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# Detection of Epstein-Barr Virus in Lower Gastrointestinal Tract Lymphomas

## A Study in Malaysian Patients

*Asmah M. Yunos,*[1] *Hasnan Jaafar,*[2] *Fauziah M. Idris,*[2] *Gurjeet Kaur*[1] and *Mohamed J.E.M.F. Mabruk*[1]

1 Advanced Medical and Dental Institute, Universiti Sains Malaysia, Kelantan, Malaysia
2 School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia

## Abstract

**Background:** Many studies in the literature have shown that Epstein-Barr virus (EBV) is associated with several human lymphoid and epithelial malignancies. However, the prevalence of EBV in non-Hodgkin lymphoma (NHL) of the lower gastrointestinal (GI) tract has not been fully elucidated.

**Aim:** The aim of this study was to determine the presence and distribution of EBV in formalin-fixed paraffin-embedded tissue samples obtained from 18 Malaysian patients diagnosed with NHL of the lower GI tract.

**Methods:** The GI tract lymphoma tissue samples analyzed for the presence of EBV were divided into the following groups: NHL of the small intestine (seven cases); NHL of the ileocecum (ten cases); and NHL of the rectum (one case). The presence of EBV-encoded RNA (EBER) in all of the above tissue samples was tested for using conventional *in situ* hybridization technology.

**Results:** Two of 18 cases (11.1%) of NHL of the lower GI tract demonstrated positive signals for EBV/EBER. In the first positive case, EBV/EBER signals were located in lymphoma cells in the serosa layer of the small intestine. In the second EBV/EBER-positive case, EBV/EBER signals were detected in diffuse B-cell lymphoma of the ileocecum.

**Conclusion:** These findings demonstrate a rare association between EBV and lower GI tract lymphomas in this group of Malaysian patients.

## Introduction

This study was initiated to determine the prevalence of Epstein-Barr virus (EBV) in lower gastrointestinal (GI) tract lymphomas.

EBV was discovered 40 years ago during examination of electron micrographs of cells cultured from a Burkitt lymphoma sample.[1] This finding became the first of an unexpectedly wide range of associations between EBV and malignancies.[2]

In general, lymphoma is defined as a primary malignant tumor of the lymphoid cells (nodal or extra-nodal). There are two types of lymphoma; Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL). Lymphomas, which are ranked the 12th most common cancer in the world, are more prevalent in men than women.[3,4]

Many published studies have shown that EBV is associated with several human lymphoid and epithelial cell malignancies.[5-7] To date, no studies have been conducted to determine the prevalence of EBV in NHL of the lower GI tract in the Malaysian population. In addition, and to our knowledge, there has been only one case study that has looked at the prevalence of EBV in NHL of the ileocecum[8] and only a few case-report studies that have looked at the prevalence of EBV in NHL of the small intestine.[9-11]

The experimental approach used in our study for the detection of EBV in NHL of the lower GI tract was *in situ* hybridization (ISH). The probe used was the EBV-encoded RNA (EBER)/EBV probe. EBER ISH is considered as the gold standard technique for detecting and localizing latent EBV in tissue samples.[12]

Here, we report the prevalence of EBV in lower GI tract lymphomas collected from 18 Malaysian patients between 1992 and 2004.

## Materials and Methods

### Tissue Specimens

This study was carried out on 18 formalin-fixed paraffin-embedded tissue samples obtained from Malaysian patients with NHL of the lower GI tract. The lymphoma tissue samples used in our study were divided into the following groups: NHL of the small intestine (seven cases); NHL of the ileocecum (ten cases); and NHL of the rectum (one case). All of these samples were collected in the Department of Pathology at the Universiti Sains Malaysia, over a period of 12 years from 1992 to 2004 and represent all of the lower GI tract lymphomas seen at our institution during this period. Patient data consisted of age, sex, clinical details, and histopathology results (see table I). The patients consisted of 11 males and 7 females, with a mean age of 44.78 years, ranging from 2 years to 77 years. None of the patients included in the present study was immunosuppressed.

