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**Dengue viral antigen and RNA localization in naturally infected human tissues
by immunohistochemistry and in situ hybridization**

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Dengue viral antigens have been demonstrated in several types of naturally infected human tissues, but little is known of whether these tissues have detectable viral RNA. We studied 13 autopsied specimens of dengue shock syndrome (DSS) cases from children at Yangon Children's Hospital, Yangon, Myanmar by immunohistochemistry (IHC) and in situ hybridization (ISH), to identify the location of dengue viral antigen and RNA, respectively. IHC was performed by using monoclonal antibody against NS1 antigen and envelop protein antigen of dengue virus on tissue specimens. For ISH, anti-sense riboprobes to the dengue E gene were applied to tissue specimens of autopsy cases in which RT-PCR was positive. Viral antigens were demonstrated in Kupffer cells and hepatocytes of the liver; macrophages, multinucleated cells, and reactive lymphoid cells in the spleen, macrophages in the lung, kidney tubules, and mononuclear cells of bone marrow. Positive-strand viral RNA was detected in the spleen. The strong, positive ISH signal in these cells indicated a high copy number of viral RNA, suggesting replication.

INTRODUCTION

Dengue infection is the most prevalent arthropod-borne viral disease in sub-tropical and tropical regions of the world [1]. The dengue virus (genus *flavivirus*, family *Flaviviridae*) consists of a single positive-strand RNA surrounded by an icosahedral nucleocapsid. There are 4 distinct serotypes of dengue virus [2]. Infection with 1 serotype provides lifelong protective immunity to that serotype, but there is no cross-protective immunity between serotypes [3]. Infection with dengue virus causes either a relatively mild disease, known as classic dengue fever (DF), or a more severe form, dengue hemorrhagic fever (DHF), a fulminating illness that is characterized by hemorrhagic manifestations and plasma

leakage and that can progress to dengue shock syndrome (DSS) and death [4].

Studies of specimens obtained from patients with DHF/DSS using immunofluorescence and immunohistochemistry (IHC) revealed the presence of viral antigens in many tissues, including liver, spleen, lymph node, thymus, kidney, lung, and skin, but mainly in mononuclear phagocytic cells [5-9]. The mere presence of viral antigens within cells, especially phagocytic cells, does not necessarily mean that the cells in question support viral replication, since antigens may represent phagocytized, killed virus or sequestered immune complexes in the process of being degraded. Evidence from *in vitro* studies suggested that other cells (e.g., hepatocytes, B and T lymphocytes, endothelial cells, and fibroblasts) could be

potential targets for virus infection and replication, but relatively little is known about the involvement of these cells in *in vivo* infections [10-21].

The *in situ* hybridization (ISH) technique allows for the precise localization of dengue viral RNA in infected tissues. If the positive staining is intense, it could suggest that viral replication may have occurred in these tissues. Indeed, this technique has shown the presence of viral RNA in the thymus and peripheral lymphocytes, suggesting that these cells support *in vivo* viral replication [16]. However, these findings were based on only a few cases and a limited range of tissue types and, thus, need to be further investigated and confirmed. The purpose of the present study is to investigate the cellular localization of dengue virus in naturally infected human tissues by applying both the IHC technique and the ISH technique to tissue specimens obtained from children died with clinically confirmed dengue infection.

MATERIALS AND METHODS

Control materials

Positive control tissue specimens

One-day-old suckling mice were injected intracerebrally with dengue 1, 2, 3 and 4. Sick mice were sacrificed, and the brains were harvested and fixed in 10% buffered formalin for at least 48 h before routine processing and paraffin-embedding. Infected cells were harvested and resuspended in PBS and, to improve cell adherence, were spotted onto slides coated with 3-aminopropyltriethoxy-silane (Sigma). The slides were air-dried, wrapped in aluminum foil, and stored at -80°C. Before use, the cells were fixed in either 4% paraformaldehyde or acetone for 20 min at 4°C.

Negative control tissue specimens

Negative controls comprised uninfected suckling mouse brain. These tissue specimens were fixed in the same manner as were the positive controls.

Test materials

All the cases in the present study were archived in the Immunology Research Division, Department of Medical Research (Lower Myanmar). Tissue specimens were obtained from 13 autopsies from patients clinically diagnosed mainly as having DHF or DSS at Yangon Children Hospital. All autopsies were done after obtaining consent from the next of kin. Autopsy tissue specimens were fixed in 10% neutral buffered formalin for 1 week before routine processing and embedding. Four-micrometer-thick sections from tissue blocks were placed on silanized slides and dewaxed for 30-60 min at 60°C, followed by serial xylene and alcohol washes before IHC and ISH.

Immunohistochemistry (IHC)

Antidengue monoclonal antibodies for 4 serotypes were obtained from Medical Molecular Biology Unit, Siriraj Hospital. These antibodies were tested for specificity and possible cross-reactivity between dengue strains and other unrelated viruses, and IHC conditions were optimized before use. The entire IHC procedure was performed at room temperature, except where stated otherwise. Deparaffinized tissue sections were washed in running water for 10 min and digested with 100 mg/ml proteinase K (Promega) in a buffer (0.01 mol/L Tris-HCl (pH 7.8), 0.005 mol/L EDTA, and 0.5% SDS) for 15 min. After a second wash with water, the sequential incubations were:

- (1) 3% H₂O₂ in absolute methanol for 20 min;
- (2) normal rabbit serum (Gibco BRL) diluted 1:20 in Trisbuffered saline (TBS), for 20 min;
- (3) primary antibody (diluted 1:500 in TBS), overnight at 4°C;
- (4) biotinylated rabbit anti-mouse antibody (diluted 1:300; Dako);
- (5) avidin biotin-horseradish peroxidase complex (Dako) and 3,3-diaminobenzidine tetrahydrochloride (Sigma), according to the manufacturers' protocols.

Between steps, the slides were washed in TBS for 5 min. Finally, the slides were washed in running water, counterstained in Harris hematoxylin, dehydrated, and mounted with DPX (Sigma).

Riboprobe preparation

Dengue viral RNA was extracted from dengue 3 and 4 infected C6/36 cells by use of TRIzol reagent (Gibco BRL), and respective cDNAs were produced by use of published specific primers to the E gene and the Access RT-PCR Kit (Promega) [23]. RT-PCR was performed in a thermocycler (DNA engine PTC-200 Peltier; MJ Research), and the conditions were the following: (1) 45 min at 48°C then 1 min at 94°C; (2) 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C, for 40 cycles; and (3) 5 min at 72°C. The 260-bp PCR fragments were purified by use of the High Pure PCR Purification Kit (Boehringer Mannheim), cloned into a suitable vector (pGEMT-Easy; Promega), and transformed into competent *Escherichia coli* cells (JM109; Promega). White and pale blue colonies were identified on X-Gal minimal agar plates and screened for the presence and orientation of the inserts by *Eco*RI restriction endonuclease digestion and by use of PCR using T7 and dengue-specific primers. Plasmids with inserts in 2 orientations were isolated by use of a modified alkaline lysis method [24]. After linearization with *Spe*I restriction endonuclease (Promega), antisense and sense riboprobes were generated by use of the DIG RNA Labeling Kit SP6/T7 (Boehringer Mannheim). Digoxigenin (DIG)-labeled riboprobes were purified as above and stored at -80°C until use.

In situ hybridization (ISH)

We used the ISH method described by Killen et al. [16]. The sensitivity and specificity of the riboprobes were tested and optimized by varying pretreatment with proteinase K, before ISH was performed on all RT-PCR positive tissue specimens. In brief, deparaffinized tissue sections were pretreated with HCl and proteinase K

(100 mg/ml, 15 min, 37°C). The hybridization and posthybridization steps were performed as described elsewhere [16]. Approximately 0.5-1.0 ng of either antisense or sense riboprobes (used as a serotype-specific probes or as single probes) was pipetted onto tissue sections, covered with diethylpyrocarbonate-treated coverslips, and incubated for 16-20 hr at 45°C. Probes that had hybridized with viral RNA were detected by use of an anti-DIG antibody conjugated to alkaline phosphatase and nitroblue tetrazolium/ 5-bromo-4-chloro-indol-3-indolyl phosphate color substrate (Boehringer Mannheim). The slides were counterstained with nuclear red stain and aqueous-mounted with glycergel (Dako).

RESULTS

Antibody specificity and sensitivity

Dengue antibody showed the strongest staining on positive controls and thus was used for all subsequent IHC assays. All the other negative controls were also found to be negative.

IHC findings

Dengue viral antigens were detected in the liver, spleen, lung, kidney, and bone marrow (Table 1). Other tissue specimens, including thymus, lymph node, thyroid, trachea, pancreas, diaphragm, heart, adrenal gland, skin, skeletal muscle, intestine, gall bladder, uterus, ovary, testis, prostate, and brain, were found to be negative. Megakaryocytes and mononuclear cells in the bone marrow were found to be positive for viral antigens.

Table 1. Immunohistochemistry (IHC) results in paraffin-embedded tissue sections

Tissues/ organs	NSI positive (%)	E protein positive (%)
Liver	10/13(77)	7/13(54)
Spleen	4/13(31)	5/13(38)
Lung	5/13(38)	0/13(0)
Kidney	1/12(8)	0/12(0)
Bone marrow	10/12(83)	2/12(17)

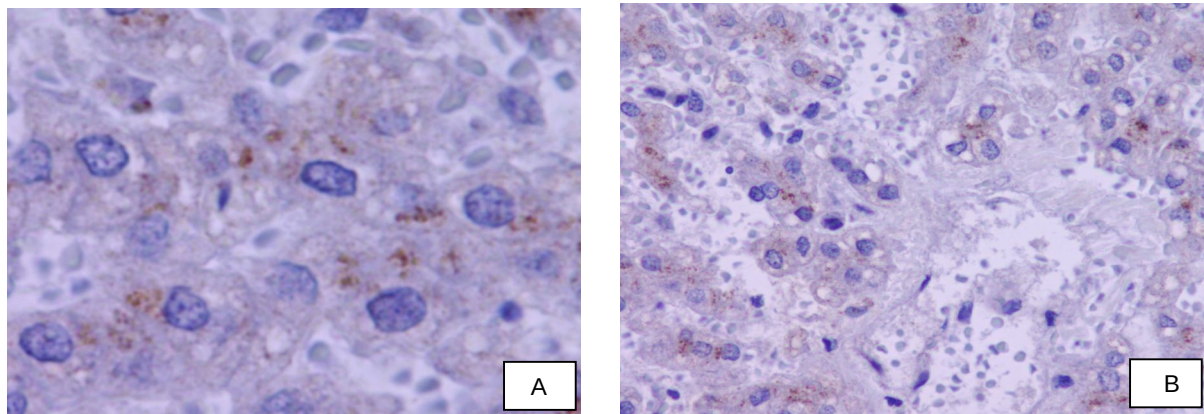


Fig. 1. Normal area (A) and, necrotic area (Dengue viral antigens (NS1) detected in the liver in Kupffer and hepatocytes) (B)

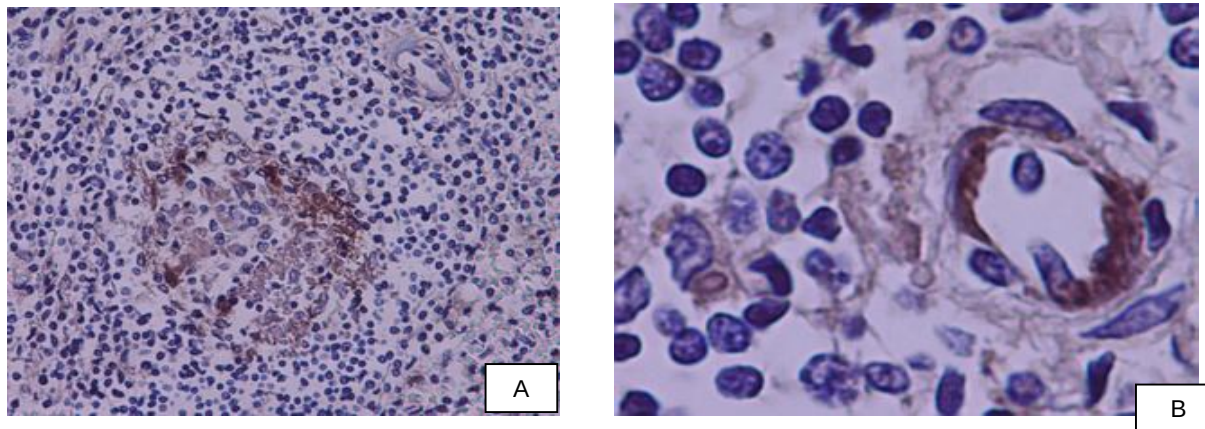


Fig. 2. Dengue viral antigens (envelop protein) detected in the spleen in white pulp (A) and endothelial lining (B)

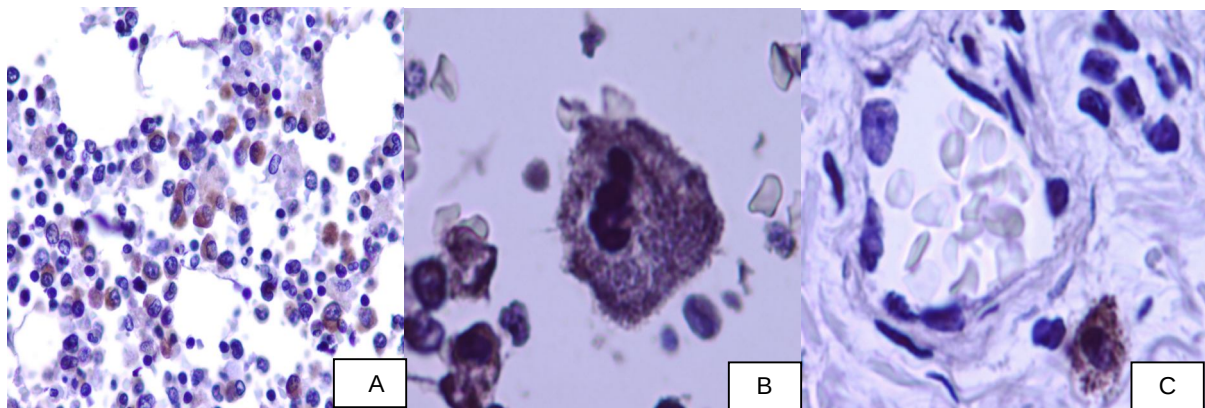


Fig. 3. Dengue viral antigens (NS1) detected in the bone marrow in mononuclear cells (A), envelop protein positive in megakaryocyte (B), NS1 positive in lung in pulmonary macrophage and negative in endothelial cells (C)

In general, antigens were immunolocalized to the cytoplasm, with no nuclear staining at all. In the liver, fine granular deposits of dengue viral antigens were localized

predominantly within Kupffer cells and hepatocytes (Fig. 1). In the spleen, viral antigens (NS1) were clearly observed in the cytoplasm of macrophages in the red

pulp, viral antigens (E protein) were present in the germinal centers of lymphoid follicles and endothelial cells in the white pulp and endothelial lining (Fig. 2 A & B). In the kidney, viral antigens were detected as discrete granular deposits within the lining cells of the tubules. In the bone marrow, viral antigens (NS1) were detected in mononuclear cells (Fig. 3 A), and envelop protein positive in megakaryocyte (Fig. 3 B). Viral antigens (NS1) positive in pulmonary macrophage and negative in endothelial cells of the lung were detected (Fig. 3 C).

Riboprobe specificity and sensitivity

DIG-labeled antisense riboprobes used in optimized ISH assays could detect dengue viral RNA in positive control tissue specimens of suckling mouse brain, giving clear signals with little background staining. Respective riboprobes were also serotype specific. Uninfected tissue showed negative staining.

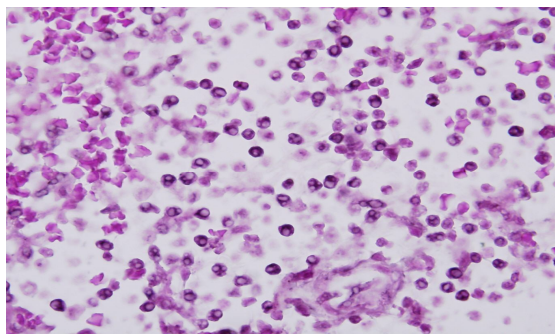


Fig . 4. ISH positive in mononuclear cells and multinucleated cells in spleen

ISH findings

In the splenic red pulp, viral RNA was localized to the cytoplasm of macrophages, reactive lymphoid, binucleated cells and multinucleated giant cells (Fig. 4). Liver Kupffer cells, hepatocytes and endothelium in these organs were found to be negative for viral RNA.

DISCUSSION

Dengue viral antigens have been detected in various human tissues in previous studies, but it is not certain whether viral RNA can

be localized to these same tissues, since little work has been done to establish this [5-9]. In fact, there are only 2 other published studies that have attempted to detect dengue viral RNA in a small number of naturally infected human tissues [16, 25]. The demonstration of positive-strand viral RNA by ISH, in reactive B lymphoid cells in the germinal centers of the spleen including centroblasts and immunoblasts and peripheral lymphocytes has been reported previously [20], but not in bone marrow. The mere presence of viral RNA does not necessarily imply that active intracellular viral replication has occurred. Nonetheless, a strong signal, indicating a high copy number, is probably good supportive evidence for infection and viral replication, particularly in cells that are not known to be phagocytic. This finding is supported by *in vitro* studies, which have demonstrated dengue virus replication in human B lymphoblastoid cells [11].

In our study, we have demonstrated for cellular localization of dengue viral RNA in splenic macrophages. Again, the strong staining obtained suggested that viral replication may have occurred in these cells. Dengue RNA has been demonstrated before in macrophages in the lymph node and skin by use of an *in situ* PCR method [25]. In *in vitro* experiments, human peripheral blood monocytes were found to support viral replication [14, 26-28]. In contrast, the failure to detect viral RNA in antigen-positive Kupffer cells and alveolar macrophages suggests viral phagocytosis or immune complex sequestration rather than viral replication. Interestingly, *in vitro* dengue virus infection of human Kupffer cells showed that these cells were not involved in productive infection [29]. It is possible that, *in vivo*, Kupffer cells and alveolar macrophages do not support viral replication but may play a protective role against dengue virus by their phagocytic function.

In the present study, the detection of viral antigens (with or without viral RNA) in

cells of the mononuclear phagocytic system confirmed previous findings [5-9]. This supports the theory that monocytes and macrophages play a significant role in the pathogenesis of DHF/DSS, in view of their possible role in the infection-enhancement phenomenon, especially in secondary infection [30, 31].

It has been postulated that infected monocytes/macrophages and activated T lymphocytes release mediators, which act directly on the vascular endothelium to increase vascular permeability, resulting in plasma leakage, shock, and death [32-36]. Direct vascular endothelium infection may also contribute to DSS. Several in vitro studies have shown that viral replication occurs in endothelial cell cultures [15-20]. However, the present study has found no evidence of viral RNA in the endothelium of the organs studied. Viral antigens have also been observed in vascular endothelium in the liver and, apparently, also in the brain [6, 7]. However, viral RNA or endothelial damage apparently has not been observed in natural human infections [32, 37]. The process of pinocytosis could explain the detection of viral antigens in vascular endothelium, since an ultrastructurally increased number of vacuoles and pinocytotic vesicles was observed in the endothelium of skin biopsy specimens from patients with DHF [37].

The absence of viral RNA in kidney tubular cells that immunolocalize for viral antigen suggests that viral replication did not occur in these cells. These antigens could represent reabsorbed immune complexes after elimination through the kidney, analogous to that in yellow fever, in which tubular cells also demonstrated the presence of yellow fever viral antigen [38]. In separate studies, viral antigens [5] and viral RNA have been demonstrated in hepatocytes by an in situ PCR method [25]. No viral antigens or RNA were detected in hepatocytes from our cases. The reasons for this may be that viral antigens seem to appear early in hepatocytes after infection,

and production of infectious particles is rather low [39]. It is interesting that there seems to be no evidence for the involvement of megakaryocytes found in the bone marrow. This suggests that thrombocytopenia, a characteristic of DHF/DSS, may not be associated with failure of platelet production. There appears to be no relationship between detection of dengue virus and the severity of clinical disease. Nonetheless, more cases have to be studied to confirm this.

