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MINIREVIEWS

Biological and historical overview of Zika virus

Najealicka Armstrong, Wangheng Hou, Qiyi Tang

Najealicka Armstrong, Wangheng Hou, Qiyi Tang, Deparment of Microbiology, Howard University, College of Medicine, Washington, DC 20059, United States

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Correspondence to: Qiyi Tang, PhD, Associate Professor, Department of Microbiology, Howard University, College of Medicine, Seeley Mudd Building, Room 315, 520 W Street, NW, Washington, DC 20059, United States. qiyi.tang@howard.edu
Telephone: +1-202-8063915
Fax: +1-202-2388518

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Abstract

The recent outbreak of the Zika virus attracts worldwide attention probably because the most recently affected country (Brazil) will host the 2016 Olympic Game. Zika virus infected cases are now spreading to many other countries and its infection might be linked to some severe medical sequelae. Since its first isolation from the infected monkey in 1947 in Uganda, only a few studies had been taken until recent outbreak. According to the history of referenced publications, there is a 19-year gap from 1989 to 2007. This might be because only mild diseases were diagnosed from Zika virus infected populations. Obviously, the recent reports that Zika virus infection is probably associated with microcephaly of the neonates makes us reevaluate the medical significance of the viral pathogen. It can be transmitted sexually or by mosquito biting. Sexual transmission of the Zika virus distinguishes it from other members of the Genus Flavivirus. Detailed information of the Zika virus is needed through a thorough investigation covering basic, epidemical, subclinical and clinical studies. Here, we reviewed the published information of Zika virus.

Key words: Zika virus; Flavivirus; Congenital infection; Outbreak; Microcephaly

Core tip: Zika virus is gaining new ground with the recent outbreaks that are starting to expand worldwide. While normally transmitted by the mosquito, other routes of transmission are being discovered. Also, other medical complications are being detected with Zika virus infections. These recent findings require the scientific community to thoroughly examine Zika virus to better understand it so that better diagnostic options, treatment, and preventative measures can be developed. In order to beat Zika virus, we must understand its history and outbreak patterns as well as gain a full understanding of

all clinical manifestations associated with this virus.

Armstrong N, Hou W, Tang Q. Biological and historical overview of Zika virus. *World J Virol* 2017; 6(1): 1-8 Available from: URL: http://www.wjgnet.com/2220-3249/full/v6/i1/1.htm DOI: http://dx.doi.org/10.5501/wjv.v6.i1.1

INTRODUCTION

The Zika virus, together with the West Nile virus, Yellow fever virus, Japanese encephalitis virus, Dengue fever virus, and many other classified and unclassified viruses, forms the genus *Flavivirus* that belongs to family Flaviviridae. The family Flaviviridae consists of many other viruses that are summarized in a 2010 review[1]. This family of viruses have enveloped icosahedral capsid that contains a single strand RNA genome (about 11000 nucleotides) with positive sense[2]. Therefore, the infected viral RNA can be directly translated to a large polyprotein precursor, which is co- and post-translationally processed by viral and cellular proteases into structural and non-structural proteins. The three structural proteins are critical for the formation of envelop and capsid, and the seven non-structural (NS) proteins play important roles in virus replication. The three structural proteins are envelope, E; membrane precursor, PrM; and capsid, C. The seven NS proteins include NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (Figure 1). The names, location in the infected cells, and functions of viral proteins are listed in Table 1. The members of the genus *Flavivirus* are characterized by similarities in genomic structure, viral protein function, pathogenesis and transmission.

The large polyprotein precursor must be cleaved to generate actively functional proteins. The cleavage of the polyprotein precursor is a sophisticated process and is completed collaboratively by cellular proteases of the PACE (Paired basic Amino acid Cleaving Enzyme)-type or other Golgi-localized proteases and the viral serine protease embedded in the N-terminal domain of non-structural protein 3 (NS3Pro), which requires NS2b for its activity[1]. A distinct feature of genus *Flavivirus* from other genera of Flaviviridae is that the 5′-end of the (+)ssRNA genome of genus *Flavivirus* is decorated with an RNA cap structure (N7meGpppA2′Ome-RNA). 5′end capping of the viral RNA is as important as that for eukaryotic mRNAs, not only to initiate the process of translation but also to protect the viral RNA from degradation by endogenous RNA exonucleases. The protein translation happens immediately after the uncoating of viral particle in the cytoplasm. The (+)ssRNA genome is used as a template not only for gene expression but also for viral genome replication. Both viral RNA replication and gene translation occur in the cytoplasm. For RNA replication, viral NS proteins and cellular proteins interact to form a replication compartment (RC). During the period of viral RNA replication in the cytoplasm, the RC consists of morphologically distinct, membrane-bound compartments that also differ with respect to both function and NS proteins composition[3]. The NS3 and NS5 proteins are central to the viral RC, as together, they harbor most, if not all, of the catalytic activities required to both cap and replicate the viral RNA. Following replication, the protected genomic RNA is packaged by the C protein to form a capsid in a host-derived lipid bilayer in which the E protein is embedded and later integrated into viral envelope. The mature particles subsequently exit from the host cell by exocytosis.

REGIONAL ISOLATION OF ZIKA VIRUS

The Zika virus is phylogenetically close to *Spondweni* virus and a member of Flaviviridae family[4]. Comparative genomic analysis revealed that coding regions of pre-epidemic and epidemic strains of the Zika virus were similar with the exception of the NS2B. Bootscan analysis and multiple sequence alignment of the Asian lineage suggested that there may be genetic recombination of a fragment (nucleotides 4237-4528) of NS2B with that of the *Spondweni virus*[5].

African countries

In 1947, a group of scientists from United Kingdom led by Haddow *et al*[4] who were investigating yellow fever isolated Zika virus from a rhesus macaque with fever in the Zika Forest in Uganda[6,7]. The isolated viral strain has been stored in ATCC (ATCC® VR84™, MR 766) and the European Virus Archive (France) and is now still used for studies. The next important step was to find out whether the Zika virus is transmitted by mosquitos. First, Boorman *et al*[8] demonstrated that Zika virus can infect and replicate in mosquitos, providing experimental evidence that Zika virus may be transmitted by mosquitos. Later, the United Kingdom *Flavivirus* research group continued their studies of arboreal mosquitos as virus vectors in Uganda. They isolated 12 strains of Zika virus from *Aedes (Stegomyia) africanus* in the Zika forest[9]. Zika virus is apparently enzootic in Zika forest, and the evidence collected by Hoddow *et al*[9] suggested that *Aedes africanus* is the primary vector and that forest-dwelling monkeys and human are, on occasion, involved. It was not clear whether the mosquito transmitted the virus to other animals because no small mammal trapped in the forest showed serum antibody against the Zika virus. The Zika virus infection in humans was first reported in 1954[10]. It has also been experimentally demonstrated *via* volunteers that the Zika virus is able to infect humans[11]. In summary, results from these investigations suggest that the Zika virus is an arbovirus, transmitted by mosquitos and infects at least monkeys and humans.

Southern Asian countries

The first isolation of Zika virus in South-Eastern Asia was reported in 1969 in Malaysia[12]. Some years later, there was another report that the Zika virus was isolated from patients in Indonesia[13]. The event occurred

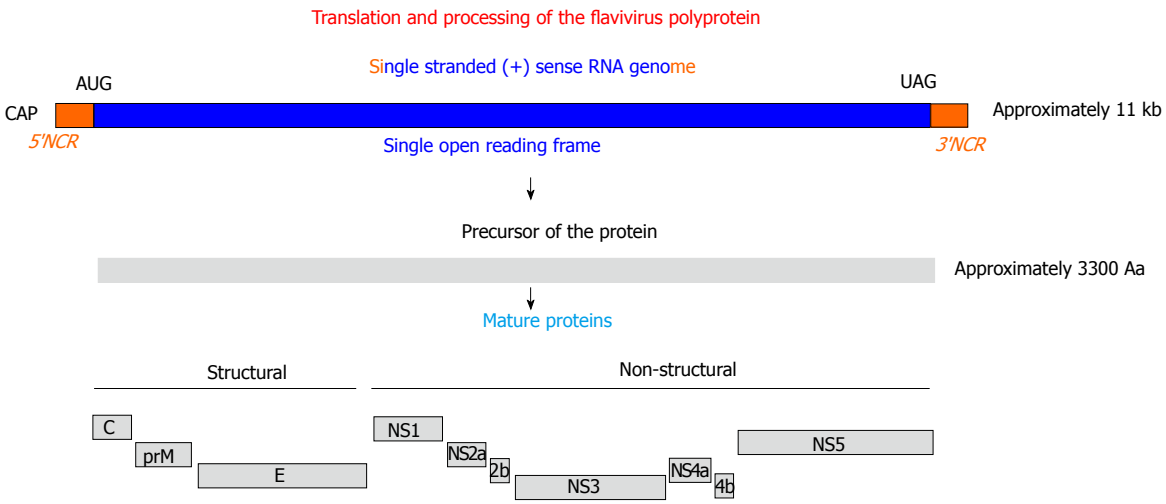

Figure 1 Genomic structure and gene production of Flavivirus. AUG: Translation start codon; UAG: Translation stop codon; NCR: Non coding RNA sequence; kb: Kilo base; Aa: Amino acid.

Table 1 Roles of viral protein and RNA during viral infection in permissive cells

Name of the vital material	Location in cell	Function
Viral genome + ssRNA (approximately 11000 nt)	Cytoplasm	Template for protein translation and for viral genome replication
Envelope, E (53 KDa)[52]	Cell membrane	Viral assembly, budding, attachment to target cells, and viral membrane fusion
Membrane precursor, PrM (20 KDa)[53]	Cell membrane	Facilitating E protein folding and trafficking, and virion maturation
Capsid, C (12 KDa)[54]	Cytoplasm	Virion maturation
NS1 (glycoprotein)[55] (46-55 kDa)	Endoplasmic reticulum vesicular compartments, cell surface	Subverting immune response virus-induced intracellular RNA replication, neurovirulence
NS2a (25 kDa)[56]	Transmembrane	Virus assembly, inhibit IFN-response
NS2b (14 kDa)[1,57]	Cytoplasm, nucleus	Viral protein cleavage
NS3 (69 kDa)[1]	Cytoplasm, nucleus	Viral protein cleavage, RNA triphosphatase, mRNA capping, RNA helicase
NS4a (16 kDa)[58]	Transmembrane	Viral RNA replication
NS4b (21.5 kDa)[59]	Integral membrane	Suppression of (IFN-α/β), suppression of the host RNAi, negatively regulate the helicase function, viral replication
NS5 (103 kDa)[60,61]	Cytoplasm, nucleus	The RNA triphosphatase, RNA-dependent RNA polymerase

during the end of the rainy season of 1977 when *Aedes aegypti* usually flourishes. Seven patients in central Java, Indonesia, appeared in the hospital with high fever, malaise, stomach ache, dizziness and anorexia. Data on these 7 Zika virus cases and several previously reported human infections indicated that clinical characteristics of infection with Zika virus appeared relatively mild, self-limiting, and nonlethal. It was suspected that the virus was transmitted by *Aedes aegypti*, which had been reported to be a probable vector in Malaysia[12]. A later investigation in Sabah, Malaysia, showed that the Zika virus infected 60 semi-captive and 84 free-ranging orangutans (Pongo pygmaeus pygmaeus)[14]. Another study conducted by the United States Naval Medical Research Unit No. 2 (NAMRU-2) isolated Zika virus in Cambodia in 2010[15]. This case was from a 3-year-old boy who had 4 d of fever, sore throat and cough as well a headache that lasted for 3 d. The studies conducted in southern Asia further confirmed that mosquitos are the vector and the primates might be the end host of viral infection.