This study was approved by the School of Medical Sciences ethical committee, Universiti Sains Malaysia, in accordance with the Malaysian guideline.

The slides stained with hematoxylin and eosin were reviewed by two independent pathologists. The phenotyping of the lymphomas was performed using standard avidin-biotin complex immunohistochemical staining, using primary antibodies (DAKO, Denmark) directed against B-cell associated antigen CD20 (L26), T-cell associated antigens CD45RO (UCHL1), and T-cell specific antigen CD3 (polyclonal anti-CD3).

### Positive Control Tissue Samples

All positive control tissue samples used were nasopharyngeal carcinoma (NPC) tissue sections, known to be positive for EBV/EBER.

### *In Situ* Hybridization

The *in situ* hybridization (ISH) probe used for the detection of EBV in all non-Hodgkin lymphomas of the lower GI tract that had been histopathologically confirmed was the peptide nucleic acid (PNA) EBV/EBER specific probe (DakoCytomation, Denmark). The detection kit for ISH was the PNA ISH Detection kit (DakoCytomation, Denmark), intended for the detection of fluorescein-conjugated PNA probes hybridized to their target RNA in cell or tissue preparations.

In each experiment a positive and negative control were included. The negative control consisted of a serial section to the test sample, treated with a negative control probe (provided by the kit manufacturer). The ISH experiments were carried out as instructed by the kit manufacturer, and all precautions were taken to avoid RNase contamination.

### Counterstaining and Mounting

The sections were counterstained with Nuclear Fast Red (DakoCytomation, Denmark), mounted with aqueous mounting solution (Merck, Germany), and a coverslip was placed on top.

Positive staining to EBV/EBER was visualized under light microscopy as a dark blue/black stain over the target site.

## Results

### Histopathology

Histopathological features for all the cases of NHL of the lower GI tract were recorded by two independent pathologists who examined slides stained with hematoxylin and eosin. The patients' details, clinical data, and histopathology are shown in table I.

### In Situ Hybridization

#### *EBV/EBER in non-Hodgkin Lymphomas of the Small Intestine*

Seven samples from patients with NHL of the small intestine were analyzed by ISH for the presence of EBV/EBER. One sample was positive for EBV/EBER (14.3%). Positive signals for EBV/EBER were located in lymphoma cells of the serosal layer (figure 1 and table I). All negative controls, from serial sections to the test samples, were tested using a negative-control probe, and proved negative for EBV/EBER (figure 1 and table I). All the positive-control tissue samples consisted of NPC, were processed at the same time as test samples, and proved positive to EBV/EBER (figure 2). This indicates that the RNA was preserved and not degraded as a result of formalin fixation.

#### *EBV/EBER in non-Hodgkin lymphomas of the Ileocecum*

Ten samples of NHL of the ileocecum were analyzed by ISH for the presence of EBV/EBER. One sample was positive for EBV/EBER (10%). Positive signals for EBV/EBER were located in scattered, diffuse B-cell lymphoma cells (figure 3 and table I). All negative controls, from serial sections to the test samples, were tested using a negative-control probe, and proved negative for EBV/EBER (figure 3 and table I). The NPC positive-control tissue samples were positive to EBV/EBER (figure 2).

#### *EBV/EBER in non-Hodgkin lymphomas of the Rectum*

The single sample of NHL of the rectum was negative for EBV/EBER. The negative-control tissue sample was negative for EBV/EBER (table I), while the positive control was positive for EBV/EBER (figure 2).

