba, 1993</td>
<td>Iraq (soil)</td>
</tr>
<tr>
<td><em>B.t. thompsoni</em></td>
<td>Manonmani and Hoti, 2001</td>
<td>India (soil)</td>
</tr>
</tbody>
</table>

More than 40,000 species of *Bacillus thuringiensis* have been isolated and identified which belongs to 39 serotypes. These include strains with various serotypes such as *Bt. canadensis*, *Bt. thompsoni*, *Bt. malayiensis* and *Bt. jegathesan*. Among these, *Bt. medellin* and *Bt. jegathesan* appear as good candidates for further characterization and investigation (Ragni et al., 1996).

These organisms are active against either *Lepidoptera*, or *Diptera* or *Coleoptera*. *Bti* was found to be specific toxic to larvae of 109 mosquito species. *Bti* has an LC50 in the range of 10–13 ng/ml against the fourth instar of many mosquito species (Federici et al. 2003). Generally, *Culex* and *Aedes* are highly susceptible compared to *Anopheles* which are less susceptible (Balaraman et al., 1983). Much higher concentrations of *Bti* are required to induce mortality in anopheline larvae than in *Aedes* species.

**Limitations:**

*B.t.* formulations produced commercially are not active against adult flies, though the proteins in the parasporal body can be able to destroy the midgut epithelium of adults. This is mainly due to inability of proteins to penetrate the cuticle. There are no available methods to induce adult flies to ingest formulations under field conditions. Therefore, *Bti* formulation commercially available at present are used as larvicides not as adulticides. It is well known that the toxicity of *Bti* lasts only a few days at most and efficacy can be reduced within 24 hours (Becker et al., 1993). In addition, *Bti* does not survive long in highly polluted water and is particularly prone to UV light inactivation in strong sunlight (Mulla, 1990).

Most of these formulations offer high levels of initial control, but with very little residual activity. This has necessitated weekly application of these formulations to keep the larval population under constant check, which would increase logistics and cost (King et al., 1997). Therefore, a new formulation with long residual life and new mode of action is necessary. Due to high cost of production compared to chemical pesticides, operational success of *Bti* against the three major vectors is only limited to temperate regions (European countries) of the world, where these vectors are considered as nuisance pests (Porter et al., 1993).

**Bacillus sphaericus (Bs):**

*Bacillus sphaericus* is another most extensively studied spore forming bacterium for its mosquitocidal properties. During sporulation, the active strains produce crystal toxin which is a binary toxin. The 51 and 42 kDa mosquitocidal crystal proteins of *B.sphaericus* are unique among bacterial insect toxins which have a low sequence similarity and are distinct from all of the cloned and sequenced insect toxins of *Bacillus thuringiensis* (Baumann et al., 1991). Upon ingestion of this toxin by mosquitoes, they bind to specific receptors present in the midgut brush-border membrane and cause damage to midgut cells and lead to death. The first reported *Bacillus sphaericus* was not effective strain but after the isolation of *B.s* from...
Indonesia (strain 1593) which is highly mosquitocidal (Charles et al., 1996). Currently nine serotypes are known to contain active strains of Bacillus sphaericus. Bacillus subsp isrealensis and B.sphaericus differ in the nature of toxin and their host range.

In general, B.sphaericus is more effective against Culex spp and Anopheles but less effective to Aedes spp. B.isrealensis subsp. remain effective against Aedes and Culex spp but not to Anopheles spp (Lacey and Undeen, 1986, Mulla, 1990). In addition, B. sphaericus has its ability to survive in polluted aquatic environments but Bti used to lose its ability in that environment (Mulla et al., 1984, Davidson et al., 1984).

Most of mosquitocidal B.s strains were isolated successfully for the past 30 years. The most active strains 1593 and strain 2362 which belong to serotype 5a5b (Charles et al., 1996, Delecluse et al., 2000).

Bacillus sphaericus VCRC-B547 isolated from excreta of arid birds has shown higher toxicity against Cx.quinquefasciatus, An.stephensi and Aed.aegypti (Poopathi et al., 2014).

The lower sensitivity of Bs may result from the fact that the protein of the bacterium is enclosed in the exosporium, whereas the delta endotoxin of Bti is uncoated. It is possible that coated spore-crystal complex is more tolerant to UV light than the uncoated protein. This feature is also responsible for the slow mode of action of products based on Bs and its potential to persist under certain field conditions (Lacey, 2007). The biolarvicide formulation from Bs strain is reported to be less effective against Anopheles culicifacies and hardly effective against Aedes aegypti (Mittal, 2003). Bs is at high risk of selecting resistance.