The Zika virus has been also isolated from animals and human in other African countries. For examples, during the years 1964 to 1970, Moore *et al*[16] isolated 171 arboviruses of 15 different types from humans in Ibadan, Nigeria. Zika virus isolation rates also varied by season, with peaks in rainy seasons (June to August) and lows in dry seasons (January to February). Viruses were isolated from all age groups, with the majority from children one to four years old. The viruses isolated in largest numbers were chikungunya and yellow fever, which caused epidemics in 1969, and dengue types 1 and 2 and Tataguine, which are endemic in Ibadan. The Zika virus was isolated at a low rate. In 1999, three strains of the Zika virus were isolated as part of yellow fever studies in the Ivory Coast[17]. In 2010, it was

reported that the Zika virus was isolated at a high rate in Cameroon. The research group investigated 102 sera from febrile patients (with negative laboratory findings for malaria and typhoid fever) at clinics in the Fako Division of Cameroon. The Zika virus was isolated at a rate of 11.4%, higher than that of any other members of Genus *Flavivirus*[18]. Therefore, following the time, the Zika virus has been spread throughout Africa.

More and more Zika virus strains have been isolated from humans worldwide[17]. Studies conducted in Nigeria during 1971-1975 isolated the Zika virus from humans. Serological experiments showed that 40% of the persons tested had neutralizing antibody to the Zika virus[16,19]. The infected populations were detected in other African countries such as Uganda, Tanzania, Egypt, Central African Republic, Sierra Leone, and Gabon, and in parts of Asia, including India, Malaysia, the Philippines, Thailand, Vietnam, and Indonesia[20]. Table 2 lists the strains that have been sequenced. The data from the viral genomic analysis support the hypothesis that the Zika viruses can be classified by origin into the Southern-Eastern Asian type and African type (Table 2). Other isolates might be derived from these types.

ZIKA VIRUS OUTBREAKS AND CLINICAL COMPLICATIONS

The Zika virus has been considered as a benign pathogen, causing asymptomatic or mild infections. Currently, there is no serological test that can clearly distinguish the Zika virus from other *Flaviviruses*. Diagnostic tests for Zika include RT-PCR, an IgM ELISA, and a plaque reduction neutralization test (PRNT). Some commercial tests have only become recently available[21]. Even a report from Olson *et al*[13] in 1981 that a cluster of 7 people with serologic evidence of the Zika virus illness in Indonesia did not attract serious attention and was not considered an outbreak due to the mildness of the associated illness. Later on, the same arbovirus research group performed a serological study that showed that 9/71 (13%) human volunteers in Lombok, Indonesia, had neutralizing antibodies to the Zika virus[22]. However, no serious cases were reported. The first outbreak of Zika virus-caused diseases was reported in 2007 on Yap Island of Micronesia. In April 2007, physicians on Yap Island characterized the disease with rash, conjunctivitis, arthralgia, arthritis, and fever. The disease affected 99 patients in 2 mo. A comprehensive study that combined analysis of patient samples, serological testing and real-time RT-PCR revealed the genetic and serological properties of the Zika virus epidemic[23]. The studies suggested that the 2007 Yap Island Zika virus is distantly related to African subclades and may be spread from Southeast Asia and the Pacific. Duffy *et al*[24] later conducted an extensive study on the Yap Island Zika virus outbreak. From 185 patients, 49 had been confirmed with the Zika virus illness, only 5 were excluded from Zika virus infection, and all others were suspected of Zika virus infection. They used survey studies in a large population, and estimated that 73% of the population of the Yap Island was infected with the Zika virus during the epidemic outbreak. Therefore, the outbreak on Yap Island in 2007 suggested that Zika virus infection has been spread outside of Africa and Asia[17]. Of course, whether or not the Zika virus was imported from Africa or Asia or other places remains to be verified.

Another Zika outbreak occurred between Oct. 2013 and Feb. 2014 in French Polynesia - like Yap Island, another island in the Pacific Ocean. In the very beginning of the outbreak, a mild dengue-like illness was observed in the patients within a family (consisting of wife, husband and their son-in-law). The symptoms included low fever (< 38 ℃), asthenia, wrist and fingers arthralgia, headache, rash, and conjunctivitis. The RT-PCR test confirmed that it was a Zika virus infection[25]. The epidemic has been spread to a large population as reported by the syndromic surveillance network (6630 suspected Zika virus infection cases), 333 of which were confirmed by real-time RT-PCR as Zika virus infections. Symptoms of most Zika virus infection cases are mild and self-limited (mean duration of symptoms is 3-6 d)[25-27]. No hospitalizations for acute infection have been reported. In contrast to the outbreak in Yap Island, some severe complications were seen in this outbreak: The first case of Guillain-Barré syndrome (GBS) was found immediately after a Zika virus infection[28], and another case of vertical transmission from an infected pregnant woman to the baby was reported in this outbreak[29].

The spread of Zika virus from the outbreak of French Polynesia has been reported. Two Japanese travelers were confirmed to be infected with the Zika virus after they returned from a trip to French Polynesia during the time of the outbreak[30]. In addition, it was found to have spread to other Pacific Islands including New Caledonia, Cook Islands, Easter Island, Vanuatu, and Solomon Islands[31]. The introduction of the Zika virus from French Polynesia into New Caledonia caused another outbreak in New Caledonia in 2014[32]; The first cases of Zika virus infection were confirmed in November 2013, and they were imported from French Polynesia. By the end of 2014, a total of 1383 cases were confirmed in a laboratory[32]. Consequently, an outbreak in New Caledonia was declared. Thus far, introduction of the Zika virus from French Polynesia to other countries has been continuously reported.

Between 1947 and 2006, < 20 cases of Zika virus infection have been reported[5]. There have been recent reports of imported cases of Zika virus infections in 18 travelers returning to the Netherlands from Surinam, which is in South America near the northern border of Brazil, and the Dominican Republic[33], 13 infections were imported from Venezuela, Fiji/Samoa, or Suriname to China[34], and 4 infections were imported from Brazil to Portugal[35]. Autochthonous cases were reported in places such as Mexico[36], Colombia[37], and Easter Island, which was the first outbreak (51 cases) reported in a territory of the Americas in early 2014[38].

Table 2 Origin of the types Zika viruses

Isolation region	Isolation year	Accession #	Strain	Ref.
Malaysia	1966	HQ234499	P6-740	Haddow *et al*[4]
Micronesia	2007	EU545988	N/A	Lanciotti *et al*[23]
Cambodia	2010	JN860885	FSS13025	Haddow *et al*[4]
Thailand	2016	KU681082	H.sapiens-tc/PHL/2012/CPC-0740	unpublished
Philippines	2016	KU681081	H.sapiens-tc/THA/2014/SV0127	unpublished
China	2016	KU744693	VE Ganxian	unpublished
China	2016	KU740184	GD01	unpublished
Nigeria	1968	HQ234500	IBH 30656	Haddow *et al*[4]
Senegal	1984	HQ234501	ArD 41519	Haddow *et al*[4]
Uganda	1947	HQ234498	MR766	Haddow *et al*[4]
Uganda	2004	NC012532	N/A	Kuno *et al*[62]
CAR	2014	KF268948	ARB13565	Berthet *et al*[63]
CAR	2014	KF268949	ARB15076	Berthet *et al*[63]
CAR	2014	KF268950	ARB7701	Berthet *et al*[63]
Senegal	2001	KF383119	ArD158084	Faye *et al*[2]
Senegal	2001	KF383118	ArD157995	Faye *et al*[2]
Senegal	2001	KF383117	ArD128000	Faye *et al*[2]
Senegal	2001	KF383116	ArD7117	Faye *et al*[2]
Brazil	2016	KU497555	Brazil-ZKV2015	Calvet *et al*[64]
Brazil	2016	KU707826	SSABR1	Costa *et al*[65]
Brazil	2016	KU527608	Natal RGN	Mlakar *et al*[48]
Brazil	2016	KU501215	PRVABC59	Lanciotti *et al*[23]
Brazil	2016	KU321639	ZikaSPH2015	Staples *et al*[66]
Brazil	2016	KU312312	Z1106033	Enfissi *et al*[67]
France	2014	KJ776791	H/PF/2013	Baronti *et al*[68]
Martinique	2016	KU647676	Martinique_PaRi_2015	Baronti *et al*[68]
Haiti	2014	KU509998	Haiti/1225/2014	Lednicky *et al*[69]

CAR: Central African Republic; N/A: Not applicable.

The recent outbreak in Brazil has attracted the most attention due to not only its growing infected population but also its likely enhanced severity of the clinical sequelae. In March of 2015, Zanluca *et al*[39] from the Molecular Virology Laboratory of Carlos Chagas Institute, Oswaldo Cruz Institute, state of Paraná, Brazil, detected the Zika virus genome by RT-PCR from 8 out of 21 acute-phase serum specimens from the patients with dengue-like symptoms. This is the first report of Zika virus outbreak in Brazil. Later, another group reported a similar detection of Zika virus cases (8 out 24 samples) by RT-PCR[40]. The virus has been assumed to have been imported from French Polynesia either by the travelers during the time of the World Cup[39] or by the teams from the Va'a World Sprint Championship canoe race that was held in Rio de Janeiro, Brazil[41]. It has been reported that the virus is carried by the travelers to other countries[42]. Genomic sequencing has been conducted to analyze the similarities between different strains isolated historically. Phylogenetic studies showed that the Brazilian strain is closely related to the one from French Polynesia, and the French Polynesia strain is likely derived from Yap Island. These strains all belong to the Asian lineage[41].

The severe clinical sequelae caused by Zika virus infection include the following. First, during the outbreak of the Zika virus in French Polynesia, the Zika virus was detected from the semen of a patient, which brought out the presumption that the Zika virus might be transmitted sexually[43]. Several cases of Zika virus infected patients have been reported to be sexually transmitted[44]. This observation implies another transmission route for the Zika virus other than through mosquito. Secondly, the Zika virus was reported to be transmitted vertically (from the infected mother to the fetus). This is a major problem for patients infected by Zika virus because the virus directly results in birth defects. Again, the first cases of congenital Zika virus infection were found during the French Polynesia outbreak[29]. Thirdly, it was reported to be related to some severe syndromes like GBS[28,45]. In addition, Zika virus infection might have been associated with microcephaly[46-51]. However, after more detailed and accurate experimental studies and clinical analysis, the number of Zika-related microcephaly dropped quickly. Therefore, all the linkages to the severe diseases are still informative not conclusive. Systemic research in different aspects for Zika virus is needed to assure that the clinical findings are explained and understood.