In conclusion, macrophages and reactive splenic lymphoid cells may be the major target cells of dengue viral replication in natural human infections. These findings have to be confirmed by examining tissue specimens from more cases of human infection. Electron microscopy and in situ PCR could be useful tools to confirm viral replication.

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Bacteriological study of squids marketed in two townships of Yangon, Myanmar

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Seafood can be contaminated with pathogens of marine aquatic environment and from primary producer to consumers by means of untreated waste water and improper handling. The consumption of contaminated raw or partially cooked seafood including squid has a direct relationship with occurrence of intestinal and extra intestinal infections. A total of 30 squid samples were studied from January 2009 to September 2009 to isolate and identify *Vibrio* species and to determine the presence of contaminating pathogenic bacteria in squids. *Vibrio* species were isolated from 14 samples. *Vibrio* species isolated were *Vibrio alginolyticus* (n=7), *Vibrio vulnificus* (n=4), *Vibrio parahaemolyticus* (n=1), *Vibrio fluvialis* (n=1), and *Vibrio mimicus* (n=1). Coliforms were isolated from 9 samples while 7 samples showed the presence of *Salmonella* species and *Shigella* species. *Proteus vulgaris*, *Staphylococcus aureus* and *Morganella morganii* were also detected in 10, 8 and 1 squid samples, respectively. The result highlighted the potential risk of infections by raw seafood consumption. This knowledge on the occurrence of vibrios and other human pathogens in squid helps in effective management and in preparation of appropriate response.

INTRODUCTION

Squids are part of various seafood dishes, breaded, deep fried as calamari, boiled, stewed, grilled, slightly boiled and make a salad. Squid is a sushi and sashimi item. Like any food item, it has the potential to cause diseases from viral, bacterial, and parasitic pathogens under certain circumstances because seafood often harbour infectious agents that are present naturally in the aquatic environment or introduced through human activities. Bacterial gastrointestinal infections continue to cause illness and death and contribute to economic loss in most part of the world. Most of the outbreaks of illnesses occur in countries where seafood is eaten raw or inadequately cooked. For example, in Japan where seafood is eaten raw, 70% of food-borne human illness is seafood associated [1].

Vibrios are the frequent isolates from seafood and are an important cause of

human food-borne illness associated with the consumption of seafood worldwide. Twelve species of the genus *Vibrio* are human pathogens [2]. The most commonly isolated *Vibrio* species from seafood are *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio mimicus* and *Vibrio fluvialis* [3].

The most commonly isolated *Vibrio* species from mussel are *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio mimicus* and *Vibrio fluvialis* [2]. *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio horveyi*, and *Vibrio vulnificus* were isolated from water, sediment and shrimp samples from shrimp farms in India [3].

Outbreaks of cholera have been associated with consumption of seafood including oysters, crabs and shrimp. *Vibrio parahaemolyticus* is a common pathogen in Asia causing 197 outbreaks and 8967 cases

in Taiwan during 1986-1995 as well as numerous outbreaks in Japan and some Southeast Asian countries. In 1990 and 1992, there were two outbreaks of gastroenteritis in Australia, caused by *V. parahaemolyticus* in chilled, cooked shrimps imported from Indonesia and there was also a death in 1992 associated with the consumption of oysters [4].

Vibrio fluvialis was isolated from more than 500 patients with diarrhoea in Bangladesh. *Vibrio hollisae* and *Vibrio mimicus* have been associated with diarrhoea in individuals who have eaten raw seafood. *Vibrio alginolyticus*, which can cause ear infections, conjunctivitis, intracranial infections and wound infections following burn and *Vibrio metschnikovii*, first isolated in 1888 from environmental sources, have also been implicated in food-borne gastro-intestinal infections. *V. vulnificus* is responsible for wound infection and septicemia following ingestion of raw seafood or contact with sea water [5].

The enteric bacteria such as *Escherichia coli*, *Salmonella* species, *Shigella* species, *Campylobacter* species, and *Yersinia enterocolitica* are present in seafood due to faecal contamination and the bacteria such as *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium perfringens* may contaminate during processing [6].

Non-typhoidal salmonellae are the most important pathogens of acute diarrhoea in children and adult in developing countries. Every year millions of infections are reported throughout the world. *Shigella* species can cause bacillary dysentery. Humans are the primary reservoir of *Shigella* species. The infection is transmitted by the excreta of infected individuals via fecal oral route [7].

Proteus spp are opportunistic etiological agents in infections of the respiratory tract and of wounds, burns, skin, eyes, ears, nose, and throat, as well as in gastroenteritis resulting from the consumption of contaminated food. Most of the Proteae are

widely distributed in nature, being frequently found in the faeces of animals and humans as well as in associated materials such as decomposing meat and sewage. *Proteus* is part of predominant microflora in fresh seafood including finfish and shellfish. The fecal contamination of seafood by microorganisms like *P. mirabilis* is one of many major problems of coastal environment. *Proteus* species and *Morganella morganii*, are believed to cause histamine formation in fish with scombroid toxicity [8].

The enterotoxigenic strain of *Staphylococcus aureus* and *Escherichia coli* were isolated from fish and seafood samples in the southern region of Brazil [9]. In a Myanmar study, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp and *Clostridium perfringens* were also isolated from 86%, 86%, 6% and 5% of raw seafood, respectively [10].

Nowadays, consumption of seafood is increasing with changing dietary habits and more people eating raw fish. Outbreak of diseases associated with consumption of seafood is a major concern to the seafood industry and public health agencies. Although public education regarding the risk of raw seafood consumption is essential to prevent infection from these virulent pathogens, detection of contamination of food with enteric pathogens is important for the perspective of public health.

Aim

- To study potentially pathogenic bacteria in squids marketed in Mingaladon and Thingangyun townships in Yangon.

Objectives

- To isolate and identify *Vibrio* species obtained in squids from Mingaladon and Thingangyun townships in Yangon.
- To isolate and identify contaminating pathogenic bacteria in squid from Mingaladon and Thingangyun townships in Yangon.

MATERIALS AND METHODS

Sample collection

A total of 30 squid samples (15 from one Mingaladon market and 15 from one Thingangyun market) were studied from January to September 2009. The samples were processed at Microbiology Laboratory, Department of Microbiology, University of Medicine (2).

Isolation and identification of *Vibrio* species

A 25 gram of squid were blended with 225 ml of alkaline peptone water with 3% NaCl and incubated at 35°C for 6 hours. After enrichment, a loopful of alkaline peptone water culture was streaked onto Thiosulphate citrate bile sucrose (TCBS) agar and incubated at 35°C for 18 hours. Morphology of colony on TCBS agar were recorded and suspected colonies were cultured onto Trypticase soy agar (TSA) and further identified by conventional biochemical tests including gram staining, oxidase test, motility, sugar fermentation, citrate utilization, urease production, indole, methyl red test, Voges Proskauer test and salt tolerance test [11].

Isolation and identification of coliforms and other pathogenic organisms

Squid samples after enrichment in peptone water overnight were subcultured onto MacConkey agar, Blood agar, Mannitol salt agar, Salmonella Shigella agar and identified by biochemical tests. Detailed identification was done according to Cowan and Steel [12]. For identification of Gram-positive organisms, catalase test, slide coagulase test and tube coagulase test were done.

RESULTS

Presence of *Vibrios* in squid samples

Vibrio species were isolated from 14 samples (7 from Mingaladon Township and 7 from Thingangyun Township). Out of 30 sample, *Vibrio alginolyticus*, *Vibrio*

vulnificus, *Vibrio parahaemolyticus*, *Vibrio fluvialis* and *Vibrio mimicus* were isolated from 7, 4, 1, 1 and 1 squid samples, respectively.

Of 15 samples from Mingaladon Township, 4 squid samples were positive for *Vibrio alginolyticus*, 2 for *Vibrio vulnificus*, and 1 for *Vibrio parahaemolyticus*. Three *Vibrio alginolyticus*, 2 *Vibrio vulnificus*, 1 *Vibrio fluvialis* and 1 *Vibrio mimicus* were the predominant *Vibrio* species isolated from Thingangyun Township (Table 1).

Table 1. Presence of *Vibrio* spp in squid samples from Mingaladon and Thingangyun townships

<i>Vibrio</i> spp	Townships n = 15 (%)		Total n=30 (%)
	Mingaladon	Thingangyun	
<i>Vibrio alginolyticus</i>	4 (26.7)	3 (20)	7 (23.3)
<i>Vibrio vulnificus</i>	2 (13.3)	2 (13.3)	4 (13.3)
<i>Vibrio parahaemolyticus</i>	1 (6.7)	-	1 (3.3)
<i>Vibrio fluvialis</i>	-	1 (6.7)	1 (3.3)
<i>Vibrio mimicus</i>	-	1 (6.7)	1 (3.3)
Total	7 (46.7)	7 (46.7)	14 (46.7)

Presence of coliforms in squid samples

Coliforms were isolated from 9 specimens (8 *Escherichia coli*, 1 *Klebsiella* spp)

Presence of other pathogenic bacteria in squid samples

Proteus spp, *Salmonella* spp, *Shigella* spp, and *Morganella morganii* were obtained from 10, 4, 3 and 1 squid samples, respectively. *Staphylococcus aureus* were also isolated from 8 squid samples.

Frequency of other bacteria in squid samples from two townships in Yangon

Escherichia coli, *Proteus vulgaris*, *Salmonella* species and *Morganella morganii* were detected in 4, 4, 2 and 1 squid specimens, respectively, from Mingaladon Township. From Thingangyun Township, *Proteus vulgaris*, *Escherichia*

Table 2. Frequency of isolation of other bacteria in squid samples from Mingaladon and Thingangyun townships

	Mingaladon No. (%)	Thingangyun No. (%)	Total No. (%)
Gram-positive bacteria (%)			
<i>Staph. aureus</i>	4 (26.7)	4 (20)	8 (22.8)
Gram-negative bacteria (%)			
<i>E. coli</i>	4 (26.7)	4 (20)	8 (22.8)
<i>Klebsiella</i> species	-	1 (5)	1 (2.8)
<i>Proteus vulgaris</i>	4 (26.7)	6 (30)	10 (28.5)
<i>Morganella morganii</i>	1 (6.7)	-	1 (2.8)
<i>Salmonella</i> species	2 (13.3)	2 (10)	4 (11.4)
<i>Shigella</i> species	-	3 (15)	3 (8.6)

coli, *Shigella* spp, *Salmonella* spp and *Klebsiella* spp were isolated from 6, 4, 3, 2 and 1 squid specimens, respectively. Four *Staphylococcus aureus* each were isolated from both townships (Table 2).

DISCUSSION

Seafood can be contaminated with pathogens of marine aquatic environment and from primary producer to consumers by means of untreated waste water and improper handling. Pathogens in seafood are acquired from three sources: (1) mainly fecal pollution of the aquatic environment, (2) to a lesser extent, the natural aquatic environment, and (3) industry, retail, restaurant, or home processing and preparation [6]. Various members of the Family *Vibrionaceae*, *Enterobacteriaceae* and *Staphylococcus* species constitute a potential health threat for consumers of raw seafood including squid.

In this study, 46.7% of squid specimens contained *Vibrio* spp. *Vibrio* species isolated were *Vibrio alginolyticus* (23.3%), *Vibrio parahaemolyticus* (3.3%), *Vibrio fluvialis* (3.3%), *Vibrio mimicus* (3.3%) and *Vibrio vulnificus* (13.3%). *Vibrio alginolyticus* was the most common organism isolated and followed by *Vibrio vulnificus*.

In a study done in Hongkong, *Vibrio alginolyticus* was the most frequently isolated species from seafood followed by *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Vibrio horveyi*, *Vibrio vulnificus*, and *Vibrio mimicus* [13].

The most commonly isolated species from mussel in Brazil were *Vibrio alginolyticus* (92-100%), *Vibrio parahaemolyticus* (67-92%), *Vibrio fluvialis* (34-67%), *Vibrio vulnificus* (8-17%), *Vibrio mimicus* (0-17%), *Vibrio furnissii* (0-17%), and *Vibrio cholerae* (0-8%) [2]. *Vibrio cholerae* was not isolated in this study. *Vibrio cholerae* non-01 and *Vibrio fluvialis* were isolated as 74% and 26% of fish samples from Pazundaung Market in Yangon, Myanmar [14]. From these studies, it can be assumed that *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio fluvialis* and *Vibrio vulnificus* are common in seafood although a variety of *Vibrio* species were isolated from different parts of the world and from different food and seafood.

Vibrio cholerae, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Vibrio fluvialis*, *Vibrio hollisae*, *Vibrio alginolyticus*, *Vibrio vulnificus*, *Vibrio furnissii*, and *Vibrio metschnikovii*, have been associated with gastroenteritis: patients present with diarrhoea and vomiting. *Vibrio vulnificus*, when ingested in raw sea food, can cross the intestinal mucosa, enter the blood stream, and cause primary septicaemia in patients with reduced stomach acidity, in people with diabetes mellitus, alcoholism, heart disease, cancer, chronic kidney disease and an impaired immune system. Most *Vibrio* species were isolated during summer in this study. This may be due to the dependence of these organisms on water temperature and salinity.

In this study, 30% of squid samples (*Escherichia* spp 8 samples, *Klebsiella* spp 1 sample) showed coliforms. The coliforms include *Escherichia* spp, *Klebsiella*, *Enterobacter* and *Citrobacter*, serve as a measure of fecal contamination, and thus

potentially, of the presence of enteric pathogens in fresh water.

Shigella was isolated from 10% (n=3) and *Salmonella* 13.3% (n=4) of squid specimens in this study. It is generally believed that organisms like *E. coli*, *Shigella* and *Salmonella* are not present in the natural aquatic environment but are derived as a result of sewage contamination. The presence of coliforms in food is highly undesirable, but it would be almost impossible to eliminate all forms.

Staphylococcus aureus is another serious cause of gastroenteritis after consumption of fish and related products. In the present study, eight *Staphylococcus aureus* (26.7%) were isolated. Consumption of mishandled seafood can result in food-borne illnesses.

Proteus spp and *Morganella morganii* were also found in 33.3% and 3.3% of squid specimens, respectively, in this study. *M. morganii* and *P. vulgaris* produce high levels of histamine, indicating the possibility of histamine formation in various fish species and seafood products. Their proliferation should be tightly monitored to prevent histamine formation during handling, processing, and distribution of seafood products [15].

These results indicate that the various seafood can be contaminated with potentially pathogenic *Vibrio* species and other pathogens. Sewage contamination of fish harvesting areas is the major reason for the presence of *E. coli*, but contamination can occur through the use of non-potable water or ice in the landing centers or fish markets. Therefore, all stages of fish production, handling and processing should be monitored for *E. coli* contamination.

The diversity of harvest methods, production areas, markets, and seafood species involved make seafood safety complex. It is imperative to have knowledge on current situation and trends with regard to occurrence and spread of human pathogens in the food production chain in order to address and manage food safety.

This knowledge on the occurrence of vibrios and other human pathogens in seafood helps in effective management and in preparation of appropriate response. This knowledge needs to be updated continuously so that appropriate responses can be prepared.

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Sociocultural and behavioural determinants of malaria and its appropriate control measures in forested areas of Central Myanmar

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A case-control study was conducted using pretested interview questionnaires in Gwegyo and Lewun villages of Pyinmana District, Nay Pyi Taw from January to October, 2009 with the main objective of determining the socio-cultural and behavioural factors of malaria among local residents in forested areas of Central Myanmar and assess the feasibility of its control. The study was preceded by epidemiological and entomological surveys and followed by indepth interviews among local key informants. The study depicted that study areas were found to be malaria meso-endemic with abundance of vectors including the main vector, *An. dirus*, in their vicinity. The determinants of malaria were male gender (OR=2.6, $p=0.004$), water fetching habit in the evening ($p<0.001$), traveling habit (OR=2.6, $p=0.006$), traveling without bednet (OR=9.6, $p<0.001$), habit of going out at dawn (OR=2.3, $p=0.01$) and low level of correct practice on malaria prevention ($p=0.007$). Most (80.52%) of participants normally used bednets but only 50% brought bednets when traveling and all used blankets. 81.17% said that they would accept if their bednets were treated with insecticide. Indepth interviews expressed some local cultural misbelieves. In conclusion, the study highlighted that it is necessary to launch an effective health education programme to remove some risk factors among the victims and it would be appropriate to provide them insecticide-treated bednets, blankets and/or mufflers to reduce the incidence of deadly disease malaria in the forested areas.

INTRODUCTION

Malaria is the most prevalent disease causing morbidity (c600,000 cases) and mortality (c3,000 deaths) each year in Myanmar [1] with subsequent outcome of increased economic burden and reduced productivity. On the disease aetiology human behaviour which is influenced by social, cultural, economic and political factors is clearly related to health. Due to AIDS epidemic medical anthropology has come of age and likewise socio-cultural and behavioural factors are considered for widespread and effective control against malaria [2]. Therefore, these factors must be sought and removed or modified by applying appropriate intervention measures

among specified populations to curb malaria. In social research considerable attempt is devoted to relate the risk of disease and socio-cultural and behavioural factors. Study concerning such factors in relation to malaria occurrence is hardly conducted in Myanmar. Therefore, the present study was carried out with the objectives of:

- (i) to determine the microscopically confirmed malaria prevalence and vector bionomics in the study area,
- (ii) to find out the socio-cultural and behavioural determinants of malaria occurrence, and
- (iii) to assess the feasibility of malaria control in terms of bednet and blanket use in the areas.

MATERIALS AND METHODS

Study design

Cross-sectional survey for finding out malaria prevalence and a case-control study for malaria determinants

Study area

The study areas are Gwegyo and Lewun villages (with 820 and 1,106 local residents, respectively), Pyinmana District, Nay Pyi Taw, Central Myanmar (c19° 90' N, 95° 85' E). The areas are characterized by extensive forests and mountains of northern Bago Mountain Range and 30-36 miles from Nay Pyi Taw. Creeks pass through the villages and locals live on semi-subsistence agriculture and forested related works like timber extraction and charcoal production. There is no permanent health staff. Malaria is perennial with a peak season at post monsoon period.

Study period

From January to October, 2009

Study population

Study population are locals aged 18 years and above for a case-control study and 2-9 years old children for spleen survey. All local Anopheline mosquitoes are studied.

Data collection instruments and technique

Epidemiological survey: In Pyinmana District, Nay Pyi Taw, its north western part which is under the health cover of Taungnyo Station Hospital is one of the malaria-endemic areas being composed of 31 villages. Out of these villages Gwegyo, Lewun and Moeswe villages were purposively selected for spleen survey. Finally two villages with same endemicity Gwegyo and Lewun villages (spleen rates, 15.94% and 13.2%, respectively) were chosen as study areas. To determine period prevalence of malaria in 10 months of study, available numbers of patients obtained during visits to the villages (numerator) were counted being based on total population (denominator) of the villages.

Entomological survey: Larval surveys were done with the help of well-trained research assistants (RAs) from Department of Medical Research (Central Myanmar) [DMR (CM)] by searching the larvae in the habitats ie sand pools in the Palwe Creek in Gwegyo Village. Adult surveys were performed by animal (cow) bait and human bait (under the cover of prophylactic antimalarials). Collected mosquitoes were identified by Assistant Entomologist of Vector-borne Disease Control (VBDC) Programme, Department of Health. During the period, ambient temperature (10-35°C) and relative humidity (5-68%) were measured.

Case-control study: To find out determinants of malaria, firstly locals as participants were collected, at the mobile clinic of research team with microscopy facility, when they saw health staff in the team to seek treatment for malaria and other ailments. The cases were microscopically confirmed malaria cases and the controls were those without malaria parasite and with no history of malaria or fever with chill and rigor within last year. Cases and controls were matched by age group. Total numbers of participants were 77 in each group.