**Limitations:**

Though, Bacillus sphaericus remain effective against Culex spp., repeated application in the field for long term effect will lead to development of resistance in target species. Persistence and recycling potential of B. sphaericus are more achievable in polluted than in clear waters (Mulla et al., 1984). Because, recycling is important phenomenon where toxin production continues during a period where several generations of target species are produced.

Table 2: Commercially available Bacillus sphaericus strains as mosquito larvicides.

<table>
<thead>
<tr>
<th>Strain number</th>
<th>source of isolation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2297</td>
<td>Sri Lanka</td>
<td>Wickremesinghe RSB and Mendis CL, 1980</td>
</tr>
<tr>
<td>1593</td>
<td>Nigeria</td>
<td>Weiser J, 1984</td>
</tr>
<tr>
<td>2362</td>
<td>China</td>
<td>Liu EY et al., 1989</td>
</tr>
<tr>
<td>VCRC-B547</td>
<td>Pondicherry</td>
<td>Poopathi et al., 2014</td>
</tr>
</tbody>
</table>

Table 3: List of Pseudomonas species reported as mosquitocidal bacterial strains

<table>
<thead>
<tr>
<th>Pseudomonas species</th>
<th>Source of isolation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>Dead mosquito larva</td>
<td>Murty et al., 1994; Prabakaran et al., 2003; Sadanandane et al., 2003; Prabakaran et al., 2009; Prabakaran et al., 2015; Pushpanathan and Selvaraj Pandian, 2008; Varun Rajan and Selvaj Pandian et al., 2008; Usharani and Paily, 2014; Lalithambika et al., 2014; Athisayamary et al., 2015; Mahamuni et al., 2015;</td>
</tr>
<tr>
<td><em>Pseudomonas pseudomallei</em></td>
<td>Soil samples (Malaysia)</td>
<td>Lee and Seenea, 1990.</td>
</tr>
<tr>
<td><em>Pseudomonas frederiksbergiensis</em></td>
<td>contaminated soil (Saudi Arabia)</td>
<td>Ahmed et al., 2014; 2015</td>
</tr>
</tbody>
</table>
in mosquito population. In fact, resistance to Bs has already been reported in field populations of Culex spp in China, Brazil, France and India (Sinegre et al., 1994, Rao et al., 1995, Silva Filha et al., 1995, Yuan et al., 2000) with resistance levels in some areas of China reported as >20,000 fold. The potential key strategy for delaying resistance to mosquitocidal proteins is to use mixture of toxins that act at different targets within the insects (Writh et al., 2005).

**Resistance against Bti and Bs:**
Due to continuous selection pressure and cross resistance, mosquito populations develop resistance against Bs binary toxin (Bin) both in the laboratory and field trials (Sinegre et al., 1994). In Brazil, it was reported that the tenfold increase in resistant population found in open drains and covered cessepits in a small area where all the breeding sites were treated during two year period with a total of 37 treatments (Writh et al., 2000). But in case of Bti strains, they have been used for mosquitoes and Black flies for about 20 years, yet no resistance to this bacterium has been reported. In contrast to their sub species only low levels of resistance was observed in the laboratory experiments. The reason behind is selection of Culex quinquefasciatus with mutants of B. thuringiensis sub species israelensis that contained different combinations of its Cry proteins and Cyt1Aa delayed the evolution and expression of resistance to mosquitocidal Cry proteins (Writh et al., 2005).

**Recombinant bacterial strains for vector control:**
Commercial products such as VectoBac and Teknar based on Bacillus thuringiensis subsp. israelensis(Bti), VectoLex based on Bacillus sphaericus are most widely used as vector control products. Even though these products gain commercial success in developed countries but their high cost of fermentation, limited their use in developing countries. Lack of persistence due to settling of the spore-crystal complexes and narrow host range compared with chemical insecticides limited the usage of wild strains of Bti and Bs (Ohana et al., 1987). Recombinant DNA technology paved the way for enhancing the synthesis of mosquitocidal proteins and by enabling new endotoxin combinations from different bacteria to be produced within single strain (Federici et al., 2003). Recombinant Bti able to produce Cyt1A, Cry proteins and Bs binary toxin, in which Cyt1A delays resistance to insecticides (Wirth et al., 2005). Higher specificity, environmental safety of the recombinants compared to synthetic insecticides with increased efficacy will provide these novel strains to be used in the future pest and vector control programmes (Park and Federici, 2009).