FUTURE DIRECTIONS

Even though the world has noticed the emergence of Zika virus infection, time is needed to achieve understanding of its pathogenesis, prevention, and treatment. A previously systemic study is lacking, so the Zika virus, from now on, will be another member of Genus *Flavivirus* to be the center of virological research. The following aspects may be very important in the near future: Animal model for Zika virus infection: It will help researchers understand

whether and how Zika virus causes neural disorder through interfering with the neural progenitor cell/neural stem cell (NPC/NSC) proliferation and differentiation; vaccine development: Like all other viruses, the best and most effective way to prevent viral infection is by vaccine. Some successful experience in Dengue virus and yellow fever virus may be useful towards developing the Zika vaccine; transmission prevention. Viral transmission needs to be studied, such as whether and how semen components enhance viral infection.

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MINIREVIEWS

Value of routine dengue diagnosis in endemic countries

James Ayukepi Ayukekbong, Olufunmilayo G Oyero, Samuel Ekpesu Nnukwu, Henry Nzike Mesumbe, Cajetang Nkong Fobisong

James Ayukepi Ayukekbong, Centre for Continuing and Online Learning, Algonquin College, Ottawa, ON K2G 1V8, Canada

Olufunmilayo G Oyero, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan PB200005, Nigeria

Samuel Ekpesu Nnukwu, Department of Medical Laboratory Science, Faculty of Allied Medical Sciences, University of Calabar, Calabar PB3651, Nigeria

Henry Nzike Mesumbe, Cajetang Nkong Fobisong, Section for Clinical Virology, Redeem Biomedical Buea, Buea SWR MILE 16, Cameroon

Author contributions: Ayukekbong JA performed the majority of the writing, prepared the figures and tables; Oyero OG performed data accusation and writing; Nnukwu SE and Mesumbe HN provided the relevant input in writing the paper; Fobisong CN designed the outline and coordinated the writing of the paper.

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Correspondence to: Dr. James Ayukepi Ayukekbong, Centre for Continuing and Online Learning, Algonquin College, 1385 Woodroffe Avenue, Ottawa, ON K2G 1V8, Canada. ja.algonquin@gmail.com
Telephone: +1-819-6391160

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Abstract

Dengue is one of the most common arthropod-borne viral diseases in humans and it is a leading cause of illness and death in the tropical and subtropical regions of the world. It is thought to account for 400 million cases annually among approximately 3.97 billion people at risk of infection in 128 endemic countries. Despite the global prevalence of the disease, the availability of a vaccine is limited in most countries in the endemic areas. Most endemic countries in South America, South East Asia and Africa serve as attractive touristic sites for people from non-endemic countries who become infected and export the virus to dengue-free regions. Dengue fever typically resembles malaria and in endemic countries most cases of dengue are treated as presumptive malaria. Consequently, routine dengue diagnosis among persons with fever will offer early treatment and reduce the burden of the disease. Also, routine testing among travellers from endemic countries will reduce importation and prevent the geographical expansion of dengue. In this essay, we seek to highlight the usefulness of routine dengue testing in endemic countries.

Key words: Dengue virus; Endemic; Mosquito; Vector-borne

Core tip: Dengue is an emerging arborvirus infection currently endemic in 128 countries in the world. In the absence of routine vaccination and specific antivirals, the main method to reduce the burden of dengue is to reduce the vector population, educate people on protective measures and timely laboratory identification. Unfortunately this routine laboratory investigation is currently neglected in most endemic countries and most cases of fevers are often misconstrued as malaria. This review provides a comprehensive summary of dengue infection and highlights the fact that routine dengue diagnosis will reduce the burden and global expansion of dengue.

Ayukekbong JA, Oyero OG, Nnukwu SE, Mesumbe HN, Fobisong CN. Value of routine dengue diagnosis in endemic countries. *World J Virol* 2017; 6(1): 9-16 Available from: URL: http://www.wjgnet.com/2220-3249/full/v6/i1/9.htm DOI: http://dx.doi.org/10.5501/wjv.v6.i1.9

INTRODUCTION

Dengue virus (DENV) is the most common arthropod-borne viral disease in humans and it is endemic in most tropical and sub-tropical countries[1]. It has been designated a major international public health concern by the World health Organization (WHO) as it accounts for 400 million cases annually among 3.97 billion people at risk of infection[1,2]. Previous phylogenetic analysis suggests that there are four distinct DENV serotypes (DENV 1 to 4)[3,4]. However, a 5th serotype associated with milder disease was isolated in 2013 in Malaysia[5]. Over the years, DENV has spread from less than 9 endemic countries to presently about 128 endemic countries[6,7]. Factors such as unrestricted large-scale international travel and trade, urbanization, global warming, virus and vector evolution contributed to its rapid spread to other regions of the World[8].

The main arthropod vectors for the transmission of DENVs are *Aedes aegypti* and *Aedes albopictus mosquitoes* are predominant in both tropical and sub-tropical regions of the world[9,10]. Infected individuals may be asymptomatic or may present with dengue fever (DF) - a mild febrile illness, dengue hemorrhagic fever (DHF) - a life-threatening complication, or dengue shock syndrome (DSS). The incubation period is between 3-15 d following an infected blood meal. Rare cases of human - human transmission *via* needle stick injuries, contaminated blood products, donor organs and vertical transmission from infected mother to an unborn child have been documented[11]. Dengue endemicity in 128 countries makes it a year round occurrence with peak prevalence during the rainy season when environmental conditions are optimal for the *Aedes* vector breeding[12]. As a result, epidemics are common during the rainy season when the vector population is high and the chances for human exposure to mosquito bites is increased[13]. In the absence of routine vaccination and specific antivirals, the main method to reduce the burden of dengue is to reduce the vector population, educate people on protective measures such as spraying of insecticides and wearing protective clothing[7,8,14]. DF typically resembles malaria and in endemic countries most cases of dengue are treated as presumptive malaria. Therefore, routine and differential diagnosis of dengue will provide a basis for evidence-based treatment and reduce the irrational use of antimalarial or antibiotics to treat febrile diseases. Routine screening in endemic countries will provide a better estimate on the burden of dengue disease for public health action.

DENGUE EPIDEMIOLOGY

Dengue is currently regarded as the most important arboviral disease internationally as over half of the world's population lives in dengue endemic countries[7]. A global estimate suggests about 50-200 million cases of dengue with 500000 episodes of DHF/DSS occur annually culminating in about 20000 dengue related deaths[15,16]. The determining factors of dengue epidemiology trends include, but not limited to: (1) rapid urban population growth and density due to rural to urban migration; (2) poor sewage disposal system and land use pattern; (3) global warming; and (4) trade necessitating movement of people[17]. It is now known that every WHO region has evidence of dengue transmission[18]. Almost 75% of the world's population at risk of dengue, live in South East Asia (SEA) and the Western Pacific region and the disease is the leading cause of hospitalization and death in children from these regions (Figure 1)[18]. Dengue is also recognized as an emerging infection in the Eastern Mediterranean region with multiple outbreaks occurring in Pakistan, Yemen, and Saudi Arabia[19]. Almost all countries in the Americas are now hyperendemic for dengue with epidemics occurring every three-to-five years especially in Latin America[16,18]. Due to the significant endemicity of malaria throughout Africa, the majority of "febrile illnesses" including dengue is likely to be mistreated as malaria. This negatively affects our understanding of the epidemiology of dengue in the region. Dengue is indeed underreported in Africa and is not a notifiable disease to WHO by most countries from the continent. A review of the subject by Amarasinghe *et al*[20] suggested that dengue is endemic in 34 countries in Africa and that the four main dengue serotypes circulate in Africa with serotype 2 responsible for most epidemics. Although the threat of dengue is rare in Europe, imported cases by European travellers to and from endemic countries continue to rise (Table 1). A report suggests the importation of dengue to 13 European countries by returning travellers[8].

CLINICAL ASPECTS OF DENGUE INFECTION

Dengue infection may present as a mild asymptomatic

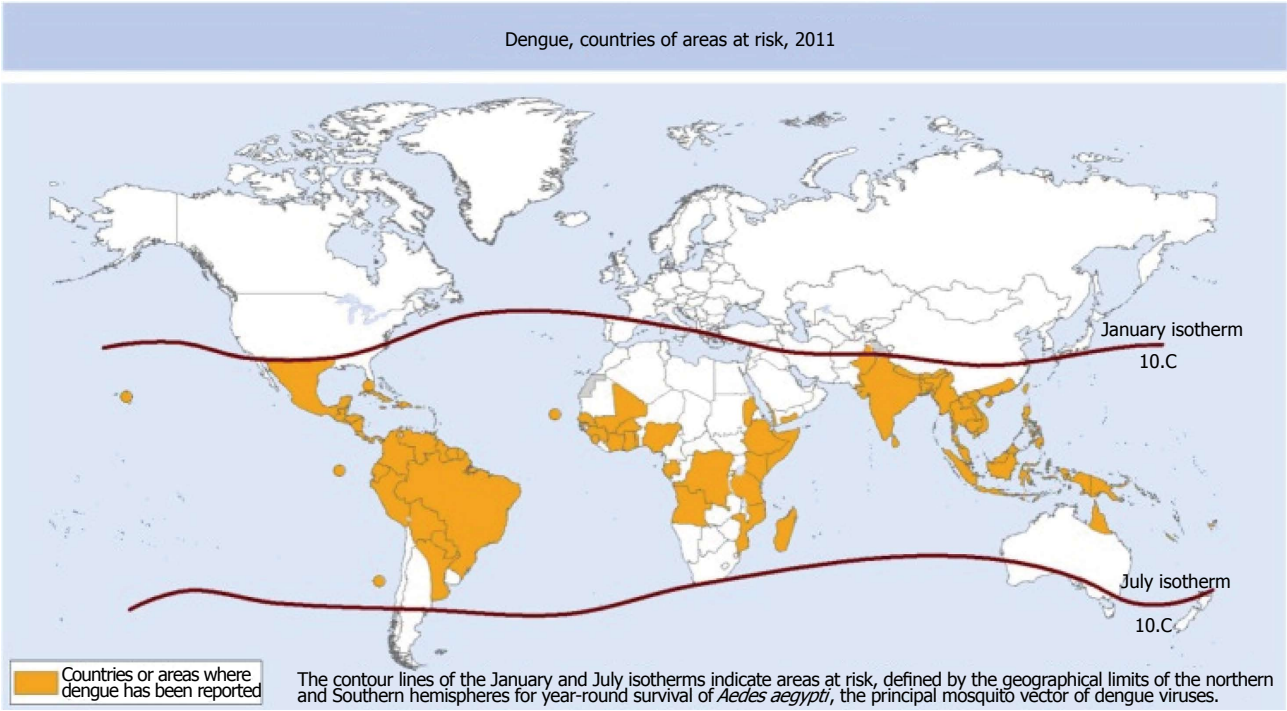

Figure 1 Countries or areas of the world where dengue was reported in 2011, as per data collected by the World Health Organization. Reprinted with permission from Murray *et al*[8]. The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted and dashed lines on maps represent approximate borderlines for which there may not yet be full agreement.

infection to severe illness that may lead to death in some cases. The disease may start as an undifferentiated febrile illness (UF), which may culminate to a diverse and complicated clinical condition such as: DF, DHF, DSS[21]. Clinically, UF illness mimics malaria and other tropical fevers and in the absence of specific serological testing, UF illness could easily be misdiagnosed or labeled as fever of unknown origin. DF is considered to be a mild disease because death is rarely reported, but may be associated with high fever, severe headache, pain behind the eyes, muscle and bone or joint pains, nausea, vomiting, rash and skin haemorrhages. Leukopenia and thrombocytopenia may also occur. The clinical presentation of DHF is similar to DF but the latter is characterized by plasma leakage resulting from alteration in microvascular permeability. The plasma leakage occurs into the pleural and peritoneal cavities that may result in pleural effusion and ascites. Typical presentation of DHF includes high fever, haemorrhagic phenomena, thrombocytopenia, hepatomegaly and circulatory failure. On the other hand, the clinical features of DSS are also similar to those of DHF but the plasma leakage is so severe that the patient develops shock[21,22]. Other signs of circulatory failure such as the skin becoming cool, blotchy, and congested; circumoral cyanosis may be observed. Also, the patients may initially be lethargic, then become restless and then rapidly enter a critical stage of shock. DSS is usually characterized by weak pulse with narrowing of the pulse, hypotension with cold, clammy skin and restlessness. Death may occur in the absence of appropriate treatment.