Secondly, all participants were interviewed using pretested interview questionnaires by well-trained RAs of DMR (CM). In the separate questionnaires to determine the level of knowledge, attitude and practice about malaria there are ten questions in each component. If a participant responded one question correctly he would gain one mark. For knowledge there are three levels of score - low (0-5 marks), medium (6-8 marks) and high (9-10 marks). Likewise, for attitude and practice the levels are - low (0-6 marks), medium (7-8 marks) and high (9-10 marks). Practice questions included (i) use of bednet while sleeping in daytime, (ii) use of bednet at night, (iii) bringing bednet while traveling, (iv) seeing a quack while falling ill, (v) seeing a traditional healer while falling ill, (vi) praying to spiritual bodies/ going to a fortune teller while

falling ill, (vii) seeing a doctor while falling ill, (viii) going to a health centre while falling ill, (ix) listening to radio/watching TV for malaria and (x) attending health talk on malaria. Then malaria cases were treated with antimalarials according to the WHO guideline.

Indepth interview: Eight local key informants were interviewed by the principal investigator using interview guidelines regarding malaria determinants and its prevention and control. Their opinions and ideas were recorded as their actual wordings and then transcribed and translated.

Consent and data analysis: Ethical approval was obtained from Ethical Committee on Medical Research Involving Human Subjects, DMR (CM). Consent was obtained from all participants. Data analysis was done by SPSS version 13.0 using the chi-squared test. Indepth interview results were also analyzed.

RESULTS

Epidemiological features

The study areas were found to be malaria mesoendemic areas (spleen rate ranged from 13.2% to 15.94%) [3]. Eighty-one adults and 12 children under 15 years were microscopically-confirmed malaria cases out of 182 adults and 21 children attending the mobile clinic during the study period (91 falciparum cases and 2 vivax cases). Malaria period prevalence in the two studied villages was 48 per 1,000 population of all ages.

Entomological features

Larvae collected from sand pools of the creek were identified as *An. Culicifacies* and *An. stephensi*. Adults collected by animal bait were made up of *An. dirus*, *An. stephensi*, *An. culicifacies*, *An. hyrcanus*, *An. Barbirostris*, *An. vagus*, *An. maculatus*, *An. Tesselatus*, and other culicine species. *An. dirus* was main vector of the study areas. There were no Anopheline mosquitoes collected

by human bait and it was probably due to bonfires made by householders nearby at the time of collection (1800-2100 hour).

Case-control study

The study investigated background characteristics, KAP scores, mosquito repelling methods and socio-cultural and behavioural variables of the participants (Table 1 & 2).

Table 1. Background characteristics, KAP scores and mosquito repelling methods among the participants in relation to malaria occurrence

Variable	Category	Participants			OR	95% CI _{OR}	p
		Case n=77	Control n=77	Total n=154			
Age group (Years)	18-19	7	7	14	-	-	-
	20-29	22	22	44			
	30-39	22	22	44			
	40-49	15	15	30			
	50-59	8	8	16			
	60+	3	3	6			
Gender	Being male	50	32	82	3	1.4-5.0	0.004
		4.94%	41.56%	53.25%			
Knowledge score	Low	44	47	91	-	-	*
	Medium	33	26	59			
	High	0	4	4			
Attitude score	Low	13	14	27	-	-	0.5
	Medium	40	33	73			
	High	24	30	54			
Practice score	Low	27	16	43	-	-	0.007
	Medium	37	31	68			
	High	13	30	43			
Use of mosquito coil		35	26	61	2	0.9-3.1	0.14
		45.45%	33.77%	39.61%			
Use of bonfire		20	24	44	1	0.4-1.6	0.48
		25.97%	31.17%	28.57%			

* χ^2 test not applicable

Majority of malaria cases were within age groups of 20-29 and 30-39 years (57.14%). Males were more affected than females and it was statistically significant in malaria occurrence [OR=2.6, 95% Confidence Interval OR (CI_{OR})=1.4-5.0; p=0.004]. Most of the cases (53.25%) had habit of water fetching in the evening and it was also statistically significant (p<0.001). Majority of cases (77.92%) had traveling habit and it was also statistically significant (OR=2.6, 95% CI_{OR}=1.3-5.3; p=0.006). Places where

they usually traveled to were the hill and forest for work.

Table 2. Sociocultural and behavioural characteristics of the participants in relation to malaria occurrence

Variable	Category	Participants			OR	95% CI _{OR}	p
		Case n=77	Control n=77	Total n=154			
Water fetching time	Morning	22	47	69	-	-	<0.001
	Afternoon	5	11	16			
	Evening	41	13	54			
	No fetching	9	6	15			
Water fetching place	In creek	56	55	111	-	-	0.55
	At well	12	16	28			
	No fetching	9	6	15			
Bath time	Morning	7	13	20	-	-	0.07
	Afternoon	15	25	40			
	Evening	38	27	65			
	Night	17	12	29			
Bath place	At home	15	23	38	-	-	*
	In creek	60	50	110			
	At well	2	4	6			
Regular bed place	Outdoor	18	16	34	1.2	0.5-2.5	0.7
	Indoor	59	61	120			
Bed place during summer	Outdoor	31	25	56	1.4	0.7-2.7	0.32
	Indoor	46	52	98			
Possession of bednet	-	62 80.52%	68 88.31%	130 84.42%	0.6	0.2-1.3	0.18
Bednet use	-	60 77.92%	64 83.12%	124 80.52%	0.7	0.3-1.6	0.42
Traveling habit	-	60 77.92%	44 57.14%	104 67.53%	2.6	1.3-5.3	0.006
Traveling without bednet	-	43 55.84%	9 11.69%	52 33.77%	9.6	4.2-21.9	<0.001
Habit of going out at dusk	-	55 71.43%	52 67.53%	107 69.48%	1.2	0.6-2.4	0.6
Habit of going out at dawn	-	36 46.75%	21 27.27%	57 37.01%	2.3	1.2-4.6	0.01

* χ^2 test not applicable

Sixty-two cases (80.52%) possessed bednets and 60 cases (77.92%) used bednets. Fifty percent of travelers (i.e. 52/104) brought bednets while traveling. 55.84% (43/77) of cases traveled without bednets when they stayed at destination overnight and it was

also statistically significant (OR=9.6, 95% CI_{OR}=4.2- 21.9; p<0.001).

Another thing is locals' habit of going out at dawn for work i.e. at about 0530-0600 hour. This habit was found to be statistically significant (OR=2.3, 95% CI_{OR}=1.2-4.6; p=0.01). Level of correct practice on malaria prevention is associated with malaria occurrence and also statistically significant (p=0.007). Other variables were not associated with malaria occurrence (p>0.05).

Forty-nine percent (38/77) of cases were agricultural workers. Family income per month ranged from 10,000 to 400,000 kyats in cases and from 20,000 to 300,000 kyats in controls. Mean±SD family income of all participants was 70,000±60,000 kyats. Forty-three percent (33/77) passed only primary school level. Most (84.42%) of participants (130/154) possessed bednet, 80.52% (124/154) used bednet and 100% used blankets when sleeping. 81.17% (125/154) accepted the insecticide treated bednets (ITNs). Concerning washing of bednet and blanket, the majority washed them at least once a month [i.e. 43.51% (67/154) and 56.49% (87/154), respectively]. Only 5.84% (9/154) had curtains at their houses.

In-depth interview

There were a particular and habit cultural misbeliefs among locals but some knew benefits of bednet utilization including ITN in malaria control and prevention.

"Majority of locals are agricultural workers - farmers. They use buffaloes in ploughing rather than oxen. The former are less tolerant of sun heat than the latter. So to avoid sun heat farmers go to the paddy field early in the morning. Due to such habit farmers are more likely to be bitten by mosquitoes and have more chance of getting malaria if they are unprotected".

(A 31-year-old medical officer of Station Hospital)

"About sixty percent of locals get malaria in the village every year. The reasons why malaria develops I think are mosquito bite, drinking of decomposed leaf contaminated water and eating of banana".

(A 51-year-old village elder)

"Those without bednet, those going to the forest without bednet are affected by malaria. Some have misbelieves that the disease is caused by evil sprits and it can be treated by traditional healers only. What I would like to suggest is it will be better if all bednets here are treated with insecticides."

(A 36-year-old village head)

DISCUSSION

Study areas are found to be malaria mesoendemic and there are no permanent health staff and no medical records about malaria. Period prevalence of malaria detected during study period (ie. 48 per 1,000 population) may be underestimated as some cases may probably seek treatment from other sources like local quacks, general practitioners in other towns and some took over-the-counter-drugs which are easily available in the villages and these cases could not be identified by the research team. This period prevalence is higher than that of Central Myanmar (around 20 per 1000 population) as the former exists as 'pocket malaria' (ie malaria occurring in a small isolated area). Among cases detected falciparum malaria predominated (*falciparum vivax* ratio is 45.5:1). Therefore, if possible a member of the health staff should be posted for these villages. Regarding bionomics of the vectors, eight species were found including the main vector *An. dirus* near the villages. Therefore, the locals should be aware of the vectors existing very close to human dwellings in order to avoid them.

Majority of cases were in age group between 20-39 years – ie active age groups which can contribute labour if they were protected from malaria. Males are 2.6 times

more prone to get malaria than females due to their nature of work. Most cases fetched water in the evening for domestic use especially from the nearby creeks which are only the water-rich sources in the villages. At that time vector may come out from their habitats and bite them causing malaria if unprotected. Such behaviour of water fetching in the evening is strongly associated with malaria occurrence. If possible water should be fetched in the morning or if not possible insecticide treated mufflers should be worn. In one of the studies impregnated scarves and hand-bands together with ITN were found to be effective in malaria control [4].

Traveling habit was one of the determinants of malaria found in the present study. Travelers have 2.6 times more chance of getting malaria when compared to non-travelers and it was found statistically significant. In addition to this traveling without bednet is strongly associated with malaria occurrence with odds of 10. Therefore, bednet is found to be an essential personal protective equipment in prevention of malaria and ITN should be provided to those without bednets after giving health education. Effectiveness of ITN in control of malaria had been searched and successful outcomes were noted in some studies [5 & 6].

The habit of going out at dawn was also found to be associated with odds of 2.3. So, persons with such habit should wear insecticide treated mufflers and long-sleeved garments.

Practice in relation to prevention of malaria is also associated with malaria. It is considered that the lower the level of correct practice, the more the chance of being affected by malaria. To raise the level or to adopt the positive health behaviour or practice an effective and sustained delivery of health education to the locals is really necessary. Such action should be made after giving the training or workshop to the health care providers according to the guidelines in Roll Back Malaria [7].

Considering the appropriate control measures against malaria, majority of participants in the present study ie 80.52% used bednet, 100% used blankets and 81.17% accepted ITN. So impregnation was possible and feasible. One point to be taken into account is frequency of washing bednet and blankets. Most of locals washed them at least once a month. So if their materials are impregnated long lasting dose should be used to leave residual effect on them. A few locals have curtains at their houses and it is impossible to use the impregnated curtains in control of malaria. Being detected from indepth interview, misbelieves about malaria among the locals should be corrected by an effective and sustained delivery of health education programme.

In conclusion, the present study expressed some sociocultural and behavioural factors conducing to malaria and these factors except the variable gender should be removed by delivery of an effective health education programme. At the same time, insecticide treated bednets, blankets and /or mufflers should be provided to the locals to use them appropriately wherever necessary. By that means the incidence of the deadly disease malaria may be reduced in the malaria endemic forested areas.

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Detection of intestinal parasitic infestation among primary school children, Magway

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Intestinal parasitic infestations contribute to childhood malnutrition and morbidity in developing countries. It may impair the physical and mental development of the afflicted children. Stool survey and treatment in addition to health education help in prevention and control of helminthic infection. The prevalence of parasitic infestation in primary school children from KanPya High School was determined. Stool specimens from 188 children between the ages of 5 years and 12 years attending KanPya High School were examined for the presence of intestinal parasites according to WHO manual. Out of 188 primary school children, only 12.8% (24/188) had intestinal parasitic infestation. Cysts of protozoa (9.6%) and helminthic eggs (3.2%) were found in their stool samples. The prevalent intestinal helminths were *Ascaris lumbricoides* (4/188, 2.1%), *Trichuris trichiura* (1/188, 0.5%) and *Hymanolepis nana* (1/188, 0.5%). Intestinal protozoa demonstrated were *Giardia intestinalis* (15/188, 8%) and *Entamoeba histolytica* (3/188, 1.6%). The highest frequency of 8% was noted for *Giardia intestinalis* among 188 school children. The highest prevalence of 9/35 (25.7%) was seen in Grade III students aged between 7 and 8 years. Parasitic infestation was more common in boys (15.1%) than in girls (10.8%).

INTRODUCTION

Intestinal parasitic infestation is still prevalent in many developing countries including Myanmar. *Giardia intestinalis* is the most frequently reported intestinal parasite worldwide. It can cause acute or chronic diarrhoea, contributing to mal-absorption and nutritional deficiency, or remain asymptomatic [1]. Infection is acquired by the ingestion of viable cysts, which are transmitted through fecal-oral contamination. Waterborne outbreaks of giardiasis have been reported. Children are more frequently infected than adults and are at high risk of chronic infection, particularly those from developing countries [1, 2]. The cysts are highly resistant, remaining viable for up to two months. Water treatments using chlorination and heating to 60°C have proven ineffective for inactivating the cysts [3]. Estimated prevalence of the disease in

developed countries varies from 2 to 5%, while in less developed countries it can reach from 20 to 30% [4].

Amoebiasis is a condition due to the infection by the protozoan parasite *Entamoeba histolytica*. The disease is still considered as a major public health problem in developing countries of the world. Amoebiasis is believed to cause between 40,000 and 100,000 deaths worldwide each year [5]. *Entamoeba histolytica* usually causes asymptomatic infection but minority causes symptoms ranging from a few loose stool to profuse bloody diarrhoea [6]. Intestinal amoebiasis due to *E. histolytica* is ranked as the third on the list of parasitic protozoan infection leading to death behind malaria and schistosomiasis [7].

The commonest causes of infection include unsanitary habits, the contamination of food and water by human faeces already infected

with the parasite as well as direct faecal-oral contact [8]. *Ascaris lumbricoides* is one of the most common helminthic human infections worldwide. It is estimated that 25 percent of the world population, ie. more than 1.4 billion people are infected with *A. lumbricoides*. Although ascariasis occurs at all ages, it is most common in children from 2 to 10 years old, and prevalence decreases over the age of 15 years. The highest prevalence of ascariasis occurs in tropical countries. The prevalence is also greatest in areas where suboptimal sanitation practices lead to increased contamination of soil and water [9].

Ascaris is acquired by the ingestion of the embryonated eggs. Individuals can be asymptotically infected and can continue to shed eggs for years. The eggs are resistant to usual methods of chemical water purification but can be removed by filtration or by boiling. Ova can survive in the environment for prolonged periods and prefer warm, shady, moist conditions under which they can survive for up to 10 years. Infections tend to cluster in families, and worm load correlates with the number of people living in a home [10, 11].

Trichuris trichiura (whipworm) is one of the most common intestinal parasites of humans in tropical areas. An estimated 1049 million persons harbour *T. trichiura*, including 114 million preschool-age children and 233 million school-age children. Majority of cases are asymptomatics. In severe cases, blood-streaked stools are observed. In the most severe cases, rectal prolapse may occur [12].

Hymenolepiasis is an infestation caused by one of two species of tapeworm, *Hymenolepis nana* or *Hymenolepis diminuta*. Humans and other animals become infected when they intentionally or unintentionally eat material contaminated by insects. *Hymenolepis nana* infection is much more common than *Hymenolepis diminuta* infection in humans [13].

Most of the intestinal parasitic infections are transmitted by ingestion of contaminated food and drinks whereas others by penetration of skin. Unhygienic habits such as improper hand washing, biting nail, playing bare-footed in the contaminated ground predispose to helminthic infections. As a result, children are the main population of parasitic infections and also source of infection in developing countries. Complications are more common in children; the most serious complications of worm infestations include intestinal perforation, intestinal obstruction, appendicitis, obstructive jaundice and vitamin deficiency. These parasitic infestations can easily be diagnosed by routine examination of stool and easily be treated with anthelmintic drugs.

The aim of this study was to study the prevalence of intestinal parasitic infestation among primary school children of Magway by stool survey and to compare the different types of intestinal parasitic infestation among school children of different grade and sex. This can give some preliminary information on types and magnitude of intestinal parasitic infestation among school children of Magway.

MATERIALS AND METHODS

Type of study and study population

A cross-sectional descriptive study was done on a total of 188 stool samples collected from primary school children of Kanpya High School at the Department of Microbiology, University of Medicine, Magway from January to February, 2010.

Procedure

Before the commencement of the study, informed consent was obtained from parents/guardians of the children. Clean dry faecal containers were distributed to them and instructed on how to collect the samples. Data concerning their sexes, ages and grades were recorded that day. Stool

samples were collected the next day and transported to the Department of Microbiology, University of Medicine (Magway) as soon as possible. Routine examination of stool samples was done by senior teaching staff according to the WHO manual [14]. All the faecal samples were examined macroscopically. The colour, odour, presence of mucus and/or blood were noted. Each specimen was prepared for wet mount examination with normal saline and iodine and examined under low and high power objectives of ordinary light microscope. The cyst and trophozoite of protozoa and eggs of helminths were examined.

Metronidazole and albendazole were prescribed to the children whose stool specimens showed protozoal cysts and helminthic eggs. Health education was given to primary school children.

RESULTS

Sex distribution of parasitic infestation in primary school children

Stool samples from 188 children were examined, 102 (54.3%) were girls and 86 (45.7%) were boys. Out of the 188 students, 24 tested school children were positive for intestinal parasites giving a frequency of 12.8%. More boys (15.1%) had infection than girls (10.8%) due to their outdoor activities and behavioral habits (Table 1).

Table 1. The prevalence rate of parasitic infestation according to sex

Sex	No. examined (%)	No. infected (%)	No. not infected (%)
Male	86 (45.7)	13 (15.1)	73 (84.9)
Female	102 (54.3)	11 (10.8)	91 (89.2)
Total	188	24 (12.8)	92 (53.2)

The prevalence rate of parasitic infestation among the studied primary school children was 24/188 (12.8%). According to sex, the prevalence was 13/86 (15.1%) and 11/102 (10.8%) for male and female, respectively.

Intestinal protozoa detected

Eighteen (9.6%) of the 188 students were proved to be infested with intestinal protozoa: fifteen with *Giardia intestinalis* (8%) and three with *Entamoeba histolytica* (1.6%). All 3 cases with amoebiasis were male students. *Giardia intestinalis* detection rate was more common in female students; out of 15 positive cases, 6 for males and 9 belonged to females. The prevalence rate of protozoa infestation in primary school children was 15/188 (8%). According to sex, the prevalence rate was 6/86 (7%) and 9/102 (8.8%) in males and females, respectively. However, overall detected rate of intestinal protozoa was slightly higher in male students (10.5% vs. 8.8%) (Table 2).

Table 2. The prevalence rate of protozoa infestation according to sex

Type of protozoal cysts detected	Male n=86 (%)	Female n=102 (%)	No. of positive cases (n=188) (%)
<i>Giardia intestinalis</i>	6 (7)	9 (8.8)	15 (8)
<i>Entamoeba histolytica</i>	3 (3.5)	-	3 (1.6)
Total protozoal cysts	9 (10.5)	9 (8.8)	18 (9.6)

Frequency of different helminthic infestation

The eggs of intestinal helminthes were detected in six out of 188 stool samples (3.2%). Four cases (2.1%) were noted for *Ascaris lumbricoides* while only one *Trichuris trichiura* infestation and one *Hymanolepis nana* infection were found among male students. No enterobiasis, taeniasis and hookworm infestation was found. Equal distribution of ascariasis in both sexes was found (Table 3). The prevalence rate of helminths infestation in primary school children was 4/188 (2.1%).

Table 3. The prevalence rate of helminths infestation according to sex

Type of helminthic eggs detected	Male (n=86) (%)	Female (n=102) (%)	No. of positive cases (n=188) (%)
<i>Ascaris lumbricoides</i>	2(2.3)	2(2)	4(2.1)
<i>Trichuris trichiura</i>	1(1.2)		1(0.5)
<i>Hymanolepis nana</i>	1(1.2)		1(0.5)
Total	4(4.7)	2(2)	6(3.2)

According to sex, prevalence rate was 2/86 (2.3%) and 2/102 (2%) in male and female, respectively.