**Clostridium bifermantans serovar Malaysia:**
The first anaerobic mosquitocidal isolate, CH18 was isolated and identified from Mangrove swamp soil from Malaysia. Hence it was named as C. bifermantans serovar Malaysia(Cbm). Another strain was isolated from the forest and reported as C. bifermantans serovar pariba(Cbp)(Seleena et al., 1997). Both these strains were active against Anopheles larvae and in increasing level of susceptibility to Aedes and Culex species. Their toxicity remains similar to Bti strains but the toxic factors are different from Bt. Though the Clostridium species includes human pathogens, the safety of Cbm strains as potential bioinsecticide is highly considerable (Thiery et al., 1992).

**Pseudomonas species:**
Pseudomonas species show remarkable and physiological versatility, enabling colonization in diverse terrestrial and aquatic habitats (Palleroni, 1992). They are generally aerobic, gram-negative bacteria, ubiquitous in agricultural soils and are well adapted to grow in the rhizosphere. Stainer et al. (1966) conducted a fundamental study on the pseudomonas that result in an extensive phenotypic characterization in which the genus was subdivided into species and species into groups. Pseudomonads possess many traits that make them well suited as biocontrol and growth-promoting agents. Many biocontrol agents from P. fluorescens are well characterized for their ability to produce antimicrobial compounds, including 2,4-diacetylphloroglucinol (DAPG), phenazines, hydrogen cyanide and surfactants (Haas and De’fago, 2005).

Some exotoxins such as Pseudomonas aeruginosa Migula have been noted to be absorbed through the cuticle of insects and act on the haemolymph proteins. Exotoxins of microbial origin, including Pseudomonas species are also known to be toxic to larvae of mosquitoes as well as lepidopteran insects (Murty et al., 1994).

The larvicidal effects of the culture supernatants of Pseudomonas fluorescens(MSS-1), originally isolated from deceased mosquito larvae reported to be active against Culex quinquefasciatus, Anopheles stephensi,
Aedes aegypti. A microbial formulation of *Pseudomonas fluorescens* (VCRC 426) was developed and formulated and tested against 4th instar larvae and pupae of three major vectors. *A. stephensi* was found to be most susceptible followed by *Culex quinquefasciatus* and *Aedes aegypti*. This was the first report that exotoxin remain effective against the pupae of the three species of mosquitoes at a very low concentration that of larvae (Prabhakaran et al., 2002). Field valuation of VCRC B426 formulation of *P. fluorescens* against *Culex quinquefasciatus* larvae and pupae showed 100% elimination of larvae and pupae at day1 after treatment and 80% reduction in pupal density (Sadanandane et al., 2003). The exotoxins produced by *Pseudomonas fluorescens* exhibited marked larvicidal and pupicidal activity against *A. aegypti* and *A. albopictus* (Pushpanathan and Selvaraj Pandian, 2008).

Binding of *Pseudomonas fluorescens* proteins to specific receptors plays an important role in the mode of action. It has been reported that binding of mosquitocidal proteins to the midgut region of treated larvae and pupae leads to considerable increase in the marker enzyme activity and Cytochrome C oxidase activity in th treated *Aedes albopictus* cell lines (Usharani and Paily, 2014). *Pseudomonas fluorescens Migula* (VCRCB426) produces secondary metabolite which is analysed and found as rhamnolipid. It is reported as first mosquito pupicidal compound which is found active against *culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti* (Prabakaran et al., 2015). The major limitation of pseudomonads as biocontrol agents is their inability to produce resting spores which remain problematic in formulation of the product. Most of commercial products of *Bti* and *Bs* have their spore-crystal complex which have longer storage facility.

**CONCLUSION**

Collectively, arthropods are responsible for the transmission of vector-borne diseases both in human and animals. Over the past 30 years, there has been a global re-emergence of infectious diseases particularly vector-borne diseases with an increased frequency of epidemic transmission and expanding their geographical distribution. Many factors directly or indirectly contribute to emergence of vector borne diseases recently. Distribution of these diseases is determined by a complex dynamic of environmental and social factors such as globalization of travel and trade, unplanned urbanization, climate change etc which are having a significant impact on these diseases transmission in recent years. These include climate change pattern, global trade, rapid unplanned urbanization, socioeconomic status, vector control programs which are highly influencing the current vector diseases epidemiology (Gubler, 2009). It has also been reported that the vectors in several countries has developed resistance to most of the highly effective class of insecticides. Hence, there remains a great challenge to control vector borne diseases. It is essential to develop a novel bioinsecticide which posses new mode of action, rapidly kills target species, high specificity and with commercial value. It is also essential to review about biocontrol agents for vector control and under laying fundamental capacities including technical expertise, stronger surveillance systems and better laboratory infrastructure facilities (WHO, 2014).

**Conflicts of interest:** The authors stated that no conflicts of interest.

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