DENGUE AMONG TRAVELLERS TO ENDEMIC COUNTRIES

The contribution of dengue expansion through international travel and intercontinental movement of goods is on the rise[23]. As the global community trades and travel more and more, so too do communicable and vector-borne diseases. Most dengue-endemic countries are popular touristic destinations and the frequency of international travel to these regions plays a role in the infection and transmission of the disease. With increasing growing markets and international trade in Africa, Asia and Latin America, the risk of dengue infection by travelers is high. It was observed in 2011 that air travel frequency was 40-times higher compared to the frequency during mid 20th century[24]. Human travel to endemic areas as well as travel of infected persons to non-endemic areas is the main driver in the global transmission and expansion of the disease. Overcrowded airports located in most tropical countries serve as ideal breeding ground and distribution source of dengue viruses and travelers contribute in the importation of the disease (Table 1)[25]. Other globalization factors such as international transport of cargo and goods, especially *via* commercial sea shipment also contribute in the importation or exportation of the dengue's primary and secondary vectors, *Aedes aegypti* and *Aedes albopictus*, respectively[26]. The transatlantic transport of used cars and tires has been linked with the introduction of exotic mosquitoes from America to Europe, which contributed to other vector-borne disease epidemics[27,28].

Table 1 Randomly selected articles revealing dengue importation by travelers from endemic countries

Year	Import country	Source country	No. of cases	Age group	Serotype	Assay	Ref.
2010	France	Benin	1	40s	Unknown	IgG/IgM seology	Gautret *et al*[54]
2001-2009	Denmark	Southeast Asia, South Asia, Central America, Africa, Caribbean, South America	114	6-79	DENV 1, 2, 3 and 4	IgG/IgM serology, PCR	Vinner *et al*[55]
2010	Italy	Caribbean, India, Indonesia, Brazil, Thailand, Venezuela, Nicaragua-Honduras	17	16-63	DENV 1, 3	IgG/IgM immunofluorescence, PCR	Pierro *et al*[56]
2013	France	Guadeloupe	1	50s	DENV 2	PCR	Marchand *et al*[57]
2010	France, Sweden	Tanzania	5	41-69	DENV 3	PCR	Gautret *et al*[58]
2012	Germany, United Kingdom	Madeira	42	20-73	Unknown	Unknown	Frank *et al*[59]
2007-2009	Sweden	Thailand	100	Unknown	DENV 2	Unknown	Heddini *et al*[60]
2009	Italy	Senegal	1	40s	DENV 3	PCR	Nisii *et al*[61]
2012	Finland	Madeira	5	50-60	DENV 3	IgG/IgM, NS1 and PCR	Huhtamo *et al*[62]
2013	Germany	Japan	1	50s	Unknown	IgG/IgM, NS1 and PCR	Schmidt-Chanasit *et al*[63]
2010	Germany	Croatia	1	72	Unknown	IgG/IgM, NS1 and PCR	Schmidt-Chanasit *et al*[64]

DENV: Dengue virus; PCR: Polymerase chain reaction.

ASSESSING DENGUE DISEASE BURDEN IN ENDEMIC COUNTRIES

While geographical expansion of dengue and its vector are evident, the true burden of the disease is underestimated due to lack of an efficient public health surveillance system for dengue. Dengue diagnosis is not routinely performed in endemic countries and most febrile illnesses are treated as presumptive malaria or fever of unknown origin. Also, most dengue cases are asymptomatic and go undetected and infected persons do not seek medical attention. Consequently, the number of dengue cases is underreported and the disease burden is grossly underestimated. However, DF, DHF and DSS cause significant humanitarian and economic hardship and it is suggested that about 3.97 billion people living in 128 endemic countries globally are at risk of dengue[7,29]. The disability adjusted life year (DALY) lost due to dengue infection globally was 700000 per year in 2009 while an estimate of aggregate annual cost of dengue was USD 2.1 billion in the Americas in 2000-2007[15,30-32]. Prior to 1990, dengue was endemic in only 9 countries but the disease is currently endemic in 128 countries across Africa, the Americas, the Eastern Mediterranean, SEA and the Western Pacific regions. A study involving twelve countries in the SEA region from 2001 to 2010 suggest an annual economic burden of US $950 million amongst the studied nation[8]. Overall, due to inadequate disease surveillance, low level of reporting, low case fatality rate, lack of routine diagnosis, the true incidence and burden of the disease is unclear.

DENGUE AND MALARIA ENDEMICITY

Mosquitoes are widespread in most tropical and sub-tropical regions of the world. Dengue vectors as well as those responsible for the transmission of yellow fever, chikungunya (*Aedes spp*) and those responsible for malaria (*Plasmodium spp*,) are known to be well established in these regions. Dengue - malaria co-infection has been recognized as an important clinical problem in endemic regions[33]. Vector expansion is driven partly by population growth, unplanned urbanization, crowded humans settlements and inadequate water, sewage and waste management[24]. These factors with the lack of effective vector control programs increase the exposure of humans to the disease vectors. Concurrent dengue and malaria co-infection has been reported in many areas of the world with predominance in the Americas, Asian tropical and sub-Saharan Africa regions[34-36]. The profound endemicity of both diseases and similar and overlapping clinical presentations often lead to misdiagnosis or misinterpretation as mono infections[37]. We previously reported that 10% of malaria patients in Ibadan, Nigeria had active dengue infection. Also, all malaria patients were positive for dengue IgG antibodies which is suggestive of a previous infection[33]. This concomitant dengue/malaria co-infection is consistent with the endemicity of both infections in the region. Despite this endemicity, routine diagnosis of dengue is often neglected and more focus is on malaria. Dengue misdiagnosis or under-diagnosis poses a great risk of increased morbidity and mortality in endemic countries[38]. Therefore, routine dengue diagnosis is very essential in endemic countries as misdiagnosis or lack of diagnosis is likely to have tremendous public health consequences in the general management of febrile conditions in these regions.

DENGUE DIAGNOSTIC METHODS

Dengue diagnosis is relevant in epidemiological sur-

veillance, outbreak control, routine diagnosis in endemic countries among people with febrile diseases as well as diagnosis among travellers visiting or returning from endemic countries. There are several diagnostic assays such as virus culture, RNA detection, antigen detection and serology. These assays are associated with many advantages and disadvantages as well as different level of specificity and sensitivity.

Virus culture

This is usually done by inoculation of samples (serum, plasma or buffy coat) into mosquitoes cell lines such as C6/36 and AP61 or mammalian cell lines such as Vero and LLC-MK2 cells[39]. Sucking mice inoculated intra-cerebrally have also been used for the isolation of DENV[40]. Autopsy tissues from spleen, liver, thymus and lymph nodes have been used to isolate the virus from fatal cases[3]. After virus isolation, serotype identification is achieved by immunofluorescence using serotype-specific monoclonal antibodies. Despite the sensitivity of this method, the routine use is limited in endemic countries due to the fact that it requires improved laboratory safety capacity. The assay is also labor intensive and time consuming requiring adequate professional training. Also, virus detection is effective mostly during the early stages of the infection. It is therefore thought that, for better results, cultures should be performed using patient sample collected during the acute phase of the infection that may contain high viral copies. Acute infection is associated with rapid viral replication with high viral load that peaks before the onset of symptoms[3]. Therefore, timing of sample collection is very important for reliable test results.

Virus RNA detection

Dengue RNA can be detected by polymerase chain reaction (PCR) from tissues, blood or sera collected during the acute phase of the infection using primers directed to serotype-specific regions of the genome[41,42]. Common genomic regions for PCR include; E, NS1, E/NS1, prM/E, NS5 and NS5/3. Viral load may be quantified by RT-PCR while strain typing could be performed by nucleotide sequencing and phylogenetic analysis[43]. However, it is essential that laboratories performing nested PCR take every precaution to prevent false-positive results that can occur as a result of contamination[40]. A recent development of real-time PCR enables a simpler and faster assay with less exposure to contamination during concurrent dengue detection and typing. The assay utilizes oligonucleotide primers and dual-labeled hydrolysis probes for *in vitro* qualitative detection of DENV serotypes in a singleplex or multiplex reactions. However, this method is hampered by late sample collection (> 5 d after onset of symptoms). Therefore a negative result does not preclude dengue diagnosis and samples should be subjected to an anti-IgM ELISA for laboratory confirmation of infection. Although highly sensitive, the method is financially prohibitive as most dengue endemic countries lack the capacity to perform such nucleic acid amplification test.

Antigen detection

The detection of viral antigen has emerged as a potential alternative to PCR and virus culture. The main antigen target is the non-structural protein 1 (NS1). The NS1 antigen is produced during viral replication and can be detected in patients with primary and secondary dengue infections up to 9 d after the onset of disease[44]. NS1 is secreted in all infected cells during the acute phase of the infection and the presence in blood stimulates a strong humoral response. Quantification of the NS1 is a prognostic maker for dengue disease and higher levels have been linked to progression to DHF[45]. A recent study that evaluates a rapid NS1 assay in both Vietnam and Malaysia revealed the following; in Vietnam the sensitivity and specificity of the test was 69.2% (95%CI: 62.8% to 75.6%) and 96% (95%CI: 92.2% to 99.8%) respectively. In Malaysia the performance was similar with 68.9% sensitivity (95%CI: 61.8% to 76.1%) and 96.7% specificity (95%CI: 82.8% to 99.9%) compared to RT-PCR[46].