Table 4. The prevalence rate of parasitic infestation in primary school children according to various grades

Grade	I (%)	II (%)	III (%)	IV (%)	V (%)
Positive/ No. examined (%)	5/46 (10.9)	2/34 (5.9)	9/35 (25.7)	5/28 (17.5)	3/45 (6.7)
Giardiasis	4 (8.7)	1(3)	6 (17.1)	2(7.14)	2(4.4)
Amoebiasis			2 (5.7)	1(3.6)	
Ascariasis	1 (2.2)		1 (2.9)	1(3.6)	1(2.2)
Trichuriasis				1(3.6)	
Hymenolepiasis		1(2.9)			

Prevalence of parasitic infestation among various grades

The highest prevalence of intestinal parasitic infection was seen in Grade III students (25.7%) followed by Grade IV students (17.8%) and Grade I students (10.9%). The highest prevalence of giardiasis was seen in Grade III students (17.1%). Amoebiasis was detected only in Grade III and IV students. Giardiasis was absent in male students of Grade IV and V. *E. histolytica* infestation was detected only in male students from Grade III and IV (Table 4).

DISCUSSION

Intestinal parasitic infestations, caused by intestinal helminths and protozoan parasites, are a global distribution although the nature and incidence of parasitic infestations vary from place to place. In developed countries, protozoan cause gastrointestinal infections more commonly than helminths. Intestinal parasites cause a significant morbidity and mortality in endemic countries. Intestinal parasitic infestations were most common in children. In this study, intestinal parasitic infestation was 12.8% (24/188) of the study population. The prevalence rate was found to be much lower than those of other countries; 54.2% in Cambodia and 71.15% in children of Kupwara, Kashmir, India [15, 16].

Giardiasis (8%) was the most common intestinal parasitic infestation in this study followed by ascariasis (2.1%) and amoebiasis (1.6%). The biannual mass chemotherapy for helminthic infection using albendazole was carried out in this population as directed by the parasitic control program of the Ministry of Health and it might account for increased giardia infestation in the study group. A similar result was obtained in a study from Bangladesh in which the prevalence of Giardia was significantly higher in the group after being treated than in group which had not been treated [17].

Prevalence rates of giardiasis range from 2-7% in developed countries to 20-30% in most developing countries. This rate of giardiasis in the present study was found to be lower than those of other countries. The prevalence rate of giardiasis among primary school children of Iran was 18.1% and that of Columbia was 27.6% [18, 19]. The prevalence of giardiasis in this study was comparable to or slightly higher than that of Thai studies where the prevalence of giardiasis in Chiangmai school children was 7.7% and Nan Province was 5.3 [20, 21].

Clinical presentations of giardiasis vary from asymptomatic carriage to acute and chronic diarrheal illness. Young children are at high risk of chronic infection resulting in nutritional malabsorption and failure to thrive. Infection is transmitted through faecal-oral contamination. Waterborne outbreaks of giardiasis have been reported and the major risk factors were sanitation and personal hygiene. Higher prevalence of giardiasis is associated with a lack of piped water inside the house, contaminated and hold back water near the dwelling, lack of sewage and defecation in open area [22]. Person-to-person transmission in crowded populations with exposure to infected persons especially in households with numerous children is well recognized. Most positive cases in the present study were asymptomatic. As giardiasis is a chronic disease, some cases might not be recent infection.

In this study, frequency of helminthic infestation was found to be lower than that of other countries. Infestation rates were 2.1% for ascariasis, 0.5% for trichuriasis and 0.5% for hymenolepiasis. The helminth parasites detection rates in Kupwara, Kashmir, India were *Ascaris lumbricoides* (69.23%), *Trichuris trichiura* (30.76%), *Enterobius vermicularis* (7.69%) and *Taenia saginata* (7.69%) [16].

In parasitological surveys of 29,846 stool specimens collected from primary school children carried out on a national scale in Laos, the cumulative egg positive rate for intestinal helminths was 61.9%, the rate for *Ascaris lumbricoides* was 34.9%, hookworm (19.1%), *Trichuris trichiura* (25.8%), *Opisthorchis viverrini* (10.9%), *Taenia* spp. (0.6%) and *Hymenolepis* spp. (0.2%) [23]. This low prevalence rate of the present study might be due to regular deworming of primary school children by School Health Team of Department of Health.

Amoebiasis is usually contracted by ingesting water or food contaminated by amoebic cysts. People with amoebiasis pass cyst and trophozoites in their faeces. About 9 out of 10 of those who have *E. histolytica* are asymptomatics. Amoebiasis can affect people living anywhere in the world, but is most common where living conditions are crowded or where there is poor hygiene and sanitation. In this present study, only 1.6% of the study population showed cyst of *Entamoeba histolytica*. This finding was comparable to that of Thai study where prevalence rate of *Entamoeba histolytica* was 1.4% [21]. However, this finding of 1.6% was much lower than that of other studies. The study conducted in a cohort of Bangladesh children found that the prevalence of *E. histolytica* in diarrhoea stool samples was 8% [24]. These discrepancies might be due to the differences in detection methods and study population. In Thailand study and the present study, microscopy was used for detection of cysts of *E. histolytica* whereas in Bangladesh study, amoebiasis was detected by rapid

antigen detection test in preschool children with symptoms.

Microscopy is not as sensitive or accurate in diagnosis as the other tests available. It is unable to distinguish the invasive parasite *E. histolytica* from the commensal parasites such as *E. dispar* and *E. moshkovskii*. As *E. dispar* is much more common than *E. histolytica* in most parts of the world and it means that there is a lot of incorrect diagnosis of *E. histolytica* infection taking place. The WHO recommends that infections diagnosed by microscopy alone should not be treated if they are asymptomatic and there is no other reason to suspect that the infection is actually *E. histolytica*.

New approaches to the detection of *E. histolytica* are based on detection of *E. histolytica*-specific antigen and DNA in stool and other clinical samples. In several studies, the *E. histolytica*-specific antigen-detection test, now commercially available, has been used and found to be sensitive and specific for the detection of *E. histolytica* [25]. To understand the real prevalence of *E. histolytica*-associated infection, a molecular method must be used for its diagnosis [26]. However, microscopy is still by far the most widespread method for diagnosis around the world. Since cysts are not shed constantly, a minimum of three stools should be examined [27]. The diagnosis and treatment of intestinal helminth infections have not been changed much, and the traditional microscopic method can be used for their diagnosis.

The present study showed the effectiveness of regular deworming; the prevalence rate of helminthiasis was lower than protozoal infestation. There is no vaccination or prophylactic drug to prevent intestinal parasitic infestation. The best method of prevention is to avoid eating or drinking food or water which may be faecally contaminated. This study emphasizes the need for improved environmental condition, i.e., clean water supplies, enhanced sanitation and chemotherapy of school-age children in rural areas.

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**Cost analysis of severe and complicated malaria cases admitted to
Medical Intensive Care Unit, No.1 Defence Services General Hospital (1000 bedded)**

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Malaria is a major global health problem and burden of malaria is multi-determined, including economic, educational and environmental factors. To assess costs of treating severe and complicated malaria, hospital-based cross-sectional descriptive study was carried out at Medical Intensive Care Unit among 20 respondents with face-to-face interview using semi-structured proforma and 6 participants for in-depth interview about illness-related burden in 2008. The mean age of respondents was 27.5 years and 16 were army soldiers with mean salary of 32,975 kyats. The currency exchange rate approved by Directorate of Military Medical Services, the reference price of National Health Laboratory and the Nutrition Department of No.1 DSGH were used in calculation of costs. The total provider cost was 1,271,930 kyats in which the total drugs cost was the highest and the total material and instrument cost was the lowest with the good quality treatment regimes were utilized. The total patient cost was 415,263 kyats in which the food cost was the highest and the cost of purchasing drugs and taking blood investigations was the lowest because of receiving free-of-charge treatment as reported by the in-depth interviews. The unit cost of treating severe and complicated malaria was 84,360 kyats for one episode of illness in which the unit cost of provider was three times higher than that of the patients. The results of study can be used to further improve the existing malaria control measures in military health care setting.

INTRODUCTION

Malaria is a major global health problem. Malaria causes about 400 to 900 million cases of fever and approximately one to three million deaths annually [1]. This represents at least one death in every 30 seconds. Indeed, if the prevalence of malaria stays on its present upward trend, the death rate could double in the next twenty years [2]. Accordingly, about 70% of reported malaria cases in Myanmar involve patients who are older than 15 years of age, and about 60% of cases are related to forestry work [3]. In addition, military personnel and front-line soldiers who are deployed in areas with high transmission are vulnerable. Early diagnosis and prompt treatment are essential to combat malaria. If a proper treatment is

not taken for malaria, the disease pattern may change into severe form of malaria.

The health consequences of malaria vary with the degrees in terms of severity, but the global impact of malaria on health, productivity, and general well-being is profound. Such being the case, scientists have attempted to set an economic value on this burden by measuring the impacts on households, health systems, and national economy [4]. The disease may account for as much as 40% of public health expenditure, 30-50% of hospital admissions and up to 50% of out-patient visits in some countries with a heavy malaria burden [2].

Therefore, the cost analysis study was conducted in tertiary hospital to estimate economic burden of hospitalized malaria

cases. It is intended to explore the health care cost of treating malaria by two sides: the provider and the patients.

Objective

The objective of the study was to assess the costs of hospitalized malaria in Medical Intensive Care Unit (Medical ICU) of No.1 Defence Services General Hospital.

MATERIALS AND METHODS

The hospital-based cross-sectional descriptive study was used to evaluate the cost of treating severe and complicated malaria and the maximum cost for treating malaria in military setting. Overall (20) patients who were admitted for cerebral malaria, severe and complicated malaria at Medical Intensive Care Unit of No.1 Defence Services General Hospital during data collection period (from September to December 2008) were included in this study.

Collection of data

Data gathering through face-to-face interview, checklist, and in-depth interview (IDI) and record review were carried out.

1. Pretested semi-structured proforma was used for hospital costing and individual patient costing. Firstly, socio-demographic characteristics of patients were obtained by interviewing; this consisted of sex, age, marital status, education, occupation, rank, and salary. After that, history of hospitalization was recorded consisting of total length of hospital-stay in Medical ICU and diagnosis for hospitalization. Then, various types of cost of illness were recorded for the period of being hospitalized in Medical ICU. These were costs of treatment, laboratory investigation and diet, which were the costs incurred by both the provider and the patients, respectively. Next, the type of travel arranged either by patient or by military services and the cost of travel were also observed through interview.

Subsequently, food costs incurred by patients were also calculated.

2. Check-lists for instruments (medical material and instruments of medical and nursing procedure) were used for hospital cost calculation.
3. By using interview guided questions, information on effect of illness, cost of episode of the illness and the burden of illness were gathered by in-depth interviews (IDI) with six patients where four patients were soldiers, one a soldier's wife, and one a civilian.

4. Following records were retrieved from record reviews of No.1 DSGH in 2008:

(a) Medical and nursing staff salaries and fringe benefits data from records of Administration Department of No.1 DSGH.

(b) Type of nutrients for patients and its expenses from records of Nutrition Department of No.1 DSGH.

(c) Total numbers of medical staff for Medical ICU from physician and medical officer rosters from Medical Wing of No.1 DSGH.

(d) Total numbers of nursing staff for Medical ICU from monthly nurses duty roster of Medical ICU.

(e) Document of malaria cases and total number of admission for malaria from admission and discharge books from Medical ICU.

(f) Uses of medical materials and instruments for patients such as drip sets, syringe and needles from daily records of Medical ICU.

(g) Uses of drugs, laboratory and X-rays investigations, medical and nursing procedures, and type of diet from malaria patients' medical and nursing records.

Analysis of data

In military health care setting, health services and treatment were provided free-of-charge for military community. Direct cost and overhead cost were calculated in the provider side. Thus, provider cost was

calculated with the following reference rate. The costs of drugs and other medical materials were based on the costs of No.1 Medical Store Depot (MSD). These costs were identified in terms of the local currency (US\$ 1.00 is equivalent to 5.55 kyats and Euro 1.00 is equivalent to 7.9 kyats at the official exchange rate of Directorate of Military Medical Services) as currency exchange rate was given approval by Directorate of Military Medical Services. The costs of laboratory investigations were calculated with reference price of National Health Laboratory. The costs of diet were calculated by the price of Nutrition Department of No.1 DSGH. Medical and nursing staff cost was calculated as the cost per patient per day. Next, this cost was multiplied by length of stay of each patient in Medical ICU. Travelling costs incurred by the military organizations, were calculated by the minimum price of public transportation consisted of by train and by bus. The following formulas were used in this study:

$$\text{Cost per admission by provider} = \frac{\text{Total provider cost}}{\text{Total number of admission}}$$

$$\text{Cost per admission by patient} = \frac{\text{Total patient cost}}{\text{Total number of admission}}$$

$$\text{Unit cost} = \frac{\text{Total cost of provider and patient}}{\text{Total number of patients}}$$

$$\text{Cost calculation for medical materials: Calculation of annual cost} = \frac{\text{The current value of item}}{\text{Annualizing factor}} \quad [5]$$

Collected data were calculated by using cost calculation and appropriate formula. Next, the calculated data were reviewed for completeness and then data entry was made. Data were analyzed by using Microsoft Office Excel 2007 and SPSS version 11.5. For the data of in-depth interview, content analysis was used. Themes and sub-themes were organized and prepared for analysis by using QSR Nvivo software.

RESULTS

Patients' background

There were twenty patients with the diagnosis of severe and complicated malaria (18) and two were severe malaria with acute renal failure (ARF). The ages of respondents ranged from 18 to 49 years (mean±SD=27.5±9.3). Out of the total 20 respondents, 17 were males and 3 were females. Twelve were single and 8 were married. Most of respondents (80%) have completed at least middle school level education. The majority, 16 (80%), were soldiers, three (15%) were dependents (soldiers' wives) and one respondent was a civilian (brother of medical officer/ a merchant). Out of 16 soldiers, 14 were from the army and 2 were from the navy with the salary ranging from 21,000 to 130,000 kyats. Eleven were categorized in the seriously ill list (SIL) and 3 were in the dangerously ill list (DIL). The length of stay in Medical ICU ranged from 3 to 15 days (mean±SD=7.4±3.29).

Cost borne by the provider side

Though health care consumers of military hospitals received their care free of charge, direct and overhead cost were borne by provider. Estimation of those costs was calculated in which drug cost and medical material and instruments cost were included in direct cost in addition to X-rays and laboratory investigation cost. Food cost and staff cost (salary and fringe benefit) were included in overhead cost borne by provider.

Regarding direct cost, the total direct cost of severe and complicated malaria was 519,862 kyats. The cost of haemodialysis was excluded in direct cost calculation, since only 2 respondents had severe malaria with acute renal failure (ARF). Haemodialysis will cost 949,862 kyats for direct cost borne by provider. The additional cost for haemodialysis during hospitalization at Medical ICU was about 430,000 kyats. It cost 140,000 kyats for first course of haemodialysis and 30,000 kyats for

the regular course of haemodialysis with reference to the price at the Department of Kidney Unit of Yangon General Hospital.

Table 1. Cost borne by the provider for severe and complicated malaria patients (in kyats) (n=20)

Cost items	Total cost	Unit cost
Direct cost		
Drugs	519,215	25,961
Material & instruments	647	32
Overhead		
Lab & X-rays	470,517	23,526
Food services	232,800	11,640
Staff	48,751	2,438
Total cost	1,271,930	

In Table 1, it was found that the total drug cost was the highest and the total material and instrument cost was the lowest among five items of provider costs.

Direct cost from patients due to illness

In Table 2, it was clear that the food cost incurred by respondents was the highest. Officers incurred more expense than other ranks in the unit cost of food. The civilian had to incur more expenses for the food, drugs and investigation. A few participants did not spend money on food:

“We don’t spend; actually we can’t spend any money for our food and drinks”.

(Su, a soldier’s wife, a malaria patient)

Table 2. Cost incurred by the patients for their illness (in kyats) (n=20)

Cost items	Total cost	Unit cost
Food	233,100	11,655
Travel	116,463	5,823
Purchase for drugs & investigation	65,700	3,285
Total	415,263	

Indirect cost from patients for illness

It was limited in terms of monetary value for indirect cost of illness, and they expressed in their own words.

1. Effects of illness on work

Most respondents were at the productivity age, thus, they were unhappy to experience

illness as they had to be absent from work.

“I could not work properly. I could not lift heavy things. I always wanted to lie in bed. When I was at the front-line I had to do sentry duty. Even though, I was feeling unwell. Sometimes I could not do that I had to report to my officers”.

(Zaw, a soldier, came from civil hospital and after being sent back from front-line)

2. Frequent attack of malaria

Soldiers suffered from malaria with frequent attack.

“One year after joining the army I was affected by malaria. In 2004, I was sent to the front-line which was malaria endemic area. After one year I returned to my battalion. Then I was affected by malaria again. In 2008, I fell again seriously ill. I could not stand it”.

(Min, a soldier)

3. Burden on care attendant

The burden of malaria on households and individuals is substantial because of the time spent caring for the ill. They expressed the need of care attendant as follows.

“My spouse is in final year Economics. It is a pity that she can't go to any tuition and she has lost her chance to sit for her examination”.

(Aung, a soldier, while he was on leave)

Table 3. Comparison between unit cost borne by the provider and the patients (In kyats) (n=20)

No.	Cost items	Unit cost	
		Provider	Patients
1.	Drugs & Lab & X-ray	49,487	3,285
2.	Food service (diet)	11,640	11,655
3.	Material & instrument	32	-
4.	Medical & nursing staff	2,438	
5.	Travel cost	-	5,823

Unit cost of severe and complicated malaria

In Table 3, two categories of food cost items were observed for the provider and the patients. When comparing these two items,

food costs borne by the provider and that by the patients, there were not much differences. But on the other hand, the provider had to spend nearly fifteen times more than the patients for the costs of drugs and laboratory investigations.

Table 4. Unit cost of severe and complicated malaria (in kyats) (n=20)

No.	Total cost (kyats)	Unit cost	Percent (%)
1.	1,271,930 (provider)	63,597	76
2.	415,263 (patient)	20,763	24
3.	1,687,193 (both provider & patient)	84,360	100

In Table 4, it was found that the unit cost of the provider was three times higher than the unit cost of the patients. Thus, it is clear that the provider spent more money than the patients. Participants also expressed their satisfaction on the curative services of military hospital. The following statement is quoted:

“No. Nothing at all. Food and drinks are provided for me and also for my mother who is attending me. Everything is OK here in Military Hospital”.

(Su, a soldier's wife, a malaria patient)

DISCUSSION

We found that a large amount of direct costs and overhead costs were borne by the provider. Among these cost items, drugs cost was the highest and the material and instrument cost was the lowest because the value of medical instruments was in US or Euro currency which are converted to domestic currency via the official exchange rate which is very much lower than the market rate. The cost of drugs was mostly calculated directly in kyats and it is used only for the individual.

The drugs cost was the highest in all levels of respondents. It was because artemisinin combination therapies (ACT) is an officially recommended treatment for malaria in military care setting. Provider

expended nearly five thousand kyats on the cost of ACT for each patient. ACTs is around 5 to 10 times more costly than the monotherapies, namely sulphadoxinepyrimethamine and quinine [6].

The expense for medical and nursing staff was the second lowest item in the provider side because the narrow down calculation of the cost of staff for severe and complicated malaria (cost per patient per day in Medical ICU) was used. The total salary and fringe benefits do not change with the increase in patients over a specified period of time or with the capacity of the work.

In cost borne by patients, the food cost was the highest and it relied on the individual expense and their status of affordable in extra food. Besides, the high level of spending on special foods was linked with the belief about diseases of heat and cold and they tried to counteract the heating effect of malaria with oranges and sweet soft drinks.

Most patients came from the front-line areas away from the tertiary hospital. It happened that people had to travel further away from their places to seek special care for the severe cases when health facilities are not available locally. Similarly, the cost of illness for patients attending outpatient malaria clinics who received early diagnosis and prompt treatment was four to seven times cheaper than the cost of illness for hospitalized malaria cases [7]. In support of equity reasons, there was a health centre accessible within 1 hour of walking to all of the covered population [8]. Thus, every person has access to a health center within 1 hour of travel time. It has reduced the high cost of travel in seeking health care.

The evidence of income loss was not found because most patients were military persons. There was no reduction in their salaries and fringe benefits by the military services. However, their productivity was reducing in military outpost duties and operation as a consequence of hospitalization owing to attack of malaria. The unit cost of severe

and complicated malaria was 84,360 kyats in which 76% were spent by the provider and 24% were spent by the patients. In conclusion, with the high cost of the provider in Myanmar military hospital effective and efficient services were given for all malaria cases in Medical ICU without fatality.

This study has the chance only to generalize the cost of severe and complicated malaria at No.1 DSGH and not for the whole military community. In the calculation of the internal cost, the cost for capital items and recurrent items (building, electricity charges, water charges, telephone charges and linen) has to be excluded because cost data were limited. Study time period was too limited to be able to cover the complete picture of these cost items.

This study has been carried out to assess only the cost of treatment for hospitalized malaria cases, even though the comparative study of cost evaluation regarding malaria treatment and prevention is more beneficial to malaria control in military setting.

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**Presence of hepatitis C virus RNA by nested polymerase chain reaction (RT-PCR)
in plasma-derived hepatitis B vaccine non-responders**

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Hepatitis C virus (HCV) has infected an estimated 170 million people worldwide. The overall prevalence of anti-HCV positivity was found to be approximately 2.8% in normal healthy persons in Yangon and 3.1% in blood donors of Central Blood Bank in Yangon General Hospital (YGH). In a clinical trial for the plasma-derived hepatitis B vaccine of DMR (LM), there were 23 vaccinees who were non-responders to four doses of this vaccine. Their sera contained anti-HBs level of less than the protective value of 10 mIU/ml. All serum samples of non-responders were negative for anti-HCV by Particle Agglutination (PAII) Method. From these sera, HCV-RNA was extracted using the chloroform and ethanol method. The extracted sera samples of non-responders were tested for HCV-RNA by using the specific primers (19, 20 for PCR1 and 21, 22 for PCR2) on 5'UTR region of genome at Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) method. One serum sample (NR21) was found to have a distinct band at the same level of HCV-RNA positive control (268 base pair) according to the 50 bp DNA ladder marker in 2% agarose gel electrophoresis with ethidium bromide staining. The diagnosis of HCV infection can be made by qualitative detection of HCV-RNA using gene amplification technique (RT-PCR). HCV-RNA can be detected in serum or plasma within 1-2 weeks after exposure to the virus and weeks before the onset of ALT (alanine aminotransferase) enzyme elevations or appearance of anti-HCV. RT-PCR assays have a lower limit of detection of 100-1,000 viral genome copies/ml. Direct detection of HCV-RNA may help in early diagnosis of acute infection during the sero-negative period in immune suppressed individuals. Rarely, HCV-RNA might be the only evidence of HCV infection. Therefore, HCV-RNA detection with RT-PCR is more direct and sensitive than anti-HCV testing and it has become one of the diagnostic standard tests for HCV infection.

INTRODUCTION

Hepatitis C virus (HCV) has infected an estimated 170 million people worldwide, more than four times as many as HIV [1]. The prevalence of anti-HCV in blood donors varies from approximately 0.3% -2% around the world [2]. The overall prevalence of anti-HCV positivity was found to be approximately 2.8% in normal healthy persons in Yangon and 3.1% in blood donors of Central Blood Bank in Yangon General Hospital (YGH) [3]. HCV is an enveloped positive-strand RNA virus

of the genus Hepacivirus in the family of *Flaviviridae*. The HCV genome consists of about 9500 nucleotides (nt) containing a single long translational open reading frame (ORF) [4]. HCV viral genome is depicted with the structural (S) and nonstructural (NS) protein-coding regions, 5' and 3'UTRs, and putative 3' secondary structure. The HCV 5'UTR is 341 nucleotides (nt) in length, highly conserved and contains multiple AUG codons upstream of the translational site [5]. HCV contains one gene, coding for a poly-protein that is subsequently spliced into at least 10 functional

proteins such as C, E1, E2, NS2, NS3, NS4A, NS4B, NS5A and NS5B [1]. There are six main genotypes of HCV ie.1, 2, 3, 4, 5 and 6. Each could be divided into subtype a and b [6]. HCV types 1, 2 and 3 appear to be in widely distribution around the world, although types 1a and 1b are the most common, accounting for about 60% of global infection [7]. In Myanmar, genotype 3b was found in 67% of chronic liver diseases in YGH [8]. Most of the HCV genome is based on reverse transcription of HCV-RNA followed by PCR amplification. HCV-RNA is directly detected by nucleic acid hybridization method and RT-PCR method [2]. Several vaccine trials carried out at the Department of Medical Research (Lower Myanmar), using plasma-derived hepatitis B vaccine has shown that there is approximately 10% non-responder rate to the vaccine. Genetic factor may play a role in non-responsiveness to this vaccine [9].

Aim and objective

- To detect the HCV-RNA in non-responders of plasma-derived hepatitis B vaccine produced at the Department of Medical Research (Lower Myanmar) using the nested R-T (Reverse Transcriptase) polymerase chain reaction.

MATERIALS AND METHODS

Of 289 apparently healthy persons who received the three doses of DMR (LM) plasma-derived hepatitis B vaccine, 38 persons did not respond to this vaccine one month after the completion of three doses. Twenty-three vaccinees did not have detectable levels (10 mIU/L) at two months after the booster vaccination of plasma-derived hepatitis B vaccine. All serum samples of non-responders were tested for anti-HCV by Particle Agglutination (PAII) test (Ortho-Clinical Diagnostics, Chiron Corporation, Tokyo, Japan). This test is based on the sensitized cells which composed of the fixed gelatin particles sensitized with recombinant HCV antigen cause agglutination in the presence of HCV

antibodies in specimen [10]. HCV-RNA was extracted from serum samples of non-responder by phenol chloroform extraction followed by precipitation with isopropanol. The resulting pellets were re-suspended in 50 µl of TE buffer that was used as a template for HCV-RNA amplification. Forty microlitre of reaction buffer was made up for PCR which contained 10X gold buffer (1X buffer containing 1.5 mM MgCL₂), 5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), 10mM dNTP mix and two pairs of PCR primers (19 and 20 for PCR1, 21 and 22 for PCR2) (Table 1), 5U of RNAase inhibitor Promega, Madison) and 200 U of Moloney murine leukemia virus reverse transcriptase (Gib-co BRL Tokyo, Japan).

Table 1. List of primer sequences for detection of HCV-RNA by nested RT-PCR

Region of gene for HCV-RNA	Level of PCR	Name of primers	Nucleotide sequences	Product size
5'-UTR	PCR 1	19 (sense)	5'-GCGACA CTCCACC ATAGAT-3'	329 bp
5'-UTR	PCR 1	20 (antisense)	5'-GCTCAT GGTGCAC GGTCTA-3'	329 bp
5'-UTR	PCR 2	21 (sense)	5'-CTGTGA GGAAC TAC GTCT-3'	268 bp
5'-UTR	PCR 2	22 (antisense)	5'-ACTCGC AAGCACC CTATCA-3'	268 bp

Thermocycler was programmed first to incubate the samples for 50 min at 37°C, then preheat at 95°C for 10 min to activate AmpliTaq Gold followed by 40 cycles consisting of 94°C for 20 sec, 55°C for 20 sec and 72°C for 7 min using a Perkin-Elmer 9700 Thermal Cycler. For the PCR2 reaction, 2 µl of the first PCR product (PCR1) was mixed with same PCR buffer and second set of each inner primer. Thermocycling for 40 cycles was done as PCR1 but the annealing temperature was set at 60°C instead of 55°C for the second round of PCR [11]. The PCR2 products of each sample were detected by 2% agarose gel electrophoresis and staining with ethidium

bromide and evaluated under UV light. The size of the PCR final product was found at 268 bp according to the migration pattern of 50 bp DNA ladder (Pharmacia Biotech, Piscataway, NJ, USA).

RESULTS

In this study, twenty-three non-responders of plasma-derived hepatitis B vaccine were not detected the level of anti-HBs (10 m IU/L) in serum samples by using the Abbott ELISA test kit , USA.

Table 2. Positivity of anti-HCV and HCV-RNA in hepatitis B vaccine non-responders

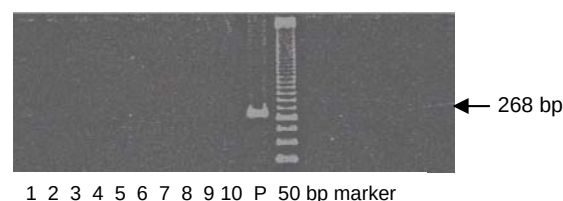
Gender	Number of vaccine non-responders	Anti-HCV by PAII test		HCV-RNA by RT-PCR	
		Positive (%)	Negative (%)	Positive (%)	Negative (%)
Male	18	-	18	-	18
Female	5	-	5	1	4
Total	23	-	23 (100)	1 (4.3)	22 (95.6)

As shown in Table 2, all serum samples of non-responders were negative for anti-HCV by Particle Agglutination (PAII) test. It showed that 100% of non-responders were negative for anti-HCV by PAII test and one vaccine non-responder (4.3% of total 23 non-responders) was positive result of HCV-RNA by nested RT-PCR method. HCV-RNA was not detected in 22 serum samples of non-responders (95.6%) by nested RT-PCR.

After running the 2% agarose gel electrophoresis with ethidium bromide staining, HCV-RNA band was not detected in first 10 samples of PCR 2 products under UV light and HCV-RNA positive control band was detected in 2% agarose gel at 268 bp according to the 50 bp marker ladder (Fig.1).

As shown in Fig. 2, one sample of non-responder (NR-12), out of last thirteen samples was found to have a distinct band at the same level of HCV-RNA positive control (268 bp) according to marker ladder (50 bp) in 2% agarose gel electrophoresis

with ethidium bromide staining after amplification of nested RT-PCR.



P= HCV positive control
1 to 10= samples of vaccine non-responders

Fig. 1. Absence of HCV-RNA in 10 serum samples of vaccine non-responders



P=HCV positive control
11 to 23= samples of vaccine non-responders

Fig. 2. Detection of HCV-RNA in one sample (NR-12) of vaccine non-responders

DISCUSSION

In this study, out of 38 vaccinees, twenty-three subjects did not respond to plasma-derived hepatitis B vaccine after booster vaccination. They did not have detectable level of anti-HBs (10 mIU/ml) by Abbott ELISA test, USA. Certain risk factors are associated with decreased antibody response such as increasing age, male gender, obesity, history of smoking and administration of vaccine in the buttock rather than deltoid muscle [12]. Poor responders to vaccine may benefit from revaccination, and genetic factors especially immune histocompatibility complex may modulate the immune response to vaccination [13].

All samples of non-responders were negative for anti-HCV antibody by using the particle agglutination test (PAII). The sensitivity of PAII test is 75% and it founds to be optimum, more than 90% in screening of strong positive samples [14]. One female non-responder (NR-12) was positive for

HCV-RNA by using nested RT-PCR method in this study. Detection of HCV-RNA is useful for diagnosis of HCV infection in early stage and HCV-RNA can be detected about 3 weeks after infection during the open window phase before anti-HCV antibody appears. Anti-HCV appears 12 weeks after infection and 6 weeks after onset of hepatitis as detected by elevation of liver enzymes [15].

Direct detection of HCV-RNA may help in early diagnosis of acute infection during the sero-negative period in immune suppressed individuals. Patients with impaired production of immunoglobulin may have HCV infections without detectable anti-HCV. The value of detecting anti-HCV IgM has been studied as a way to shorten the time window between initial HCV infection and detection of anti-HCV antibody, during which period HCV-RNA may be detected [2]. RT-PCR assays have a lower limit of detection of 10^{-10} RNA molecules [16]. Rarely, RT-PCR is more direct and sensitive than anti-HCV testing and it has become one of the diagnostic standard tests for hepatitis C infection [17].

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Production, quality control and immune response of the plasma-derived and the yeast-derived recombinant hepatitis B vaccines locally developed at the Hepatitis B Vaccine Plant, DMR (LM): Comparative study

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The DMR (Lower Myanmar) produced the plasma-derived and yeast-derived (recombinant) hepatitis B (HB) vaccines under the supervision of scientists from the CJ Pharmaceutical Corporation, Republic of Korea at the GMP standard DMR Vaccine Plant, Hlegu Township in 2003. This was a non-intervention, comparative study carried out by reviewing the two methods regarding production, quality control and immune response of plasma-derived and recombinant HB vaccines locally produced during the period of 2003 to 2006. For production process, 35 liters of high titre HBsAg-positive plasma were needed as a starting volume per lot and a total yield of 225 mg purified HBsAg with 100,000 vaccine dosages was obtained within a month for plasma-derived HB vaccine production, whereas only a single ampoule of Master Cell Bank (MCB) containing the HBsAg protein-expression gene is required as a starting material and a total yield of 5 gm purified HBsAg with 500,000 vaccine dosages can be produced per lot within a month for recombinant HB vaccine production. Regarding the quality control, more biological tests were needed for plasma-derived vaccine testing with 95-97% purity whereas more chemical tests were required for testing the recombinant purified bulk which is 98-100% purity on SDS-PAGE. Results of the clinical trial conducted on neonates revealed that 90.7% of neonates vaccinated with plasma-derived vaccine showed sero-conversion with anti-HBs geometric mean titre of 415 mIU/ml whereas 100% of vaccinees showed sero-conversion with geometric mean titre of 610.7 mIU/ml in recombinant vaccine group. Since the major obstacle for plasma-derived vaccine production is the very limited supply of the starting raw material i.e. HBsAg-positive blood, annual production capacity could not fulfill the countrywide requirement in Myanmar. Therefore, it is suggested that recombinant HB vaccine production, which has an unlimited availability of starting material (MCB) and an annual production capacity of 5 million doses at the Vaccine Plant, is the appropriate and acceptable method for large scale production of HB vaccine in the future.

INTRODUCTION

Hepatitis B (HB) viral infection is an important health problem worldwide because the infection can cause significant liver diseases. Since there is no cure for chronic HB viral infection, prevention is very important. Globally, the first HB vaccine derived from plasma became commercially available in 1981 and the recombinant HB vaccine entered the market

in 1986 [1]. Myanmar is hyperendemic for HB viral infection as research studies have shown that the HB carrier rate is 10-12% and infection rate, 35-65% [2, 3]. In 1997, small scale production of plasma-derived HB vaccine was successfully carried out locally at the Department of Medical Research (Lower Myanmar) (DMR, LM) in collaboration with WHO and UNDP [4]. For large scale production, the contract for development of the plasma-derived and

yeast-derived recombinant HB vaccines was signed between the governments of the Union of Myanmar (DMR, LM) and Republic of Korea (Samsung/ CJ Corporation) under the EDCF Loan in 2001. According to the contract, the supplier built a WHO Good Manufacturing Practices (GMP) standard HB vaccine plant which has an annual capacity to produce 2 million and 5 million dosages of plasma-derived and yeast-derived recombinant HB vaccines, respectively, also with the objective to apply for certificate of WHO GMP approval.

In 2003, test production of both HB vaccines was carried out by scientists from DMR (LM) under supervision and guidance of Korean scientists at the DMR HB Vaccine Plant, Myanmar and was completed in 2004. The products of both plasma-derived and recombinant vaccines production passed the quality control tests (recommended by WHO) performed in Myanmar and Korea FDAs. Clinical trials of both vaccines were carried out on healthy newborns (target population) at the Thingangyun Sanpya Hospital and South Dagon Hospital, Yangon in 2006. The results showed that after completion of third doses of HB vaccination, sero-conversion rates (i.e. development of anti-HBs) in these neonates were found to be satisfactory without undesirable side effects [5, 6].

In this study, different production processes, quality control test results and immune responses of the plasma-derived and recombinant HB vaccines were compared with an aim to determine the most appropriate types of vaccine and acceptable method for further large scale production of HB vaccine to fulfill the requirement of our country in the future.

MATERIALS AND METHODS

This is a non-intervention, comparative study carried out by reviewing two methods; plasma-derived and recombinant HB vaccines regarding production, quality control and immune response of lot no.

P-01-1/04, P-01-2/04 and P-01-3/04 of plasma-derived and R-01/04, R-02/04 of recombinant HB vaccine produced during the period of from 2003 to 2006 in the GMP standard HB Vaccine Plant, DMR (LM) were systematically reviewed and analyzed. In addition, starting materials, purity, total protein and HBsAg contents of purified bulk in quality control testing, yields of vaccine doses and time consumed per lot and results of clinical trials on healthy newborns in both plasma-derived and recombinant HB vaccination were also compared.

RESULTS

Flow diagrams of manufacturing processes of plasma-derived and recombinant vaccines are compared and shown in Fig. 1. In plasma-derived HB vaccine production, major steps are: collection of HBsAg-positive blood, separation of high-titre HBsAg-positive plasma, precipitation by using hydrochloric acid (HCl), polyethylene glycol (PEG), hydroxyapatite (HAP), dialfiltration and concentration, potassium bromide (KBr) gradient fractionation, ultracentrifugation at 60,000 rpm for 17 hours, dialysis, flash heat inactivation followed by pasteurization. Major steps involved in recombinant HB vaccine production are: cultivation of master cell bank (MCB) and working cell bank (WCB) in seed and main fermentation, dialfiltration and concentration, homogenization, pH precipitation, ultracentrifugation at 40,000 rpm for 40 hours, gel chromatography followed by sterile filtration. In formulation procedure, aluminium phosphate gel is used in plasma-derived vaccine production whereas aluminium hydroxide powder is used in recombinant vaccine production.