Serological methods

Serology is based on screening for dengue IgG/IgM antibodies. It is suggested that IgM production occurs 4-8 d after the onset of fever and last for a couple of weeks. On the other IgG production is low after primary infection but matures slowly within weeks and months and may last for several years[47]. ELISA-based IgM assays have become an important tool for the surveillance of dengue. Although these IgM-based assays are a useful diagnostic tool, results from these tests should be interpreted with caution. In addition, there is cross reactivity with other flaviviruses including West Nile virus (WNV), St. Louis encephalitis virus (SLE), Japanese encephalitis virus (JEV) and yellow fever virus (YFV). Therefore, during interpretation of results, patient's past medical history, recent travel history, and vaccination record (especially yellow fever vaccination) should be reviewed in order to determine the likelihood that the current acute febrile illness is due to an infection with DENV. There may also be false-negative results due to an extended seroconversion period[48]. The presence of anti-dengue IgM suggests recent infection while IgG antibody detection may be used for the classification of both primary and secondary infection[49]. That is a ratio of IgM/IgG greater than 1.78 represents primary infection and lesser than 1.78 represents secondary infections[48]. Also, the diagnostic value of IgA has been suggested and it has been shown that significantly higher levels of IgA antibodies occur in DHF/DSS than in DF cases[50]. The sensitivity and specificity of IgM-based assays is influenced by the quality of the antigen used and can vary greatly between commercially available products.

ROUTINE DENGUE DIAGNOSIS IN ENDEMIC COUNTRIES

In spite of the methods listed above, their routine use

in dengue endemic countries is limited due to either lack of laboratory capacity or skilled personnel. An ideal routine diagnostic test in endemic countries would fulfill the ASSURED criteria: (1) Affordable by those at risk of infection; (2) Sensitive; (3) Specific; (4) User-friendly; (5) Rapid and robust; (6) Equipment-free; and (7) Delivered to those who need it. The recent developments in rapid point-of-care (POC) immunochromatograhic tests (ICT) offer hopes for improved diagnosis of early dengue infection[51]. ICT for the detection of DENV NS1 antigen, IgG, IgM, and IgA antibodies promises to offer tremendous opportunities for the rapid detection of DENV in clinical samples. These ICT are manufactured in lateral flow cassettes and strips and allows the flow of sample by capillary action[52]. Considering that the majority of patients in developing countries are treated in primary health centers without the availability of a laboratory, POC testing may offer a unique advantage in the routine diagnosis of DENV among febrile patients. The tests can be performed in approximately 10-15 min and requires no specialized equipment or training.

PREVENTION AND CONTROL OF DENGUE

In the absence of specific antivirals and a vaccine, the main method of dengue control is to reduce the vector population and to educate people in endemic countries as well as travellers to these regions on basic protection measures such as wearing protective clothing and the use of anti-insecticide sprays.

Effective dengue control in endemic countries also requires more governmental, public and other stakeholder commitment and intervention at all levels. We recommend the following areas for action: Institution of policies for dengue to be a notifiable disease and the provision of POC diagnosis of dengue in febrile patients in endemic regions. Vector reduction activities by sustained environment and space spraying with larvicide as complementary measure. Unfortunately, there is lack of key indicator measurements for vector control programs at national surveillance systems in most tropical countries. Mobilization for public awareness on dengue control and the need to establish a sustained and integrated disease surveillance-response information and knowledge generation programs in vulnerable countries[53]. Effective waste disposal and water supply system management to reduce vector breeding grounds. Operational research is needed to generate evidence-based and cost-effective knowledge for innovative policies to outwit dengue from the region. Innovative approach on genetically modified mosquitoes to reduce vector population and interrupt transmission. Availability of the dengue vaccine, Dengvaxia (Sanofi Pasteur) for use in 9 to 45 years old persons in hotspots areas as well as accelerating dengue drug discovery and the availability of treatment.

CONCLUSION

This review outlined some of the basic public health issues associated with dengue control in endemic countries. Despite the increasing contribution of DENV as a major cause of febrile disease in developing countries, there is a high rate of misdiagnosis and underreporting from endemic countries, as well as lack of routine surveillance and public health prioritization. In summary, we suggest the need for public health commitment to include dengue as a notifiable disease, implement routine laboratory diagnosis and personnel training in endemic countries. Also, dengue NS1 ICTs or IgM antibody tests should be available at all primary health care centers to enable early detection of cases. Travellers visiting dengue endemic countries should be fully informed on symptoms of dengue and strongly urged to do a dengue test prior to departure or immediately after entering their own country, if they suspect infection by the virus, to reduce the risk of importation of the disease.

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ORIGINAL ARTICLE

Basic Study

Expression of hepatitis B virus surface antigens induces defective gonad phenotypes in *Caenorhabditis elegans*

Yi-Yin Chen, Li-Wei Lee, Wei-Ning Hong, Szecheng J Lo

Yi-Yin Chen, Li-Wei Lee, Szecheng J Lo, Department of Biomedical Science, College of Medicine, Chang Gung University, Tao Yuan 333, Taiwan

Yi-Yin Chen, Wei-Ning Hong, Szecheng J Lo, Graduate Institute of Biomedical Science, College of Medicine, Chang Gung University, Tao Yuan 333, Taiwan

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Correspondence to: Szecheng J Lo, PhD, Professor, Department of Biomedical Science, College of Medicine, Chang Gung University, #259 Wen-Hwa 1st Road, Tao Yuan 333, Taiwan. losj@mail.cgu.edu.tw
Telephone: +886-3-2118800-3259
Fax: +886-3-2118392

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Abstract

AIM

To test whether a simple animal, *Caenorhabditis elegans* (*C. elegans*), can be used as an alternative model to study the interaction between hepatitis B virus antigens (HBsAg) and host factors.

METHODS

Three plasmids that were able to express the large, middle and small forms of HBsAgs (LHBsAg, MHBsAg, and SHBsAg, respectively) driven by a ubiquitous promoter (*fib-1*) and three that were able to express SHBsAg driven by different tissue-specific promoters were constructed and microinjected into worms. The brood size, egg-laying rate, and gonad development of transgenic worms were analyzed using microscopy. Levels of mRNA related to endoplasmic reticulum stress, *enpl-1*, *hsp-4*, *pdi-3* and *xbp-1*, were determined using reverse transcription polymerase reaction (RT-PCRs) in three lines of transgenic worms and dithiothreitol (DTT)-treated wild-type worms.

RESULTS

Severe defects in egg-laying, decreases in brood size, and gonad retardation were observed in transgenic worms expressing SHBsAg whereas moderate defects were observed in transgenic worms expressing LHBsAg and MHBsAg. RT-PCR analysis revealed that *enpl-1*, *hsp-4* and *pdi-3* transcripts were significantly elevated in worms expressing LHBsAg and MHBsAg and in wild-

type worms pretreated with DTT. By contrast, only *pdi-3* was increased in worms expressing SHBsAg. To further determine which tissue expressing SHBsAg could induce gonad retardation, we substituted the *fib-1* promoter with three tissue-specific promoters (*myo-2* for the pharynx, *est-1* for the intestines and *mec-7* for the neurons) and generated corresponding transgenic animals. Moderate defective phenotypes were observed in worms expressing SHBsAg in the pharynx and intestines but not in worms expressing SHBsAg in the neurons, suggesting that the secreted SHBsAg may trigger a cross-talk signal between the digestive track and the gonad resulting in defective phenotypes.

CONCLUSION
Ectopic expression of three forms of HBsAg that causes recognizable phenotypes in transgenic worms suggests that *C. elegans* can be used as an alternative model for studying virus-host interactions because the resulting phenotype is easily detected through microscopy.

Key words: Hepatitis B virus; *Caenorhabditis elegans*; Green fluorescence proteins; Endoplasmic reticulum stress; Gonad retardation; Surface antigens

Core tip: In the past, mouse and cell culture models have been used for studying the effects of hepatitis B virus antigens (HBsAg) on hosts. Both models have advantages and disadvantages in terms of economic and time concerns. In this study, we provide an alternative animal model, *Caenorhabditis elegans (C. elegans)*, to demonstrate that SHBsAg can induce observable phenotypes which has never been reported in mouse and cell culture models. We suggest that *C. elegans* can serve as a new plateform for studying various viral pathogenesis.

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INTRODUCTION

Human hepatitis B virus (HBV), a member of the family *Hepadnaviride*, is a partially double-stranded DNA virus. The genome of HBV contains approximately 3200 nucleotides that encodes four open reading frames, namely surface (S), core (C), polymerase (P) and X in an overlapping but frame-shifted manner[1-3]. Infection with HBV inducers a broad range of clinical outcomes, from asymptomatic hepatitis to fulminant hepatitis. Chronic hepatitis B carriers are highly associated with the development of liver cirrhosis and hepatocellular carcinoma[4,5]. Molecular biology analyses from tumor samples have revealed that HBV DNA integration could activate genes associated with the cell cycles, leading to abnormal cell proliferation[6,7]. Pathogenesis and etiology studies have found that X and truncated preS proteins play oncogenic roles[8-10].

Woodchucks were the first animal model for studying liver carcinogenesis caused by natural woodchuck hepatitis virus (WHV) infection[11,12]. A high incidence of liver tumor formation occurs in newborn woodchucks infected with WHV. Molecular dissection has revealed that *c-myc* oncogene level are highly elevated in liver tumors[13,14]. Later, the transgenic mouse model was applied to express an individual viral protein, such as the large hepatitis B surface antigen (LHBsAg) and X protein, which are driven by the albumin promoter for specific expression in the liver, to study the mechanisms of liver carcinogenesis induced by viral proteins[15,16]. In combining molecular biology analyses of the HBV X (HBx) gene in transfected cells, numerous studies have elucidated that the X protein is multifunctional and induces transactivation activity, signal transduction and cell death[9,17,18]. Recently, Geng *et al*[19] employed *Caenorhabditis elegans* (*C. elegans*), a soil nematode, to express HBx under a heat shock control and found that HBx induced cell apoptosis and necrosis through the interaction of HBx and CED-9, a human homolog of Bcl-2.

C. elegans was first used as a model organism for studying development and the nervous system because the species is transparent throughout its life span and in its adult form, possesses approximately 300 neurons out of 1000 somatic cells[20,21]. Because of its short-life cycle, simplicity, numerous available mutated forms, and ease of handling for knocking-down specific gene, *C. elegans* is now a model for studying various biological topics, such as aging, human diseases, host-pathogen interaction and viral pathogenesis[22-25]. Because of a high percentage of genes in numerous cellular pathways is conserved across nematodes to vertebrates, a study of PEG-mediated *Poxviridae* infection in *C. elegans* revealed that the core genes of apoptosis (*ced-3* and *ced-4*) control vaccinia virus replication in worms[26,27]. Therefore, *C. elegans* could serve as a new platform for virologists to study virus-host interaction and pathogenesis in addition to the currently used cell culture and mammalian models. In this study, we expressed three forms of HBsAg in *C. elegans* to determine different degrees of defects in gonad development.