Table 1 summarizes the specifications of quality control tests performed for plasma-derived and recombinant HB vaccines. During the in-process control of production, more biological tests such as determination of anti-HCV, anti-HIV, anti-syphilis, blood

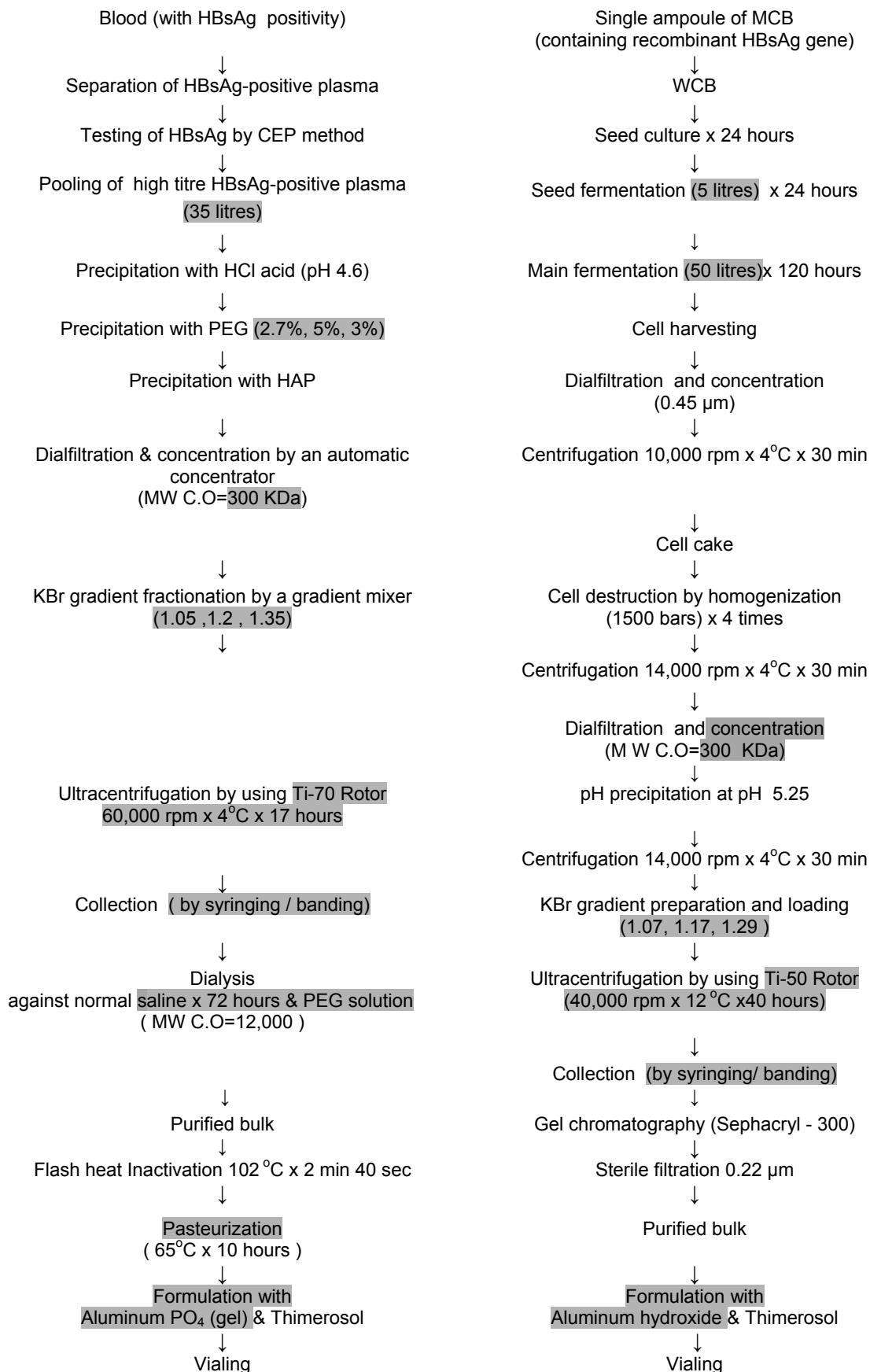


Fig. 1. Comparison of the flow diagrams of production processes between the plasma-derived and recombinant hepatitis B vaccines

Table 1. Comparison of quality control tests performed for purified bulk, final bulk and finished products between the plasma-derived and recombinant hepatitis B vaccines

Test items	Specifications	
	Plasma-derived vaccine	Recombinant vaccine
1. Characteristics	White turbid solution	White turbid solution
2. pH	5.9 - 6.9	5.9 - 6.9
3. Sterility	No evidence of bacteria, fungi and mycoplasma contamination	No evidence of bacteria, fungi and mycoplasma contamination
4. Adventitious viruses	No evidence of viral infection	*
5. Abnormal toxicity (General safety)	At least 80% of animal must survive	At least 80% of animal must survive
6. Protein content	Not more than 40 µg/ml	Not more than 40 µg/ml
7. Antigen content	Not less than 4 µg/ml	Not less than 20 µg/ml
8. Aluminum content	Not more than 1.25 mg/ml	Not more than 1.25 mg/ml
9. Thimerosal content	0.007 - 0.012 w/v %	0.007 - 0.012 w/v %
10. Bromide content	Not more than 5 µg/dose	Not more than 25 µg/100 µg of protein
11. Sodium chloride content	0.85 - 0.95%	*
12. Polysaccharide content	*	Not more than 10 µg/100 µg of protein
13. Lipid content	*	Not more than 100 µg/100 µg of protein
14. Tween	*	Not more than 50 µg/100 µg of protein
15. Antigen purity (SDS-PAGE)	Identified at about 24 KDa	Identified at about 24 KDa
16. Immunoblot	*	Identified at about 24 KDa
17. Pyrogenicity	The summed increase temperature is not more than 1.3°C	The summed increase temperature is not more than 1.3°C
18. Potency (antigenicity)	The lower value of 95% confidence limit is not less than 1	The lower value of 95% confidence limit is not less than 1
20. Specific activity test	*	More than 1x10 ⁷
21. Test for HBV-DNA detection	Not more than 1 pg/50 dose	*
22. Test for host-derived DNA detection	*	100 pg/dose
23. DNA polymerase	Not detected	*
24. Blood groups	Not detected	*
25. Test for completeness of antigen adsorption to adjuvant	Antigen is not less than 98% in sediment	Supernatant is negative for antigen
26. Filled volume	As specified	As specified
27. Leak test	No leak is detected	No leak is detected
28. Foreign matter	No foreign matter detected	No foreign matter detected
29. Test for glass vial	Colourless & transparent with no toxicity	Colourless & transparent with no toxicity
30. Rubber insert test (Fragmentation test)	*	No fragment of rubber detected

* indicates 'not done'

Table 2. Comparison of the starting volumes, final yields and sero-conversion rates between the plasma-derived and yeast-derived recombinant hepatitis B vaccines

Items of comparison	Plasma-derived (n=3)	Yeast-derived (n=2)
Starting volume per lot	35 liters (Plasma)	WCB 1 vial (single colony)
Total protein of purified bulk per lot*	250 mg	5 gm
Total antigen of purified bulk per lot *	225 mg	5 gm
Purity (SDS-PAGE)(range)*	95-97%	98-100%
Antigen content in finished product vial*	3 µg/ml	20 µg/ml
Total yields (pediatric doses/ lot)*	112,500	500,000
Time consumed per lot (average) *	4 weeks	4 weeks
Immunogenicity on clinical trial (% of sero-conversion)		
After 2nd vaccination	63.4%	86.57%
After 3rd vaccination	90.7%	100%
Anti-HBsAg titre (mean±SD) in vaccinees		
After 2nd vaccination	203.5±236.1 mIU/ml	129.8±175.5 mIU/ml
After 3rd vaccination	415±256 mIU/ml	610.7±177 mIU/ml

*average

group antigens need to be carried out in plasma-derived vaccine production whereas in recombinant HBsAg purified bulk, more chemical tests such as determination of polysaccharide, lipid, Tween were performed.

Comparisons of the starting materials, final yields and sero-conversion between plasma-derived and recombinant vaccines are depicted in Table 2. A total of 35 liters of high titres HBsAg-positive plasma are needed for a yield of 250 mg protein with purity of 95-97% HBsAg and vaccine doses of 100,000 per lot within a month. For recombinant vaccine production, a single ampoule of lyophilized yeast (MCB) containing HBsAg-expressed gene is required for 50 liters cultivation in a main fermentor and a total of 5 gm protein, about 100% purified HBsAg with a yield of 500,000 dosages per lot within a month. The anti-HBs sero-conversion rates with geometric mean titres were found to be 90.7% with 415 mIU/ml and 100% with 610.7 mIU/ml in newborns vaccinated with plasma-derived and recombinant HB vaccines, respectively.

DISCUSSION

Plasma-derived HB vaccine is prepared from surface antigen of human HB virus (HBsAg) i.e. purified 22 nm sub-viral particles or subunit derived from the surface antigen which is present abundantly in the plasma of HB virus carriers. This plasma contains surface antigen, Dane particles plus a complex mixture of proteins and other normal plasma components. The objective of vaccine preparation is to extract the HBsAg in its purified form, to free it from infectious viral components and to formulate it in a stable immunologically potent medium. The procedures used to ensure these properties include a complex mixture array of purification, inactivation, formulation and testing processes. This native HBsAg is encoded by envelope gene sequences; S plus pre-S, in the viral DNA [7].

Recombinant HB vaccine is produced by mass cultivation in a fermentor, of the recombinant yeast cells which contain the HBsAg protein-expression gene in their genomes, prepared by insertion of the gene-coding for HBsAg into yeast cell by using recombinant DNA technology. These yeast cells are then processed under concentration, dialfiltration, homogenization, purification, gelfiltration and sterile filtration to obtain purified HBsAg protein for further formulation process. Since there is no pre S sequence in the HBsAg-coding gene, recombinant vaccine contains only the S-gene-encoded peptide of the HB virion. However, the HBsAg particles produced in yeast cells are morphologically similar to those isolated from human plasma, the mean particle size of the former may be slightly smaller, and the protein is not glycosylated [7].

In our study, for production of one lot of the plasma-derived HB vaccine, 35 liters of high titre HBsAg-positive plasma were needed as a starting volume per lot and a total yield of 225 mg purified HBsAg with

about 100,000 vaccine dosages were obtained within a month. This amount of plasma can be obtained by a 6-month collection from various hospitals and blood banks in Yangon area. Therefore, only a total dose of maximum 200,000 vaccine doses can be produced within a year which can not fulfill the annual requirement of HB vaccine in our country. Although it has been proven that the two-step of inactivation, ie. 102°C for 2 min 40 second of Flash heat and 65°C for 10 hours pasteurization procedures, have shown to inactivate all major groups of pathogenic viruses, including HIV, some people are still afraid of contracting the other potential viruses.

For recombinant HB vaccine production, only a single ampoule of Master Cell Bank (MCB) i.e. transformed *Hansenula polymorpha* yeast cells containing the recombinant HBsAg protein-expression gene is required as a starting material and a total yield of 5 gm purified HBsAg with 500,000 vaccine dosages can be produced per lot within a month. Therefore, by using 10 vials of working cell banks (WCB), prepared from a single ampoules of MCB, 5 million dosages of HB vaccine can be produced within a year, thus capable of providing the annual requirement of HB vaccines in our country. In addition, recombinant vaccines, made of HBsAg particles similar to those found in the plasma of human carriers, contain only the S-gene-encoded peptide of the HB virion and thus, do not need the inactivation steps as required for plasma-derived vaccine.

Regarding the quality control testing in our study, the final bulk obtained from one lot of plasma-derived HB vaccine is 95-97% purity on SDS-PAGE. Since human blood and plasma may harbor infectious agents, including HIV which is the cause of acquired immunodeficiency syndrome (AIDS), particular attention must be given to selecting the donors of plasma, to the process of separation of the antigen and to inactivation procedures to ensure that any

potential infectious agents that may still be present after the purification of the antigen have been inactivated. Therefore, biological tests for the safety of plasma-derived vaccine consist of: the screening of transfusion transmitted infections in starting plasma, *in vitro* and *in vivo* assays for viral and microbial sterility (in suckling mouse, adult mouse, chicken embryo), rabbit pyrogen test, mouse immunogenicity test, HBV DNA and blood group substances determination. More biological tests were needed for plasma-derived vaccine testing and these biological tests are conducted at the individual plasma unit, starting plasma pool, purified inactivated bulk antigen and final products.

For recombinant HB vaccine production, the purified bulk obtained is 98-100% purity on SDS PAGE. Since more biologically active extraneous components such as DNA and endogenous proteins from the host-cell system may be found in the final product and unwanted gene products may also be co-expressed unexpectedly with the HBsAg, several chemical tests for determination of polysaccharide, lipid, Tween and yeast-derived DNA were required for the quality control testing of recombinant vaccine.

On study of immune response of both vaccines, results of the clinical trial conducted on neonates revealed that after completion of 2nd and 3rd doses of vaccination, 63.4% and 90.7% of neonates vaccinated with plasma-derived vaccine showed sero-conversion with geometric mean titre of 205.5 mIU/ml and 415 mIU/ml, respectively, whereas 86.57% and 100% of vaccinees showed sero-conversion with anti-HBs geometric mean titres of 129.8 mIU and 610.7 mIU/ml, respectively, in recombinant vaccine group. The obvious differences were noted in both sero-conversion time as well as rates and anti-HBs mean titres showing the apparent superiority of recombinant vaccine. The finding is in accordance with those of WHO in 1987 [7]. This could be an important advantage in certain high-risk settings. The yeast-derived

recombinant vaccine is immunologically similar to the plasma-derived vaccines both *in vitro* and *in vivo* and have similar protective efficacy [8].

Based on the above findings, although plasma-derived vaccines have shown to be safe and effective, the supply of vaccine is potentially limited by available sources of HBsAg-positive plasma. In addition, extensive processing and safety testing are necessary to ensure production of a pure and safe vaccine that is free of any extraneous living agents that might have originally been present in the plasma. Since the yield of production capacity per lot is 10 times lower than that of the recombinant vaccine, the cost for one vial of end product is relatively expensive. These difficulties associated with their preparation as well as their acceptance by the public have prompted the development of a vaccine, based on recombinant DNA technology in Myanmar since a decade ago.

Furthermore, the source of the vaccine produced in yeast is constant and well-characterized and the vaccine may be produced in unlimited quantities. A recombinant HB vaccine is not only antigenically similar to the plasma-derived vaccine but also elicits a similar immune response in human. Therefore, it is suggested that recombinant HB vaccine production, which has an unlimited availability of starting material WCB/MCB and an annual production capacity of 5 million doses at the Vaccine Plant, is the appropriate and acceptable method for large scale production of HB vaccine to fulfill the

annual requirement of our country in the future.

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Safety and immunogenicity of live Japanese encephalitis vaccine SA14-14-2

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Live Japanese encephalitis vaccine SA14-14-2 is currently used worldwide as a safe and immunogenic vaccine but it has never been introduced into Myanmar before. This study was to conduct the safety and immunogenicity of SA14-14-2 in Myanmar people. It was manufactured by Chengdu Institute of Biological Products, China. The *in vitro* sterility test was done to detect the presence of microorganisms in the vaccine. Pyrogen test and abnormal toxicity test were performed in adult rabbits and laboratory mice, respectively. All the results were within normal range after the incubation period. Twenty-one healthy adult volunteers were recruited from Department of Medical Research (Lower Myanmar) and they were checked for medical fitness (physical examination and laboratory tests) before and three days after vaccination. After vaccination, all the volunteers were checked for immediate reaction and side effects of vaccination for 5 days. There were no significant changes regarding medical fitness before and after vaccination, but one volunteer complained of mild pain at the injection site. Booster dose was given ninety days after the primary dose. Five volunteers complained of febrile reaction on the next day. One month after the booster dose, sera were collected from 19 vaccinees and tested for the presence of neutralizing antibodies. All the vaccinees had antibody titers ranging from 38 to 1321. The protective antibody titer for Japanese encephalitis disease is more than 10. In conclusion, live-attenuated JE vaccine (SA14-14-2) can be regarded as a safe and immunogenic vaccine.

INTRODUCTION

Japanese encephalitis virus (JEV) is the most important cause of epidemic encephalitis worldwide, with an estimated 35,000 to 50,000 cases and 10,000 deaths annually. The virus is a member of the JE serogroup of the genus *Flavivirus*, family *Flaviviridae*, and is transmitted between vertebrate hosts by mosquitoes, principally by *Culex tritaeniorhynchus* [1]. JE virus is transmitted in an enzootic cycle involved in birds. Pigs can become infected and act as amplifying host bringing the virus close to human especially in parts of Asia where pigs are kept near homes. [2]. There are about

27 countries worldwide with epidemic or endemic JEV, and Myanmar is included in the list [3]. Most of the countries have data on prevalence rate or case fatality rate due to JEV. The case fatality rate of JE virus infections is approximately 25%, with 50% of survivors developing permanent neurological and psychiatric sequelae [4].

In Myanmar, a study on vector, amplifier, and human infection with JEV in a Rangoon community was done in 1982. In that study, JEV infection was detected in 52.1% of the pigs. The known JEV vector mosquito species were found out in the study area but no concurrent human JEV infections were elicited [5].

In another study from 2003 to 2006, among 161 sera samples from patients presenting with symptoms of encephalitis from the Yangon Children's Hospital, 11% were positive for JE IgM antibodies. However, there is no currently known hospital-based laboratory data from adults presenting with symptoms of encephalitis [6].

Since the virus and its vectors and host are ubiquitous in rural Asia, most of the population is exposed during childhood as shown by serological studies though the disease develops in only a small proportion of infected persons. Various measures have been used to try to control the spread of the virus. Still the best hope of controlling Japanese encephalitis lies in vaccination [2]. A killed mouse brain-derived JE vaccine with 91% efficacy (95% confidence interval [CI], 70%-97%) that is manufactured by Biken (Osaka, Japan) is available internationally, although quantities are insufficient to meet the need worldwide. The price is about US\$ 5/dose, with a three-dose primary vaccination recommended and yearly boosters administered in some countries. Thus, the expense and additional rare occurrence of hypersensitivity and neurological reaction hinder the immunization efforts.

In 1998, the Chinese licensed an inexpensive (US\$ 0.75/dose) live-attenuated primary hamster kidney-derived JE vaccine (SA 14-14-2). Prior to licensure, trials conducted in highly endemic area indicated 95% efficacy after a single dose. The short-term safety of a live-attenuated JE vaccine (SA 14-14-2) was done among children in China, 1997. In that study, fever was the most common adverse event (4.9%) that was followed by irritability (3.7%), cough (3.45%) and rash (2.2%) [7].

It can be assumed that JE outbreaks can happen anytime in Myanmar as the *Culex* spp. which can carry JEV and amplifying hosts such as pigs are of abundance. Therefore, it is better to immunize the high-risk persons such as workers from pig farms,

farmers, residents from border areas and medical researchers dealing with JE virus and mosquitoes (entomologists). This study was to check the safety and immunogenicity of live JE vaccine (SA14-14-2) in Myanmar people which will be commercially available in Myanmar soon.

MATERIALS AND METHODS

In vitro sterility and *in vivo* safety tests

The Myanmar FDA (Food and Drugs Administration) had done *in vitro* sterility test by using bacterial culture medium to detect the presence of any bacteria or fungi in the vaccine prepared.

In vivo laboratory safety tests include pyrogen test and abnormal toxicity test. For pyrogen test, healthy adult rabbits of either sex, weight not less than 1.5 kg showing no loss of body weight during the preceding test were selected and initial rectal temperature of each rabbit was recorded. The vaccine was diluted with 0.5ml sterile water and injected slowly into the marginal vein of the ear of each rabbit over a period not exceeding 4 minutes. Then, the rectal temperature was measured hourly for three consecutive hours.

Abnormal toxicity test in laboratory mice was performed as follow. Seven mice of five weeks old, 17~22 gm, having no signs of disease and a normal increase in body weight during a quarantine period for at least 5 days were used, and were weighed before the test. Two mice were selected as control and five were injected intraperitoneally with 0.5 ml of vaccine. Then, all mice were weighed daily for 7 consecutive days.

Study volunteers

The protocol was reviewed and approved for implementation by the Institutional Ethical Committee of the Department of Medical Research (LM). Exclusion criteria for screening potential volunteers included acute febrile illness, malignancy,

pregnancy, allergy to vaccine components, chronic cardiac, hepatic, renal, or immunologic diseases, immunosuppressive therapy, and history of receiving JE vaccine. Laboratory investigations were ECG, CP, ESR, Urea, Creatinine, AST, ALT and Urine RE, and participants were checked two times (5 days before and 3 days after vaccination). Twenty-one healthy adult volunteers (age range between 24 and 57 years of 13 men and 8 women) were recruited from three divisions, Virology Research Division, Immunology Research Division and Medical Entomology Research Division of Department of Medical Research (Lower Myanmar).

Vaccine/ Vaccination schedule

Live-attenuated primary hamster kidney-derived Japanese encephalitis vaccine (SA14-14-2), manufactured by Chengdu Institute of Biological Products, China, brand name – CD JEVAX.

Single-dose vials of lyophilized vaccine were stored at 2-8°C. Each dose was reconstituted immediately before administration with 0.5 ml of sterile water solvent supplied by the manufacturer. Vaccine was given subcutaneously in the deltoid skin-fold. Recipients were observed for 20 min after each injection. Primary dose of JE live vaccine was given on Day 0 and booster dose was on Day 90.

Symptoms/ Adverse reactions

Participants were specifically questioned about local reactions (soreness or redness at the site of injection) and systemic reactions (fever, headache, and rash). Participants reporting symptoms were asked to estimate the severity and duration in hours of each. A symptom was defined as mild if it was barely noticeable, moderate if it was present but did not interfere with work, and severe if it interfered with work. Participants were given a record card after each vaccination so that symptoms following each dose of vaccine were noted down for five consecutive days. Record

cards were collected after 5 days of each vaccination.