MATERIALS AND METHODS

Plasmid constructions

P_{fib-1}::gfp::icr::SHBsAg: A 1.5 kb fragment excised from the P_{fib-1}::gfp::LD plasmid[28] by cutting with *Hind*Ⅲ and *Age* Ⅰ and was isolated and then inserted into the *Hind*Ⅲ and *Age* Ⅰ sites of pPD95.75 to generate P_{fib-1}::gfp. P_{fib-1}::gfp was then cut with *EcoR* Ⅰ and ligated with a 0.8 kb of icr::SHBsAg fragment which was isolated from P_{fib-1}::LD::icr::SHBsAg to generate a 6.8 kb of P_{fib-1}::

gfp::icr::SHBsAg. When microinjection of $P_{fib-1::}$gfp::icr::SHBsAg into N2 strain, worms expressed green fluorescence proteins (GFP) and HBV small surface antigens (SHBsAg).

$P_{fib-1::}$gfp::icr::linker: A linker was designed to contain *EcoR* Ⅰ, *Not* Ⅰ, *Bgl* Ⅱ, *Sal* Ⅰ, *Nsi* Ⅰ and *Sac* Ⅰ cutting sites. This linker was ligated to $P_{fib-1::}$gfp and generated plasmid $P_{fib-1::}$gfp::linker. $P_{fib-1::}$gfp::linker was cut with *EcoR* Ⅰ *and Not* Ⅰ and ligated with an icr fragment which was isolated from $P_{fib-1::}$gfp::icr::SHBsAg to generate a 6.2 kb of $P_{fib-1::}$gfp::icr::linker plasmid.

$P_{fib-1::}$gfp::icr::MHBsAg: A 680 bp of MHBsAg DNA fragment was amplified from pMH3/3097[29] using primers HBVs(M)-Not Ⅰ-F and HBVs-Sal Ⅰ-R. This fragment was ligated with $P_{fib-1::}$gfp::icr::linker to generate a 7.0 kb of $P_{fib-1::}$gfp::icr::MHBsAg. Transgenic worm carrying $P_{fib-1::}$gfp::icr::MHBsAg expressed GFP and MHBsAg.

$P_{fib-1::}$gfp::icr::LHBsAg: A 1.1 kb of LHBsAg DNA fragment was amplified from pMH3/3097[29] using primers HBVs(L)-NotI-F and HBVs-Sal Ⅰ-R. This fragment was ligated with $P_{fib-1::}$gfp::icr::linker to generate a 7.3 kb of $P_{fib-1::}$gfp::icr::LHBsAg. When microinjection of $P_{fib-1::}$gfp::icr::LHBsAg into N2 strain, worms expressed GFP and LHBsAg.

$P_{myo-2::}$gfp::icr::SHBsAg: The fragment of *fib-1* promoter was cut from P_{fib-1}GFP-icr-SHBsAg by digestion with *Hind* Ⅲ and *Age*I, and replaced with the *myo-2* promoter which was isolated from $P_{myo-2::}$gfp::icr::DsRed::LD[28] to create a 6.6 kb of $P_{myo-2::}$gfp::icr::SHBsAg. Transgenic worms carrying this plasmid expressed both GFP and SHBsAg in pharynx.

$P_{mec-7::}$gfp::icr::SHBsAg: The plasmid was generated by substitution of the *fib-1* promoter of $P_{fib-1::}$gfp::icr::SHBsAg with the *mec-7* promoter which was isolated from $P_{mec-7::}$gfp::icr::DsRed::LD[28] to create a 6.3 kb of $P_{mec-7::}$GFP::icr::SHBsAg. Transgenic worms carrying this plasmid expressed GFP and SHBsAg in neurons.

$P_{ges-1::}$gfp::icr::SHBsAg: The plasmid was generated by substitution of the *fib-1* promoter of $P_{fib-1::}$gfp::icr::SHBsAg with the *ges-1* promoter which was isolated from $P_{ges-1::}$gfp::icr::DsRed::LD[28] to create a 6.6 kb of $P_{ges-1::}$gfp::icr::SHBsAg. Transgenic worms carrying this plasmid expressed GFP and SHBsAg in intestinal cells.

Primers used in this study

For plasmid constructions and RT-PCR analyses, the following paired primers were used: linker-F: 5′-aat tcaaaaagcggccgcagatctgtcgacatgcatgagctc-3′; linker-R: 5′-gtttttcgccggcgtctagacagctgtacg tactcgagttaa-3′; HBVs(L)+Not Ⅰ-F: 5′-gggaacaagag cggccgcatggggcag-3′; HBVs(M)+Not Ⅰ-F: 5′-acactc atcgcggccgcatgcagtgg-3′; HBVs+Sal Ⅰ-R: 5′-gttttgtgtcgacttaaatgtataccc-3′; eft-2-F: 5′-ggtggtcaaatcatcccaac-3′; eft-2-R: 5′-tcc-tcgaaaacgtgtcctct-3′; endoplasmin-F: 5′-t gaaaa cctccaacagcaca-3′; endoplasmin-R: 5′-gcagtttccttg agccagtc-3′; hsp-4-F: 5′-ttttcgaggttcttgccact-3′; hsp-4-R: 5′-tctccggtattttcgacacc-3′; PDI-F: 5′-gccgtttcca aca aagaaga-3′; PDI-R: 5′-cccttgagc ccatcagtaga-3′; xbp-1-F: 5′-cgtcgtcta cgaagaagaagtcgtc-3′; xbp-1-R: 5′-gatg ata gttagatacatatccacactg-3′.

Worm strains and culture

N2 (wild-type) worm was obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota) and cultured on Nematode Growth Medium (NGM) following standard methods[20]. Images of transgenic worms were acquired using Leica DM2500 equipped with CoolSNAP K4 (photometrics) and processed with a MetaMorph (version 6.1).

Microinjection

Plasmid DNA was prepared by using QIAprep spin miniprep kit and the concentration was adjusted at 100 ng/μL in injection buffer (20 mmol/L potassium phosphate, pH 7.5, 3 mmol/L potassium citrate, pH 7.5, 2% polyethylene glycol, M.W. 6000). The injection mixture also contained pRF-4 which was included as a screening marker. Worm was placed onto 2% agarose pads and injected by capillary needle loaded with DNA mixture using a FemtoJet system (Eppendorf AG, Hamburg, Germany). The glass capillaries were purchased from World Precision Instruments (Kwik-Fil™, borosilicate 16 glass capillaries, item number 1B100F-6, United States) and pull by Flaming/Brown micropipette puller (MODEL P-97, Sutter Instrument Co., United States).

Measurement of egg-laying activity and brood size

Worms were first synchronized and placed one worm per a single plate. The offspring in each plate were counted every day.

Microscopy

For visualization of GFP expression in transgenic worms, an upright fluorescence microscope (Leica DM2500) was used. For visualization of gonad structure and development a differential interference contrast (DIC) microscope was used and images were captured using a cool CCD (CoolSNAP K4).

Reverse transcription

The total RNA was extracted from transgenic worms expressed both GFP and HBsAgs with TRIzol reagent. The reverse transcription reaction was first carried out with 4 μg of RNA, 2 μL of dNTP (10 mmol/L), 2 μL of Oligo-dT (10 mmol/L), and added DEPC H_2O to 12 μL. After incubated at 68 ℃ for 5 min, the mixture was then added 4 μL of 5 × first-strand buffer (invitrogen), 2 μL of DTT (0.1 mmol/L, invitrogen), 1 μL of RNase inhibitor (invitrogen) and 1 μL of Reverse Transcriptase (invitrogen) and incubated at 42 ℃ for 50 min, and then 70 ℃ for

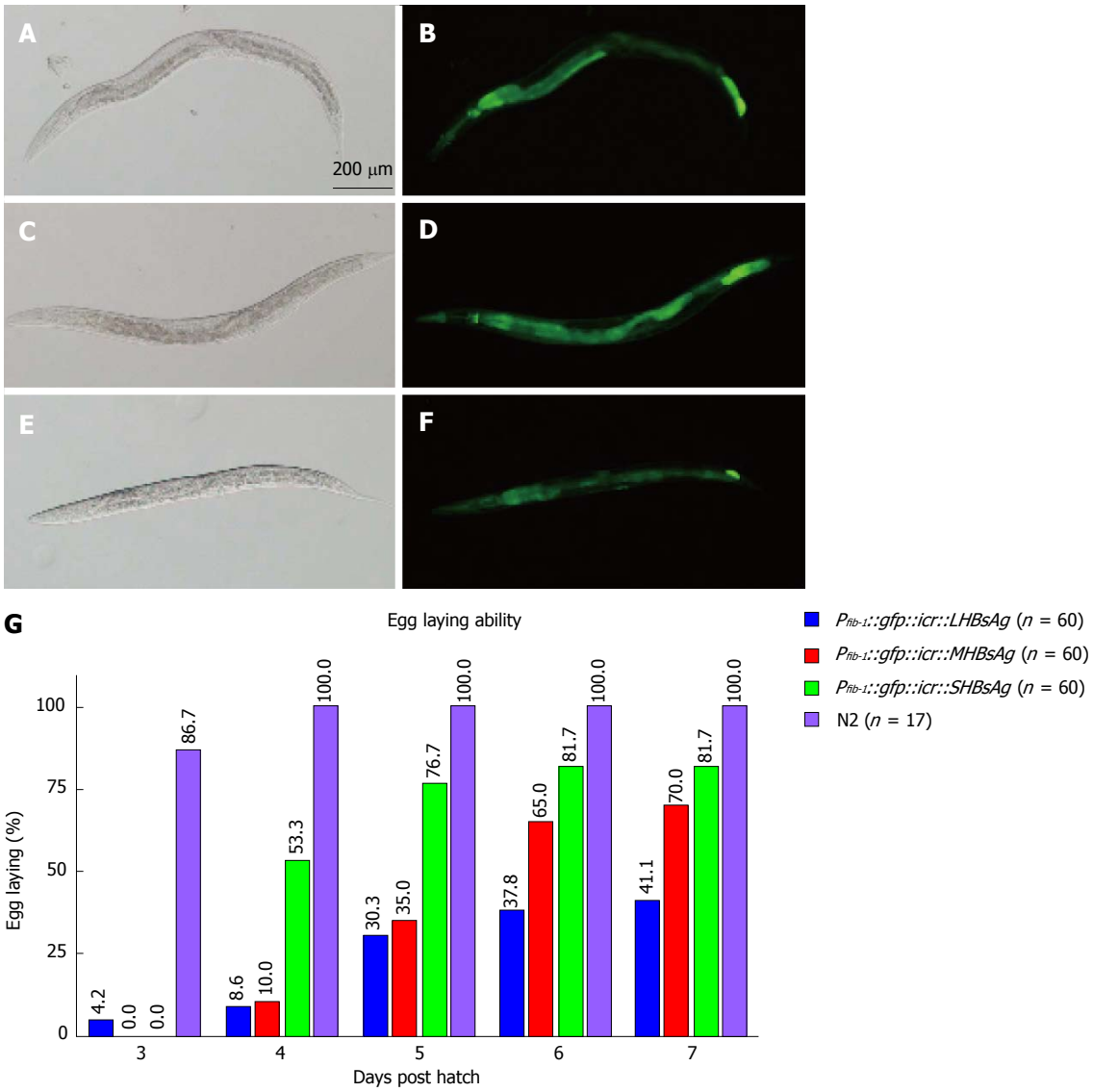

Figure 1 Expression of various lengths of hepatitis B virus antigens in whole worms induced defects in the rate of egg-laying. A-F: Micrographs of transgenic worms expressing LHBsAg (A and B), MHBsAg (C and D), and SHBsAg (E and F) were captured under a bright-field microscope (A, C, and E) and a fluorescence microscope (B, D, and F). The heads of the worms are shown toward the left. The scale bar indicates 200 μm. G: Egg-laying capability of three lines of transgenic worms and wild-type worms (N2) shown using various color bars. The rate of egg-laying in 3 to 7 d post-hatching is shown above the bar. HBsAgs: Hepatitis B virus antigens.