Record card on side effects of JE vaccine

Name Age... Sex - M/F Division.....
Date of vaccination.....
Type of vaccination - primary/ booster

	D1	D2	D3	D4	D5	Remarks
Systemic reactions						
Fever (°C)						
Cough						
Irritability						
Headache						
Nausea						
Vomiting						
Diarrhoea						
Axillary lymph node tenderness						
Others						
Local reactions	D1	D2	D3	D4	D5	
Pain at the site of injection						
Tenderness						
Swelling						
Warmth						
Erythema						
Size of erythema						
Others						

Immunogenicity test/ Neutralizing antibody test (PRNT test)

Separated sera were kept under -20°C before testing. The assay was performed according to the method described by Russell *et al.* [8]. Plaque count was determined by using LLC-MK2 plaque assay single overlay technique. Briefly, sera were thawed, diluted, and heat-inactivated by incubation at 56°C for 30 min. Serial ten-fold dilutions of serum were made (1:10, 1:100 and 1:1,000). JE virus, diluted to contain about 40-50 PFU/well, was added to each serum dilution tube. Following incubation at 37°C for 60 min, 0.2 ml was removed from each tube and inoculated onto triplicate 6-well plates of confluent LLC-MK2. Each plate was incubated at 37°C for 90 min and mono-layers were then overlaid with 4 ml of 3.5% carboxy methyl

cellulose/ MEM. Plates were incubated for 7 days at 37°C with 5% CO₂. Plaques were counted and PRNT₅₀ were determined by using SPSS program. The neutralization end-point in the protocol was a 50% reduction in plaque formation. Antibody titer above 1:10 was considered positive and protective [9]. PRNT test was done at the Center for Vaccine Development, Mahidol University, Salaya, Thailand.

RESULTS

In vitro sterility test and in vivo safety tests (pyrogen and abnormal toxicity)

In vitro sterility test showed no growth of bacteria or fungus after an incubation period. For pyrogen test, after 3 hours of observation period, the total increased temperature of three rabbits was not exceeded 1.3°C (that is 1.0°C) and so the pyrogen test was passed (Table 1).

Table 1. Pyrogen test results in three rabbits

Rabbit no.	Weight (kg)	Rectal temp (°C)	Rectal temp. after injection (°C)			Increased temp (°C)
		Just before	1 hr	2 hr	3 hr	
1	1.5	39	39.5	39.5	39.5	0.5
2	2.0	39	39.5	39.5	39.5	0.5
3	1.5	40	40.0	40.0	40.0	0
Total increase in temperature of 3 rabbits						1.0

For abnormal toxicity test, laboratory mice were weighed daily for 7 consecutive days. After the observation period of seven days there were no abnormal signs and normal increase in body weight so that the test was within acceptable criteria (Table 2).

Safety and immunogenicity in human volunteers

After the *in vitro* and *in vivo* safety tests had been done, volunteers were screened for eligibility and only two of the volunteers had ischemic pattern in ECG results, but others had no abnormal findings in laboratory results. So, those volunteers were given primary dose of JE live vaccine

Table 2. Weight recorded during seven-day observation period of two control and five test mice.

Item	Mouse no.	Weights (gm)						
		Pre-test	D1	D2	D3-4	D5	D6	D7
Test	1	20	20	20	Holiday	20	20	21
	2	17	17	16	Holiday	17	18	19
	3	19	19	18	Holiday	19	19	19
	4	17	17	17	Holiday	18	19	20
	5	18	18	17	Holiday	18	19	20
Control	1	22	22	22	Holiday	21	23	22
	2	20	20	20	Holiday	20	22	22

D=Day

subcutaneously over deltoid muscle of left or right arm on Day 0. One female volunteer complained of moderate pain at the injection site, immediately after the injection, but it was spontaneously relieved within fifteen minutes, while others gave no complaints. On the same day, volunteers were given a medical record card to note down the local and systemic reactions up to five days. Three days later after the primary dose, the volunteers were checked again for laboratory investigations. There were no significant changes in laboratory results when comparing the data before and after the vaccination. One volunteer complained of scaling off skin on Day11, but after investigation that reaction was due to soap allergy. Booster dose was given on Day 90 and the medical record cards were also given, same as primary vaccination. On reviewing the record cards, five volunteers complained of mild febrile reaction on the next day but there was no complaint from others during the following 5 days (Table 3).

Table 3. Adverse events observed in 21 vaccinated volunteers at day1, 2, 3, 4 & 5 after JE vaccination

Vaccination	Event	No. of subjects experiencing event
Primary	Injection site pain	1/21
Booster	Febrile reaction	5/21

One month after the booster dose, blood was drawn from the nineteen volunteers (two

Table 4. JE PRNT titers of vaccinees 1 month after the booster dose

Sr. no.	Code	Reciprocal JE PRNT
1	VJE 001	132
2	VJE 002	386
3	VJE 003	526
4	VJE 004	190
5	VJE 005	863
6	VJE 006	49
7	VJE 007	1321*
8	VJE 008	611
9	VJE 009	162
10	VJE 010	105
11	VJE 011	268
12	VJE 012	38*
13	VJE 013	403
14	VJE 014	554
15	VJE 015	225
16	VJE 016	118
17	VJE 017	190
18	VJE 018	299
19	VJE 019	288

*The highest and lowest antibody titers of vaccinees

were on field survey) and then plaque reduction and neutralization test (PRNT) was done to check the immunogenicity. After the test, all the vaccinees had neutralizing antibody titers ranging from 38 to 1321 (Table 4).

DISCUSSION

Discovered 125 years ago, JE has spread widely in the 20th century. Almost half of the human population now lives in countries where the disease is endemic. JE is a vector-borne epidemic with several features that are typical of an emerging infectious disease. The failure to halt the spread of JE in Asia and Pacific region, despite the availability of an effective and inexpensive vaccine for 40 years, is of considerable public health concern.

High quality data on transmission and incidence of JE are lacking in various countries. Although clinical and serologic methods to diagnose and monitor JE are available, health systems in many developing countries are unable to differentiate encephalitis diagnoses. Information regarding the distribution and public health

importance of JE in Bangladesh, Cambodia, Indonesia, North Korea, Laos, Myanmar, Papua New Guinea, and Pakistan is inadequate. Examples of countries with successful JE control programs are Japan and South Korea. The following key control strategies and developments might explain the successful decline in these countries:

- 1) large-scale immunization programs for humans,
- 2) pig immunization and the separation of pig rearing from human settlements,
- 3) changes in practices (e.g., enhanced mechanization and decrease of irrigated land), and
- 4) improved living standards (e.g., better housing and urbanization) [10].

For Myanmar being a developing country, application of the first key control strategy (i.e. large-scale immunization programs for humans) might be the best to control JE. Studies already highlighted the safety and immunogenicity of live-attenuated SA14-14-2 JE vaccine, and low vaccine cost favors availability of the vaccine in developing world. In our study, the sterility test and animal safety tests were within the acceptance criteria. Medical check-up results before and after first dose of vaccination showed no significant changes. In addition to the side effects from record cards, there were no complaints up to a year after the first dose.

The most common adverse events in our study were: fever 5/21 and injection site tenderness 1/21, which were higher events than those in China study of 13/266 and 1/266, respectively [7]. That higher incidence may be due to small sample size. Although fever was common (5/21) in this study, it was mild. Advisory Committee on Immunization Practices (ACIP) recommended that the protective antibody (neutralizing antibody) titer for JE is 10 [9]. In our study, all the vaccinees had neutralizing antibody titers ranging from

38 to 1321 one month after the booster dose so that immunogenicity of SA14-14-2 is also acceptable.

In conclusion, the results indicated that the vaccine is not associated with serious adverse events in 5 days following vaccination and is immunogenic. This provides reassuring evidence of the safety and immunogenicity of an affordable vaccine with known effectiveness.

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Study of serum leptin level in obese subjects

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The aim of the present study was to determine the serum leptin level in obese Myanmar subjects. In this study, middle-aged obese subjects were recruited from North Okkalapa Township. Anthropometric parameters and serum leptin level of 66 obese men and women were determined. BMI was 28.3 ± 3.46 kg/m² for obese men (n=26) and 28.97 ± 4.38 kg/m² for obese women (n=40). Obese subjects were selected according to WHO (2000) classification of BMI and waist circumference in adult Asians. Serum leptin concentration was determined by radioimmunoassay at Nuclear Medicine Research Division, Department of Medical Research (LM). It was found that weight, waist circumference and waist-hip ratio were significantly higher in obese men than in obese women ($p < 0.01$). However, total fat percent and serum leptin concentration were significantly higher in obese women than in obese men ($p < 0.05$); $38.55 \pm 5.27\%$ vs. $27.19 \pm 4.3\%$ for total fat percent and 26.78 ± 21.06 ng/ml vs. 14.97 ± 13.4 ng/ml for serum leptin concentration. In those with BMI exceeding 30, serum leptin concentration was higher in women than in men (43.41 ± 22.1 ng/ml vs. 23.64 ± 12.7 ng/ml, $p < 0.05$). It was concluded that serum leptin was higher in obese women than in men. Sex difference in serum leptin was also found when BMI was ≥ 30 . The study highlights that serum leptin level varies with sex and BMI.

INTRODUCTION

Obesity, in simple terms, may be defined as a state of imbalance between calories ingested versus calories expended which would lead to excessive or abnormal fat accumulation [1]. The operational definitions of obesity and overweight are based on body mass index (BMI) which is closely correlated with body fatness and BMI has been the most accepted parameter for defining overweight [2]. Leptin is a product of the *ob* gene and its level increases exponentially with increasing fat mass [3]; leptin production is higher in subcutaneous than in visceral fat depots [3-6].

Adiposity and gender are the major determinants of leptin concentration. Some investigators reported that this sex difference is not related to sex hormones or fat distribution, but possibly to differences in hypothalamic regulation of leptin

production or in adipose tissue biological characteristics. Leptin's sexual dimorphism suggests that women may be resistant to its lipostatic actions and that it may have a reproductive function [7]. Besides its role in the regulation of energy balance, leptin seems to be involved in linking energy stores to the reproductive system. A gender-dependent difference exists in plasma leptin concentration and leptin messenger ribonucleic acid expression in rodents and humans [8]. Therefore, we evaluated the gender difference of serum leptin concentration on body mass index (BMI) among adult obese men and women.

MATERIALS AND METHODS

Subjects

Obese subjects were recruited from North Okkalapa Township. Obese subjects were selected according to WHO (2000) classification of BMI and waist circum-

ference in adult Asians. Out of 60 participants, 40 were women and 26 were men.

Anthropometry

Weight and height of the subjects were measured. The subjects were in light clothes without any footwear and body weight was taken to the nearest 0.5 kg using a bathroom scale. Standing height was measured using stadiometer to the nearest 0.5 cm. Body mass index (BMI) was calculated by dividing body weight in kilograms by the height in meter square [9].

The waist circumference was measured at the specific level half way between the superior iliac crest and lower border of rib cage at the mid-axillary line. Hip circumference was measured as the maximum hip circumference to the nearest centimetre. Waist-hip ratio (WHR) was calculated by waist circumference divided by hip circumference in centimetre [10]. Anthropometric measures reflect both total adiposity as well as abdominal fatness [11]. The body fat percentage was calculated using the following equations [12].

$$\text{Body fat \%} = 1.2 (\text{body mass index}) + 0.23 (\text{age}) - 10.8 (\text{gender}) - 5.4$$

Gender = 1 for men

Gender = 0 for women

Normal range

Adult men = 15-20%

Adult women = 25-30%

Procedures

A written informed consent was obtained from all subjects before the study. History taking, physical examination and anthropometric measurements were done on Day 1. After that, subjects were instructed to fast overnight (from 8:00 pm to 8:00 am) and come at 7:30 am the next day (Day 2) to the Laboratory of Physiology Department, University of Medicine (2). Then, about 3cc of fasting blood sample were collected from each subject at 8:00 am.

Blood analysis

Blood samples were transported to Nuclear

Medicine Research Division, Department of Medical Research (Lower Myanmar) where blood was separated into serum, and then stored at -20°C. Then, serum leptin was determined using radioimmunoassay (RIA).

Statistical analysis

Data were analyzed by using the computer based statistical package of statistical product and service solution (SPSS) version 11.5. Results were reported as mean±SD. Correlation between serum leptin and anthropometric parameters was done by Pearson correlation. Differences of leptin in relation to sex and onset of obesity were calculated by independent sample 't' test.

RESULTS

Mean values of all parameters are shown in Table 1. Anthropometric values of obese men were significantly higher than those of obese women except hip circumference (Table 1).

Table 1. Comparison of study population between men and women

	Men (n=26)	Women (n=40)
Age (year)	40.96±7.23	39.95±6.32
Weight (kg)	81.42±14.61 **	70.23±10.46
Body mass index (kg/m ²)	28.3±3.46	28.97±4.38
Waist circumference (cm)	101.88±10.38 **	89.23±7.06
Hip circumference (cm)	101.3±9.02	105.58±9.44
Waist-hip ratio	1±0.04 ***	0.84±0.05

**=p<0.01

***= p<0.001

Significant correlations were found between serum leptin concentration and body fat distribution (r=0.5, p<0.01) except waist-hip ratio in both obese men and women (Figure not shown). Serum leptin concentration was significantly higher in obese women than in obese men (26.78±21.1 ng/ml vs. 14.97±13.4 ng/ml; p<0.05). Mean total fat percent of obese women (38.55±5.2) was significantly higher than that of obese men (27.19±4.3) (p<0.001) (Figure 1).

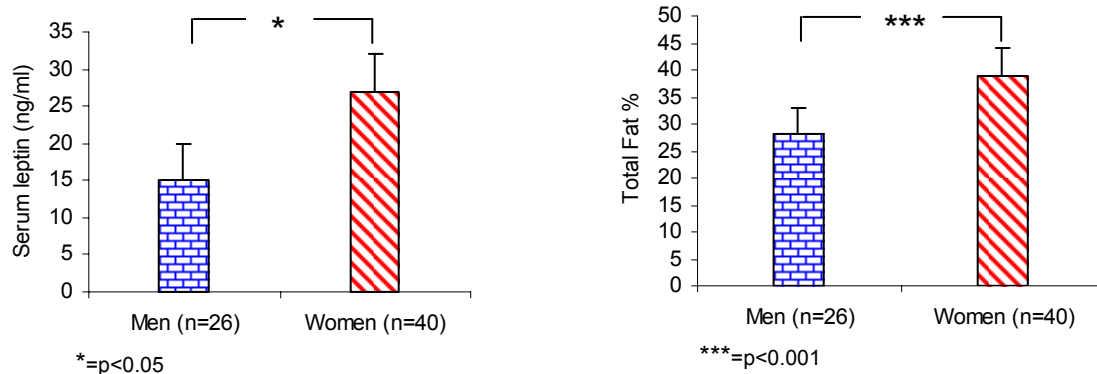


Fig.1 (A & B). Serum leptin level and total fat of obese men and women

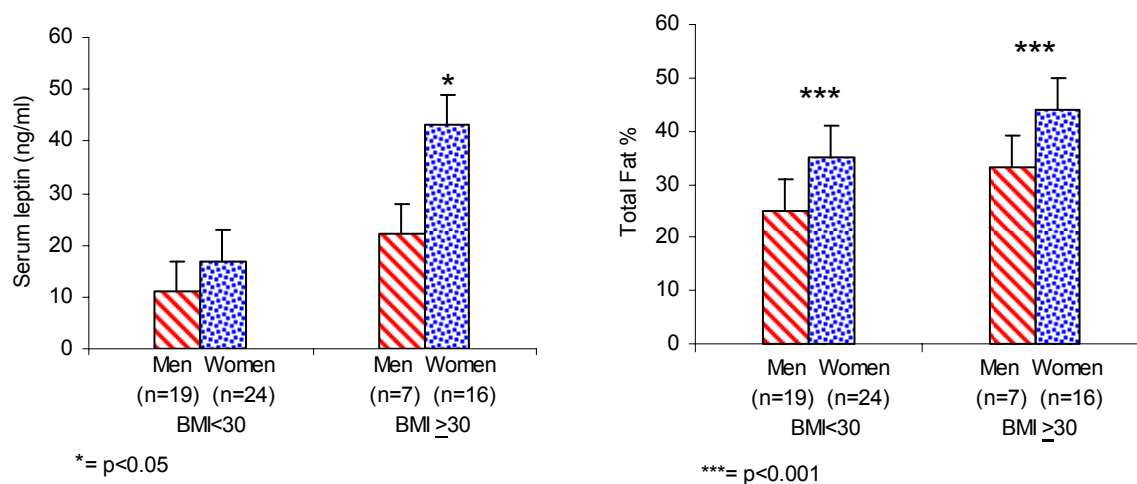


Fig. 2. (A & B). Serum leptin level and total fat percent of obese men and women with BMI <30 and ≥30

In the BMI ≥30 group, serum leptin concentration was significantly higher in women than in men (43.41 ± 22.1 ng/ml vs. 23.64 ± 12.7 ng/ml; $p < 0.05$) whereas in the BMI <30 group, there was no difference in serum leptin concentration between men and women. However, total fat percent of both sexes was significantly different between men and women whose BMIs were either <30 or ≥30 ($p < 0.001$) (Fig. 2).

DISCUSSION

In Asians, the cut-offs for overweight (≥ 23 kg/m²) and obesity (≥ 25 kg/m²) were lower than the WHO criteria [13]. In the present study, obesity assessment was performed according to classification of

above WHO criteria. Although body fat distributions were higher in men than in women, serum leptin and total fat percent were higher in women than in men. That was more pronounced when BMI was greater than 30. Significantly higher serum leptin level in obese women could be due to significantly higher total fat percent which reflects total fat mass in women because BMI was not different between two groups. It seems that total fat percent might be a better indicator of serum leptin concentration. The finding of present study was in agreement with that of a study in which serum leptin concentrations were higher in obese women than in obese men before and during weight loss, and the topography of fat tissue influences serum

leptin concentrations. Base-line finding of above study was 35.7 ± 14.4 ng/l vs. 19.8 ± 6.9 ng/l and the present finding was 26.78 ± 21.1 ng/ml vs. 14.97 ± 13.4 ng/ml [14]. Other researchers studied the sex difference of serum leptin level at puberty age. Pubertal girls had two to three folds greater serum leptin concentrations than pubertal boys after correction for adiposity [15-17].

The lower serum leptin concentration in males may also be due to an androgen induced reduction in leptin production [18]. One study reported that serum leptin levels were positively correlated with subcutaneous adiposity but negatively correlated with androgen levels [19]. It could be suggested that factors other than sex steroids such as body fat patterning, energy expenditure and aerobic fitness may also influence serum leptin concentrations [19].

In the present study, mean value of serum leptin level was significantly higher in obese women than in obese men, particularly when BMI is equal to or exceeds 30. That finding might suggest that association between increase in BMI and body fat mass is greater when BMI exceeds 30.

The association at the cut-off point seems to be strong because the number of obese persons with BMI ≥ 30 was less than that with BMI < 30 , and yet serum leptin was significantly correlated with body fat distribution including total fat percent in both obese men and women. The above findings might suggest that serum leptin was absolutely dependent on total fat percent.

Many observations suggested that two and possibly three critical periods existed for the development of obesity and its complications. Those included the period of gestation, early infancy and adolescence. Obesity that began at those periods appeared to increase the risk of persistent obesity and its complications. The mechanisms that accounted for an increase in obesity associated risk at these ages remained unclear [20].

There was also a report on sex predisposition to serum leptin in which high leptin levels were found to be associated with excessive future weight gain only in girls. Plasma leptin levels were significantly higher in obese girls compared with obese adolescent boys. Multiple linear regression analysis revealed that high basal leptin levels were positively associated with greater changes in BMI Z-score only in girls [21].

It was concluded that serum leptin was higher in obese women than in men. Sex difference in serum leptin was also found when BMI was ≥ 30 . Therefore, it could be interpreted as serum leptin level is influenced by both sex difference and body fat mass since BMI also represents the total fat mass and fat percent.

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Patients' perspectives on choosing either public or private sector for malaria treatment in Upper Myanmar

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In spite of vigorous activities of Vector Born Disease Control Program making available rapid diagnostic tests and anti-malarial drugs down to community level free-of-charge, a considerable number of patients are purchasing anti-malarial treatments at private sector. Thus, this study identified the determinants of choosing either public or private sector for malaria treatment from the consumer side. A community-based cross-sectional, analytical study was conducted at Moegoke, Kyaukse and Myitthar townships of Mandalay Division. A total of 200 uncomplicated malaria patients, 100 each from public and private sector, were interviewed within one-year study period from September 2008 to September 2009. In the final multivariate model, significant factors influencing the choice of public sector included: poor socio-economic status [adjusted odds ratio (AOR)=5.0], residing close to health center (AOR=14.0), treatment cost ≤1000 kyats (AOR=24.5), sympathy and humanity of provider (AOR=6.2 and 3.9), acceptable waiting time (AOR=3.0), domiciliary treatment service (AOR=20.4) and knowledge on availability of free-of-charge treatment at public sector (AOR=4.1).