15 min. The primers used in PCR analyses were listed as above.

RESULTS

Expression of three forms of HBsAg reduces egg-laying capability

To determine whether *C. elegans* can be a new platform for studying virus-host interaction we ectopically expressed three lengths of HBsAg (SHBsAg, MHBsAg, and LHBsAg) in worms under the control of the ubiquitous promoter fibrillarin (*fib-1*). Three HBsAg gene sequences were individually placed in a bicistronic vector behind a reporter gene, green fluorescence protein (GFP), which was used as a selection marker[30]. Transgenic worms were selected for the expression of GFP (Figure 1A-F) and maintained to characterize phenotypes. After synchronization, transgenic worms were singled out and placed on single plates, and the numbers of eggs produced by individual worms were counted every day. The results showed different egg-laying averages in a total of 60 transgenic animals in three groups expressing SHBsAg, MHBsAg, and LHBsAg (Figure 1G). On the fourth day after hatching, the wild-type worms (N2) displayed an egg-laying rate of 100% whereas those worms expressing SHBsAg, MHBsAg, and LHBsAg demonstrated laying-egg rates of approximately 8.6%, 10%, and 53.3%, respectively. Although the egg-laying rates of the three lines of transgenic worms increased in the following days, the maximum egg-laying rate was 41.1% for worms expressing SHBsAg, 70% for worms expressing MHBsAg and 81.7% for worms expressing LHBsAg at 7 d post-hatching (Figure 1B). The reduced rate of egg-laying in the three lines of HBsAg-expressing

Table 1 Comparison of egg-laying ability and brood size among various transgenic worms

Construct	Strain	Ecotopic proteins	Protein expression site	Egg-laying ability (%)	Brood size
	N2			100 (n = 17)	290 ± 15 (n = 17)
Pfib-1::gfp::icr	N2	GFP	Whole worm	100 (n = 10)	268 ± 29 (n = 18)
Pfib-1::gfp::icr::SHBsAg	N2	GFP, SHBsAg	Whole worm	9 (n = 60)	66 ± 15 (n = 14)
Pfib-1::gfp::icr::MHBsAg	N2	GFP, MHBsAg	Whole worm	10 (n = 60)	175 ± 50 (n = 15)
Pfib-1::gfp::icr::LHBsAg	N2	GFP, LHBsAg	Whole worm	54 (n = 60)	239 ± 14 (n = 15)
Pmyo-2::gfp::icr::SHBsAg	N2	GFP, SHBsAg	Pharynx	83 (n = 60)	163 ± 20 (n = 26)
Pges-1::gfp::icr::SHBsAg	N2	GFP, SHBsAg	Intestine	97 (n = 60)	203 ± 50 (n = 32)
Pmec-7::gfp::icr::SHBsAg	N2	GFP, SHBsAg	Neuron	100 (n = 60)	270 ± 42 (n = 26)

HBsAgs: Hepatitis B virus antigens; GFP: Green fluorescence proteins; SHBsAg: Human hepatitis B virus small surface antigens; MHBsAg: Human hepatitis B virus middle surface antigens; LHBsAg: Human hepatitis B virus large surface antigens.

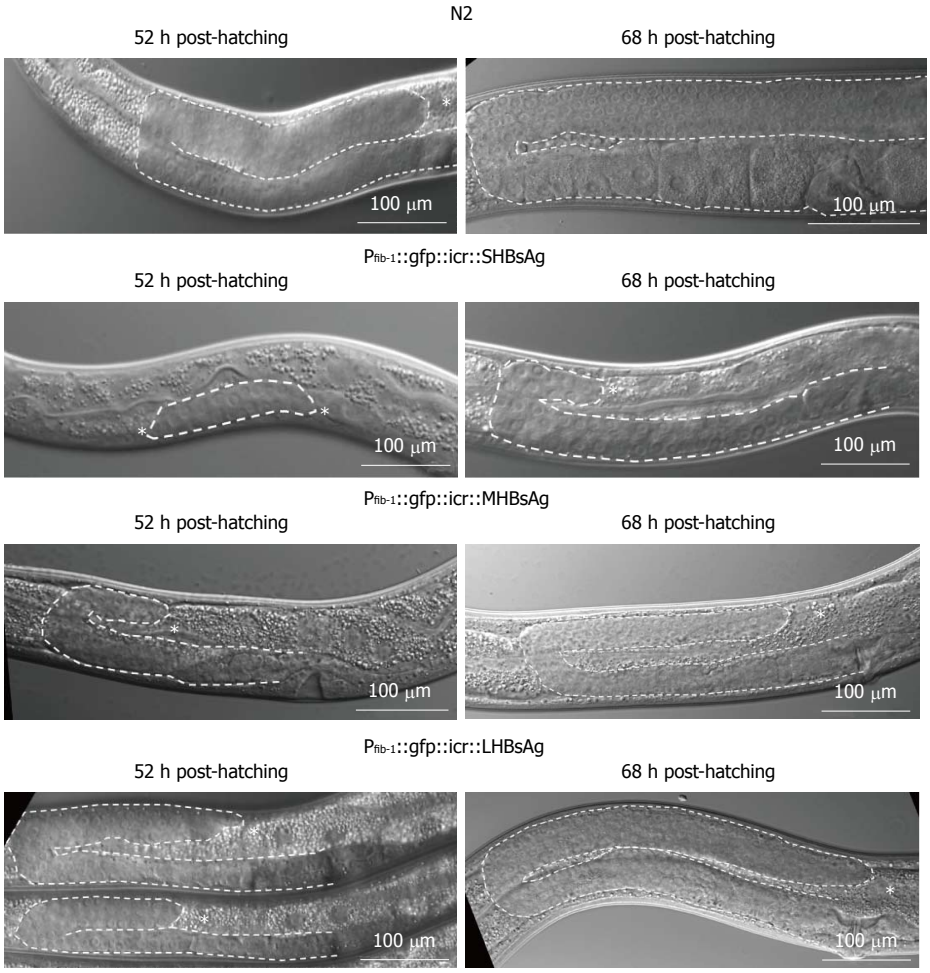

Figure 2 Differential interference contrast micrographs of gonad development in transgenic worms at various post-hatching times. The upper row shows a wild-type worm (N2); the second row shows a transgenic worm expressing SHBsAg; the third row shows a transgenic worm expressing MHBsAg; and the bottom row shows a transgenic worm expressing LHBsAg. The right column shows the gonads at 52 h post-hatching and the left column at shows gonads at 68 h post-hatching expect the worm expressing SHBsAg is at 72 h post-hatching. The gonad contour is indicated with dotted lines and the tip (distal end) is marked by asterisk.

worms was unlikely to have caused by the ectopic expression of GFP because worms carrying a plasmid with the sole function of expressing GFP throughout the body exhibited egg-laying capability of 100% (Table 1).

Expression of SHBsAg causes the most severe gonad retardation

To understand why the expression of various lengths of HBsAg in transgenic worms caused a reduction in egg-laying capability, we examined the gonad development of the three types of transgenic worms under a DIC microscope. As shown in Figure 2 (upper two rows), at 52 h after hatching, wild-type worms had nearly completed the gonad development; by contrast, worms expressing SHBsAg exhibited a dramatic retardation of gonad development in larval stage 3 (L3). The process of oogenesis was observed in N2 worms 68 h post hatching whereas the gonads of worms expressing SHBsAg were

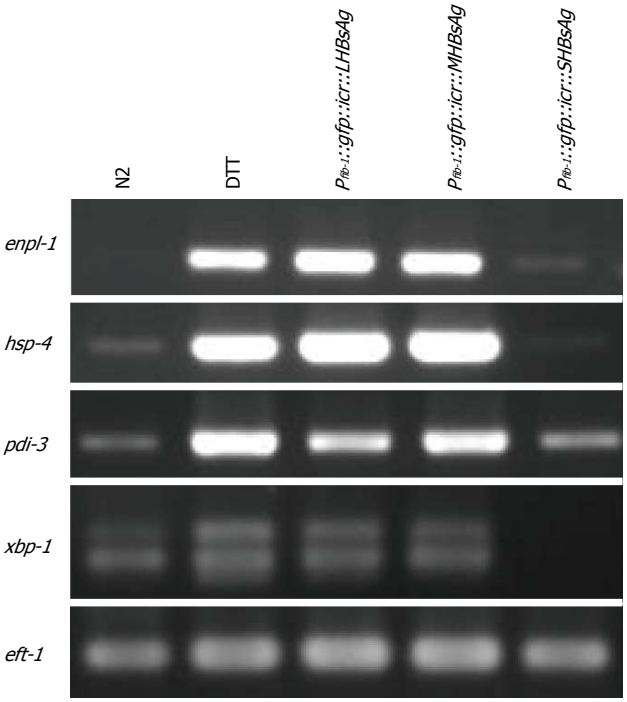

Figure 3 Reverse transcription polymerase reaction analyses of mRNA levels in various transgenic worms. The four transcripts (*enpl-1*, *hsp-4*, *pdi-3*, and *xbp-1*), ER-stress markers, from wild-type worms (N2) with or without DTT pretreatment, an ER stress inducer, and three lines of transgenic worms were analyzed using RT-PCR and gel-electrophoresis. The transcript of the translation factor (*eft-2*) served as a loading control. N2 worms treated with and without DTT and transgenic worms are indicated above the gel. DTT-treated worms severed as positive controls of ER-stress responses. DTT: Dithiothreitol; RT-PCR: Reverse transcription polymerase reaction.

only just beginning to turn as mid-stage of larva 4 as 72 h post-hatching. Oogenesis was observed in some worms expressing SHBsAg until 96 h post-hatching (data not shown). Worms expressing MHBsAg and LHBsAg at 52 h and 68 h post-hatching showed a retardation of gonad development that was less severe than that observed in worms expressing SHBsAg at a similar stage (Figure 2, lower two rows). The severity of gonad retardation clearly reflected the reduced percentage of egg-laying (9%, 10%, and 54%, respectively) and average brood size (66, 175, and 239, respectively) in the three lines of transgenic worms, as shown in Table 1.

Gonad retardation caused by SHBsAg may operate through unknown pathways

A previous study reported that endoplasmic reticulum (ER) stress could cause retardation of gonad development[31]. To determine whether the defective phenotypes of the three lines of transgenic worms resulted from ER stress, we performed RT-PCR analysis of ER stress markers. Total RNA from the three lines of transgenic worms and N2 worms with or without DTT treatment was isolated and analyzed for the expression levels of *enpl-1*, *hsp-4*, *pdi-3* and *xbp-1* through RT-PCR. The results of the gel-electrophoresis of RT-PCR products indicated that the levels of *enpl-1, hsp-4* and *pdi-3* were substantially elevated in worms expressing LHBsAg and MHBsAg, being similar to those N2 worms pretreated with the ER stress-inducer DTT whereas the *xbp-1* level had increased only slightly (Figure 3). Only a slight increase in the level of *pdi-3* was observed in worms expressing SHBsAg compared with that of wild-type worms, and no obvious elevation of other ER stress-related transcripts was detected. We concluded that the defective phenotypes caused by the expression of LHBsAg and MHBsAg were likely attributable to ER stress signals. By contrast, the defect induced by the expression of SHBsAg might have been caused by other unknown pathways.

Expression of SHBsAg in the pharynx and intestines induces defective phenotypes

To determine which tissues expressing SHBsAg were responsible for gonad retardation, we substituted the *fib-1* promoter with *myo-2*, *ges-1*, and *mec-7*, to express SHBsAg in the pharynx, intestines, and neurons, respectively. Transgenic worms expressing GFP in the pharynx, intestines, and neurons were selected (Figure 4A) and analyzed for egg-laying capability and gonad development. As shown in Table 1, 100% and 97% egg-laying capability were found in worms expressing SHBsAg in the neurons and intestines, respectively, compared with 83% egg-laying capability in worms expressing SHBsAg through *myo-2*. When examining the gonad development of the three lines of transgenic worms using DIC, we found no obvious retardation in worms expressing SHBsAg in neuron at 52 h and 68 h post-hatching and a moderate level of gonad retardation in worms expressing SHBsAg in the pharynx and intestines (Figure 4B). This suggested that cross-talk between the digestive and reproductive system may be triggered by secreted SHBsAg, causing defects in a small portion of the population.

DISCUSSION

In this study, we demonstrated that transgenic worms expressing three forms of HBsAgs throughout the body exhibited lower rates of egg-laying, reduced brood sizes and retardation of gonad development to various degrees. Unexpectedly, worms expressing SHBsAg displayed the most severe defects (Table 1). No study has yet reported that the expression of SHBsAg can induce detectable phenotypes in cultured cells or animals; however, ER-stress and tumor formation have been observed in cells and animals expressing LHBsAg and MHBsAg[16,32]. Consistent with previous studies, worms expressing LHBsAg and MHBsAg were found to possess higher levels of *enpl-1*, *hsp-4*, *pdi-3* and *xbp-1* transcripts as did N2 worms pretreated with DTT (Figure 3). Because the gonad is the organ most sensitive to environmental changes[33], we suggest that ER-stress signal occurring autonomously or non-autonomously in the gonad can lead to gonad retardation, a reduced rate of egg-laying, and a smaller brood sizes in transgenic worms expressing LHBsAg and MHBsAg.

The unexpected results of the most severe pheno-

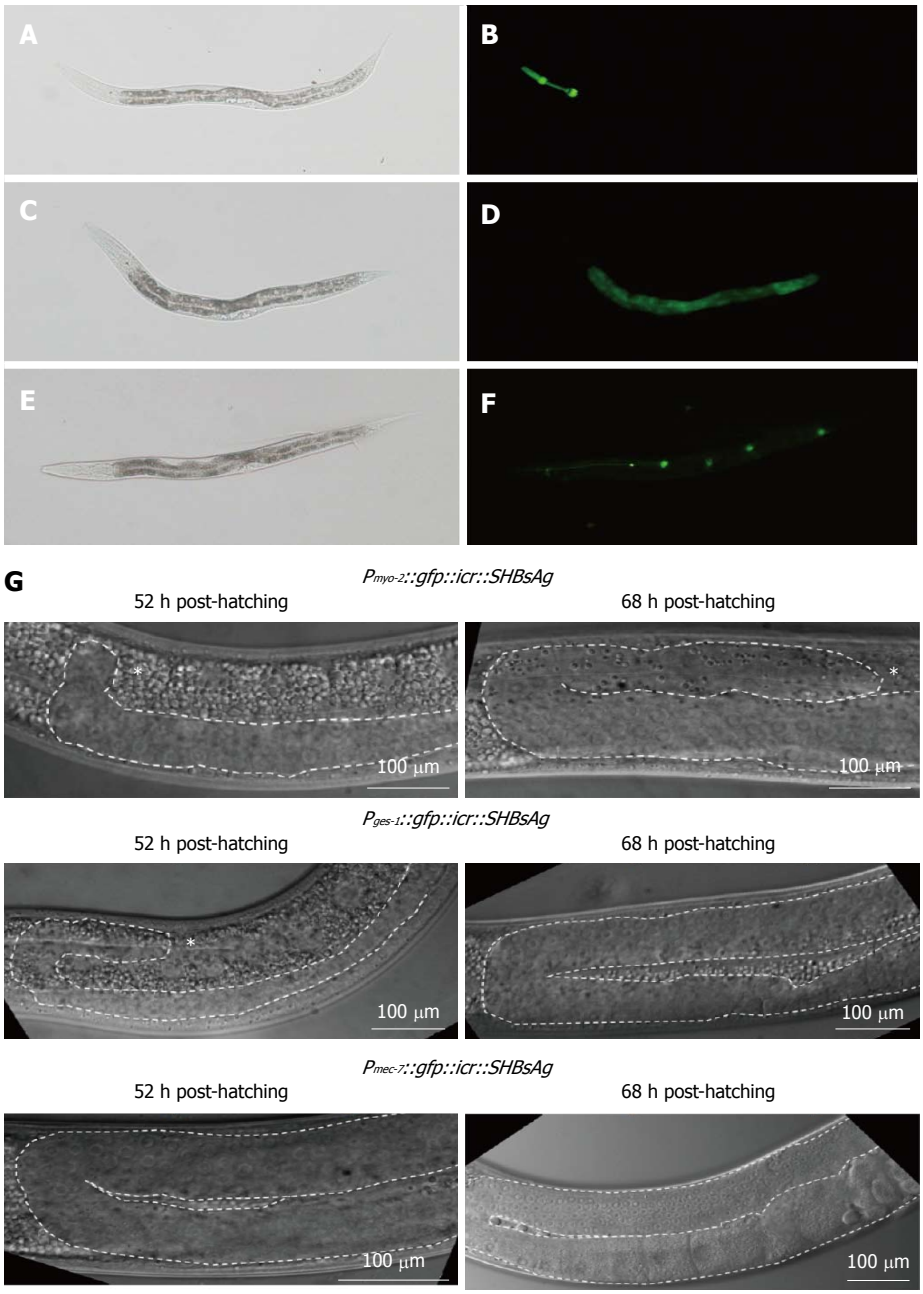

Figure 4 Features of transgenic worms expressing SHBsAgs in different tissues. A-F: Micrographs of transgenic worms expressing SHBsAgs in the pharynx (A, and B), intestinal cells (C and D), and neurons (E and F) were captured under a bright-field microscope (A, C, and E) and a fluorescence microscope (B, D, and F). The heads of the worms are shown toward the left. The scale bar indicates 200 μm; G: Gonad development in transgenic worms: The upper row is a transgenic worm expressing SHBsAg in the pharynx; the middle row is a transgenic worm expressing SHBsAg in intestinal cells; and the lower row is a transgenic worm expressing SHBsAg in neurons. Images were captured under a DIC microscope. The right column shows the gonads at 52 h post-hatching and the left column at 68 h post-hatching. The gonad contour is outlined by dotted lines and the tip (distal end) is marked by asterisk. HBsAgs: Hepatitis B virus antigens.

types induced by the expression of SHBsAg might be explained by the different nature of the three forms of HBsAg. In general, SHBsAg can form subviral particles of approximately 22 nm and be constantly secreted outside of cells whereas MHBsAg is less efficiently secreted and LHBsAg is usually retained in the ER[29,32]. This hypothesis is supported by the results shown in Figure 3, namely that four ER-stress related transcripts (*enpl-1*, *hsp-4*, *pdi-3* and *xbp-1*) were substantially elevated in worms expressing LHBsAg and MHBsAg but only one transcript (*pdi-3*) displayed a slight elevation in worms expressing SHBsAg. The secretion of SHBsAg might either trigger signals inhibiting gonad development or titrate out secretion factors that are required for gonad development, although ER-stress signals might also play a minor role (Figure 4). Nevertheless, the underlying mechanism that leads to the most severe phenotypes in worms expressing SHBsAg remains unknown and will be elucidated by performing rescue and genetic cross experiments in the future.

C. elegans has been used for studying viral pathogenesis and virus-host interaction for more than a

decade[27,34]. In comparison with the number of publications using *C. elegans* study viral, bacterial and fungal pathogenesis, relatively few papers have focused on viral pathogenesis and virus-host interaction in the past 10 years. The bottleneck could be due to the difficulty of creating transgenic worms expressing viral antigens. Currently, two methods for delivering ectopic genes into *C. elegans* are microinjection and gene bombardment, neither of which are easily achievable in general biology laboratories. To use *C. elegans* as a platform for studying virus-host interaction, virologists must collaborate with worm scientists. Alternatively, virologists could engineer the three viruses (Orsay, Santeui, and Le Blanc virus[35,36]), that naturally infect *C. elegans* to become versatile vectors for the easy deliver of different viral genes into worms through infection.

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COMMENTS

Background

The purpose of this research was to develop a model for human hepatitis B viral antigen interaction with host factors in the standard model animal *Caenorhabditis elegans* (*C. elegans*).

Research frontiers

The hepatitis B viral surface proteins (large, small and middle sized) (LHBsAg, MHBsAg, SHBsAg) are essential in viral assembly and infection. Here the three viral surface proteins were synthesized in *C. elegans* after microinjection of bacterial plasmids containing the genes for the proteins expressed under the control of a ubiquitous animal promoter. Severe reduction in egg laying and brood size as well as gonad retardation occurred with expression of the small hepatitis B virus (HBV) surface antigen in the worm. Smaller effects were found with the middle and larger sized surface antigens.

Innovations and breakthroughs

The specific effects of human HBV surface antigen expression of the *C. elegans* worm demonstrates the worm as a useful model for understanding viral infection and its effects on animal tissues.

Applications

Simple and rapid animal cell and model animal systems are essential for understanding of the infection process and disruptions caused by important human disease agents such as HBV. Here a new and useful model is established and its characteristics presented.

Terminology

HBV is the familiar abbreviation for human hepatitis B virus, although with HAV and HCV, a major cause of liver damage and morbidity. LHBsAg, MHBsAg and SHBsAg are the abbreviations for the large, middle-sized and small versions of the viral surface antigen, the proteins involved in the initial phase of virus surface attachment and infection.

Peer-review

The paper is well written.

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