INTRODUCTION

Early diagnosis and prompt adequate treatment is one of the main strategies in malaria control. Provision of early diagnosis and prompt treatment to uncomplicated malaria cases will reduce duration of illness and severity of disease preventing progression to severe malaria and death [1]. Malaria treatment in public sector was much improved by giving training on new antimalarial treatment guidelines to basic health staff (BHS), as well as supplying rapid diagnostic test kits (RDT) down to sub-center (SC) levels. Public sector is providing antimalarial treatment free-of-charge at the community level. However, a considerable number of patients are purchasing anti-malarial treatments at private sector especially from general practitioners (GPs). This study attempted to find out factors to improve

the public sector according to expectations from the consumer side.

MATERIALS AND METHODS

A community-based cross-sectional, analytical study was conducted using quantitative and qualitative approaches at malaria endemic areas of Moegoke, Kyaukse and Myitthar townships in Mandalay Division from September 2008 to September 2009. Patients taking anti-malarial treatment in either public or private healthcare facility at the time of survey or (one week before the survey) were included in this study.

Public health facility: The list of rural health centers (RHCs) in the study townships was prepared as a sampling frame. For 3 townships, 12 RHCs were situated in the study area. Among them, we selected 5 RHCs randomly. Each RHC

has 4 SCs on average. Therefore, there was a total of 20 SCs included in this study.

Private health facility: The list of registered GPs in the study townships was prepared as a sampling frame. From approximately 60 GP clinics, across 3 study townships, a total of 20 GP clinics were selected randomly.

Among randomly selected 20 SCs and 20 GP clinics, at least 5 malaria patients per facility were interviewed for this study. Therefore, a total of 200 uncomplicated malaria patients, 100 each from public and private sector, participated within one-year study period. Face-to-face interviews with malaria patients were conducted using pre-tested, structured closed-ended questionnaires.

For each RHC, in-depth interviews (IDIs) using pre-tested guidelines were done with one male and one female patient who could converse fluently. Therefore, 10 IDI sessions: 5 male and 5 female patients were conducted at SCs. For each township, IDIs were conducted with two male and two female patients taking treatment at GP who could converse fluently. Therefore, 12 IDI sessions: 6 male and 6 female patients were conducted at GP clinics.

Quantitative data were entered into computer using Epi-data version 3.02 software. Double data entry was done to check completeness and accuracy. Any discrepancy was corrected by reviewing the raw data. Data analysis was performed using R statistical software. The significant level was set at p value <0.05. For qualitative data, IDIs were tape-recorded, transcribed and analyzed across themes and sub-themes for triangulation with quantitative data.

Ethical considerations

This study was approved by the Institutional Ethical Review Committee of Department of Medical Research (Upper Myanmar) and written informed consent was taken from each and every participant.

RESULTS

Social and demographic characteristics of malaria patients

The significant differences in gender, education of patients, decision maker to choose health facility, occupation, personal possession and family income of patients were found between public and private sectors. Firstly, average family income per month of all patients was computed and categorized into dichotomous variables based on average to minimize the over influence of this variable. Next, socio-economic status was determined using socio-economic index derived from scores adding up occupation, personal possession, type of housing and categorized income variables. Finally, socio-economic status was categorized into dichotomous variables including poor and rich using the average score as cut-off point. The patients with low socio-economic status were more likely to use the public sector than private sector.

Environmental factors

The mean distance between residence and health sector was 3.1 ± 1.2 kilometers (km) which ranged from 1 to 6 km. The average distance between residence and health sector was 3.5 ± 1.3 km (range: 1 to 6 km) in private users and it was 2.6 ± 0.9 km (range: 1 to 5 km) in public users ($p=0.001$). In public sector, 46% received domiciliary care and the rest came to health sector on bicycle (25%), by motorized vehicles (22%), and on foot (7%). Among private sector users, only 9% received domiciliary care and the rest came to health sector by motorized vehicles (74%), on bicycle (12%), and on foot (5%). The modes of travel to reach health sector were different in malaria patients using public and private sectors ($p=0.0005$).

Social factors

The proportion of patients who reported that healthcare provider was polite was (65% vs. 49%), sympathetic was (77% vs. 60%), humane was (51% vs. 32%) and good in social dealing was (78% vs. 63%) in

public and private sectors, respectively ($p<0.005$). All of the healthcare providers in public sector were midwives (MWs), while most of the GP doctors (73%) were males. Majority of male patients (62%) were not reluctant to seek treatment at MWs while majority of female patients (66.7%) went to male GP doctors for malaria treatment ($p<0.001$).

IDI results for social factors revealed:

“Sayarma is very polite in taking care of patients. Moreover, I believe in Sayarma that she can treat malaria.”

(a 27-year-old male at SC)

“Sayar (GP doctor) is kind-hearted. However, he seems to be angry with patients who do not bring the treatment record book. But, it is patients’ fault.”

(a 32-year-old female patient at GP)

Service factors

The majority of MWs (40%) were available at their SCs at any time. On the other hand, 32% of the MWs were available only at day time and 28% were available during office hours only. In the private sector, majority of GP doctors (45%) gave service during clinic opening hours. Approximately one-third (33%) were available during day time and 22% of them gave 24-hour service.

The waiting time of less than one hour to receive treatment in public and private sectors was 55% vs. 36%, 1 to 2 hours (26% vs. 33%) and more than 2 hours (19% vs. 31%), respectively ($p=0.022$). Domiciliary service was provided by 46% of MWs and 9% of GP doctors ($p<0.000$). Most of the patients were diagnosed as malaria by RDT (51.5%), followed by microscopically (30%) and clinical background (18.5%). Methods of diagnosis were similar in two health sectors ($p=0.08$).

Cost

The average traveling cost was 500 ± 277 kyats in public sector users (range: 200 to 1,500 kyats) and it was $1,494\pm537$

kyats in private sector users (range: 500 to 3,500 kyats). There was a significant difference in traveling costs in two sectors ($p=0.036$). Among patients in public sector, 36% took anti-malarial treatment at BHS free-of-charge and the remaining had to pay for ancillary treatments. The average treatment cost among public users was $1,546\pm602$ kyats (range: 500 to 2,500) and it was $4,330\pm2,774$ kyats in private users (range: 1,000 to 10,000; $p=0.000$).

Among all patients, majority (71.5%) had to pay extra-cost during treatment. The patients who had to pay extra-cost were higher in private sector than public sector (78% vs. 65%; $p=0.042$). Among the patients in private and public sectors who paid extra-cost, the cost included for registration (28.2% vs. 35.4%), investigations (28.2% vs. 32.3%), for record-book (19.2% vs. 24.6%) and for other costs such as transport fees to reach diagnostic center, incentives for technicians etc (24.4% vs. 7.7%), respectively ($p=0.068$).

IDI results for cost revealed:

“She (MW) said malaria drugs are free-of-charge because I got them from the program. However, you need to pay for other fever releasing drugs because I bought those drugs. anyway, it is OK. I can afford to give 500 kyats.”

(a 34-year-old female at SC)

“I can afford the treatment cost. I prefer good drugs. I know, good drugs are expensive (ဆေးကောင်းလို့ ဈေးကောင်းတာ).”

(a 28-year-old male at GP)”

Knowledge on availability of free anti-malarial treatment at BHS

Among all patients, only 30.0% knew that public sector was giving anti-malarial treatment free-of-charge. The public users had more knowledge on availability of free anti-malarial treatment at BHS than private users (38% vs. 22%; $p=0.014$).

Table 1. Factors influencing choice of health sector

Factors	Public sector	Private sector	Crude OR 95%CI	p value	Adjusted OR 95%CI	p value
Social and demographic characteristics of patients						
Age (years)						
>35	38	43	1	0.471	NA	-
≤35	62	57	0.8 0.5-1.4			
Gender						
Female	27	15	1	0.037	NA	-
Male	73	85	2.1 1.0-4.2			
Marital status						
Single	24	15	1	0.108	NA	-
Married	76	85	1.8 0.9- 3.7			
Education						
High	61	37	1	0.001	1	0.559
Low	39	63	2.6 1.4-4.9		1.2 0.2-10	
Decision-maker to choose health sector						
Family	61	27	1	0.000	1	0.18
Husband	39	73	4.2 2.2-8		0.4 0.1-1.5	
Socio-economic status of patients						
Rich	65	36	1	0.000	1	0.000
Poor	35	64	3.3 1.8-6.1		5.0 1.1-16.7	
Environmental factors						
Distance to health sector (km)						
>3	66	27	1	0.000	1	0.001
≤3	34	73	5.2 2.8-10.1		14.0 3.4-56.8	
Mode of transportation						
Other transports	91	54	1	0.000	NA	-
Domiciliary	9	46	8.5 3.8- 21.4			
Social factors of healthcare provider						
Polite						
No	51	35	1	0.022	1	0.079
Yes	49	65	1.9 1.1-3.6		2.8 0.9-9.1	
Sympathy						
No	40	23	1	0.01	1	0.016
Yes	60	77	2.2 1.2-4.3		6.2 1.3-29.7	
Humanity						
No	68	49	1	0.006	1	0.042
Yes	32	51	2.2 1.2 - 4.1		3.9 1.1-14.4	
Social dealing						
Unsatisfac- tory	37	22	1	0.02	1	0.577
Satisfactory	63	78	2.1 1.1-4.1		1.4 0.4-4.7	
Service factors						
24-hour service						
No	78	60	1	0.006	NA	-
Yes	22	40	2.4 1.2 - 4.6			

Continued:

Waiting time						
>half hour	64	45	1		1	
≤half hour	36	55	2.2 1.2-3.9	0.007	3.0 1.2- 10.8	0.048
Domiciliary treatment service						
No	91	54	1		1	
Yes	9	46	8.5 3.8- 21.4	0.000	20.4 3.3-124.9	0.001
Diagnosis methods						
Other	56	41	1			
RDT	44	59	1.8 1.0- 3.3	0.034	NA	-
Cost						
Traveling cost (kyats)						
>1000	65	48	1			
≤1000	35	52	2.0 1.1 -3.7	0.015	NA	-
Treatment cost (kyats)						
>1000	59	10	1		1	
≤1000	41	90	12.8 5.8- 30.9	0.000	24.5 5.7-103.8	<0.001
Extra-cost during treatment						
Yes	78	65	1			
No	22	35	1.9 0.9 - 3.8	0.06	NA	-
Patient's knowledge on malaria and anti-malarial treatment						
Availability of anti-malarial treatment						
Don't know	88	51	1		1	
Know	12	49	6.9 3.3-15.8	0.000	4.1 1.2-13.8	0.004

Patients' implication on anti-malarial treatment

"I can't resist the malaria attack. I usually try some injection at the available clinic whoever the doctor is (ငှက်ထလာရင်တော့ မခံနိုင်ဘူး။ တွေ့ရလူနဲ့ထိုးရတော့တာဘဲ။)"

(a 27-year-old female patient at SC)

"I would like to get good treatment as soon as possible for any attack."

(a 34-year-old male patient at GP)

"I don't mind charges. But, I like to cure with only one shot of injection (only one visit)."

(a 40-year-old female patient at GP)

"Malaria attack is very terrible. I couldn't go to clinic. I'm very grateful to Sayarma for coming to me."

(a 28-year-old male patient at SC)

Factors influencing the choice of health sector

In bivariate analysis, there were no significant differences in the choice of health sector by age and marital status of patients. The patients were more likely to choose public sector if they were males (OR=2.1; 95% CI 1.0 to 4.2; $p=0.037$), lower education (illiterates and primary school) (OR=2.6; 95% CI 1.4 to 4.9; $p=0.001$), poor socio-economic status (OR=3.3; 95% CI 1.8 to 6.1; $p<0.001$) and decision maker to choose health sector was husband (OR=4.2; 95% CI 2.2 to 8.0; $p<0.001$). Residing close to health center (≤ 3 km) (OR=5.2; 95% CI 2.8 to 10.1; $p<0.001$), politeness (OR=1.9; 95% CI 1.1 to 3.6; $p=0.022$), sympathy (OR=2.2; 95% CI 1.2 to 4.3; $p=0.01$), humanity (OR=2.2; 95% CI 1.2 to 4.1; $p=0.006$) and good social-dealing (OR=2.1; 95% CI 1.1 to 4.1; $p=0.02$) of provider, convenient clinic opening time (24-hour service) (OR=2.4; 95% CI 1.2 to 4.6; $p=0.006$), acceptable waiting time ($\leq$ half hour) (OR=2.2; 95% CI 1.2 to 3.9; $p=0.007$), domiciliary treatment service (OR=8.5; 95% CI 3.8 to 21.4; $p=0.000$), diagnosis by RDT (OR=1.8; 95% CI 1.0 to 3.3; $p=0.034$), affordable traveling cost (≤ 1000 kyats) (OR=2.0; 95% CI 1.1 to 3.7; $p=0.015$), cheaper treatment cost (≤ 1000 kyats) (OR=12.8; 95% CI 5.8 to 30.9; $p=0.001$) and knowledge on availability of free-of-charge treatment at public sector (OR=6.9; 95% CI 3.3 to 15.8; $p<0.001$) were significant factors to choose public sector.

In best fit multivariate model, significant factors influencing choice of public sector included: poor socio-economic status (AOR=5; 95% CI 1.1 to 16.7), residing close to health center (AOR=14; 95% CI 3.4 to 56.8), cheaper treatment cost (AOR=24.5; 95% CI 5.7 to 103.8), sympathy and humanity of provider (AOR=6.2; 95% CI 1.3 to 29.7), (AOR=3.9; 95% CI 1.1 to 14.4), acceptable waiting time (AOR=3.0; 95% CI 1.2 to 10.8), domiciliary treatment service (AOR=20.4; 95% CI 3.3 to 124.9)

and knowledge on availability of free-of-charge treatment at public sector (AOR=4.1; 95% CI 1.2 to 13.8) (Table 1).

DISCUSSION

The malaria control program made accessible to early diagnosis as well as prompt and adequate treatment in the community free-of-charge. Considering price as well as technical and structural quality of healthcare providers, patients rationally should have a higher preference to public sector. However, a considerable number of patients were still purchasing anti-malarial treatment at private sector [2-4]. This study attempted to find out the underlying factors associated with choosing private or public sector for anti-malarial treatment.

In our findings, about half of the patients (49.5%) were in low socio-economic status. The patients with low socio-economic status were more likely to use public sector than private sector. Socio-economic conditions playing an important role in choosing health providers were reported in studies conducted in Indonesia [5], Nepal [6] and Malaysia [7].

In this study, 30.5% of patients knew that proper anti-malarial treatment could be obtained from BHS with free-of-charge. This figure becomes higher than the previous study which reported that only 18.3% of respondents knew the availability of anti-malarial treatment at BHS with free-of-charge [8].

Our result showed that choice of health care provider also associated with the distance between the residence and health sector. Studies in Thailand, Tanzania and Guinea reported that distance and transportation were identified as reasons for utilization of different facilities [9-11].

In this study, although the treatment cost was free-of-charge, patients need to spend traveling cost, ancillary treatment cost, and some extra-costs in both sectors.

In Bangladesh, one study reported that financial factors such as free consulting fees, lower medicine fees, little or no traveling cost and no diagnostic costs determined the patient's health care seeking in rural area [12].

Most of the patients in this study appreciated domiciliary care: home visit by GP or MW to give anti-malarial treatment. A study in Nigeria also reported that community appreciated the domiciliary provision of anti-malarial treatment [13]. In our study, patients preferred clinic waiting time of less than 30 minutes. A study in rural areas of Bangladesh also reported that convenient waiting time (<1 hour) as a determinant of the choice of healthcare provider [12].

In conclusion, factors influencing malaria patients' choice of healthcare provider were multi-factorial. Among them, actions to improve more accessible to public sector should include ensuring actual free-of-charge treatment, publicizing free availability of malaria diagnosis and treatment down to community level, encouraging domiciliary care, reducing clinic waiting time, and considering equal gender distribution of healthcare provider in public sector.

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SHORT REPORT

Seroprevalence of HBsAg and anti-HBs among people living with HIV/AIDS (PLWHA)

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Approximately 400 million persons worldwide have chronic hepatitis B virus (HBV) infection and up to 1 million die annually of HBV related liver disease. The most common risk factors for acquiring HBV, similar to HIV, are illicit drug use and sex including both heterosexual and homosexual activity. Although the prevalence of past exposure is high (90-95%), active infection with HBV is much less common and occurs in 10-15% with HIV infection [1].

Because HIV and HBV have overlapping transmission routes, many people are infected with both viruses. Co-infection with HBV and HIV is a rapidly growing public health concern. A majority of HIV-negative people infected with HBV in adults spontaneously clear the virus without treatment, but 5%-10% develop chronic infection lasting more than 6 months. But studies showed that HIV-positive people were more likely to develop chronic infection [2]. The natural history of HBV is known to be complicated by HIV co-infection, with a higher rate of chronic hepatitis, greater levels of HBV replication and lower incidences of spontaneous loss of HBV e antigen (HBeAg) or HBsAg. Additionally, the incidence of cirrhosis and mortality attributable to liver disease are significantly increased when HBV infection is complicated by HIV infection [3].

Myanmar is hyper-endemic for HBV infection, and research studies showed that the HB carrier rate and infection rate in general population were found to be 10-12% and 35-60%, respectively [4].

It was reported that HB carrier rate and infection rates for HBV in people living with HIV/AIDS (PLWHA) were 14.3% and 92.0%, respectively [5]. There were lots of international papers regarding the HIV/HBV co-infection. In Myanmar, there were many studies regarding the prevalence of HBV infection in healthy adults. However, information is very scarce on the prevalence of HBV infection among PLWHA patients. Therefore, this study was conducted with an aim to determine the prevalence of HBV infection among PLWHA with clinical staging I. It was a cross-sectional descriptive study and data were collected according to the proforma, and data entry was analyzed manually. This study was part of preliminary investigation leading to advanced research on HBV and HIV co-infection in Myanmar.

In this study, there were 122 HIV-positive stage I patients including 26 males and 96 females aged ranging between 21 and 62 years participated in this study. All sera samples were tested for the presence of HBsAg using Acon HBsAg rapid test kit, USA. Among them, 10 subjects (8.2%) were found to be seropositive for HBsAg. Those who were negative for HBsAg were further tested for the presence of anti-HBs antibodies using SDICT test device, Republic of Korea and 27 subjects (22.13%) were found to be seropositive for antibody to HBsAg (Table 1).

So, seroprevalence rate of HBsAg and anti-HBs among PLWHA with clinical stage I was found to be 30.33% (i.e. 37/122). Most of them said that they acquired

Table 1. Prevalence rate of HBsAg and anti-HBs among PLWHA stage I

	Positivity (%)	Negativity (%)
HBsAg	10 (8.2)	112 (91.8)
Anti-HBs	27 (22.1)	95 (77.9)

HBV/HIV co-infection through sexual contact. It was noted that HBV carrier rate was lower when comparing with the findings of similar research in 1992. This study was just the prevalence of HBV infection among PLWHA stage I patients who had the CD4+ count of $400 \times 10^6/L$ and above. The prevalence rate may be higher among PLWHA in all stages because of the similar risk factors and transmission routes. In those with presence of HBV surface antigen, it is important to establish the activity of HBV to differentiate those with active viral replication (HBV-DNA positive with or without HBeAg) from chronic carriers (HBV-DNA negative, HBeAb positive). HBV infection increases significantly liver-related mortality in HIV-positive patients. This implies that every HIV-positive patient should be screened for HBV and advised for HB vaccination if they do not have HBsAg and anti-HBs antibody. Interventions to decrease mortality related to liver disease in HIV-positive patients

with chronic HBV, such as early initiation of HAART regimens targeted against both HBV and HIV, deserve further investigation.

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