**Table I.** Demographic details, clinical data, and histopathology results for all patients diagnosed with non-Hodgkin lymphoma (NHL) of the lower gastrointestinal tract

| Patient no. | Age (yrs) | Sex | Site of lymphoma | Histopathology diagnosis (Working Formulation / WHO classification) | Phenotype | Results of *in situ* hybridization analysis for Epstein-Barr virus (EBV)/ EBV nucleic acid |
|---|---|---|---|---|---|---|
| 1. | 38 | Male | Duodenum | Small lymphocytic lymphoma / lymphocytic | T cell / low grade | Negative |
| 2. | 61 | Female | Jejunum | Diffuse large B-cell lymphoma / centroblastic-polymorphic | B cell / high grade | Negative |
| 3. | 2 | Male | Jejunum | Anaplastic large cell lymphoma / pleomorphic, medium and large cell | T cell/ high grade | Negative |
| 4. | 66 | Male | Ileum | Diffuse large B-cell lymphoma / centroblastic | B cell / high grade | Negative |
| 5. | 77 | Male | Ileum | Anaplastic large cell lymphoma / anaplastic | T cell / high grade | Negative |
| 6. | 29 | Female | Ileum | Diffuse large B-cell lymphoma / centroblastic | B-cell/ high grade | Positive |
| 7. | 38 | Male | Ileum | Diffuse large cell lymphoma / immunoblastic | T cell / high grade | Negative |
| 8. | 11 | Female | Ileocecum | Burkitt lymphoma / Burkitt's | B cell / high grade | Negative |
| 9. | 3 | Male | Ileocecum | Burkitt lymphoma / Burkitt's | B cell / high grade | Negative |
| 10. | 75 | Female | Ileocecum | Diffuse large B-cell lymphoma / centroblastic, centrocyctic | B cell / high grade | Negative |
| 11. | 73 | Male | Ileocecum | Diffuse large B-cell lymphoma / centroblastic | B cell / high grade | Negative |
| 12. | 43 | Male | Ileocecum | HPE-diffuse large B-cell lymphoma-immunoblastic variant / immunoblastic NHL | not available | Negative |
| 13. | 50 | Male | Ileocecum | Diffuse large B-cell lymphoma / centroblastic, centrocyctic | B cell / high grade | Negative |
| 14. | 66 | Female | Ileocecum | Diffuse large B-cell lymphoma: anaplastic large B-cell variant / anaplastic | B cell / high grade | Negative |
| 15. | 34 | Male | Ileocecum | Diffuse large B-cell lymphoma/ centroblastic | B cell / high grade | Negative |
| 16. | 29 | Female | Ascending colon | Diffuse large B-cell lymphoma / centrocyctic | B cell/ high grade | Positive |
| 17. | 48 | Male | Ileocecum | Diffuse large B-cell lymphoma: anaplastic large B-cell variant / Ki 1 lymphoma | B cell / high grade | Negative |
| 18. | 63 | Female | Rectum | Metastatic NHL | Not available | Negative |

## Discussion

Most of the cases of NHL of the lower GI tract analyzed in the present study were high-grade B-cell lymphoma. B-cell lymphomas make up 80–85% of NHL at all anatomical sites. T-cell lymphomas form the large majority of the remainder of NHL.[13]

The results of our study demonstrate a rare association between EBV and lower GI tract lymphoma, based on these tissue samples obtained from Malaysian patients. Only 2 of 18 cases of lower GI tract lymphomas were positive for EBV/EBER. In positive cases, EBV/EBER-positive signals were detected only in cases of high grade B-cell lymphoma.

Fig. 1. *In situ* hybridization detection of Epstein-Barr virus (EBV)/EBV-encoded RNA (EBER) in a tissue sample of non-Hodgkin lymphoma (NHL) of the small intestine. **a & b** illustrate tissue stained with hematoxylin and eosin (original magnifications: a = × 100; b = × 200). **c & d** illustrate the corresponding serial tissue section to which the EBV/EBER probe was applied, showing EBV/EBER staining as blue/purple color (arrow) in the lymphoma cells of the serosal layer (original magnifications: c = × 100; d = × 400). **e & f** show the same serial section, to which a negative control probe was applied with no staining present (original magnifications: e = × 100; f = × 400).

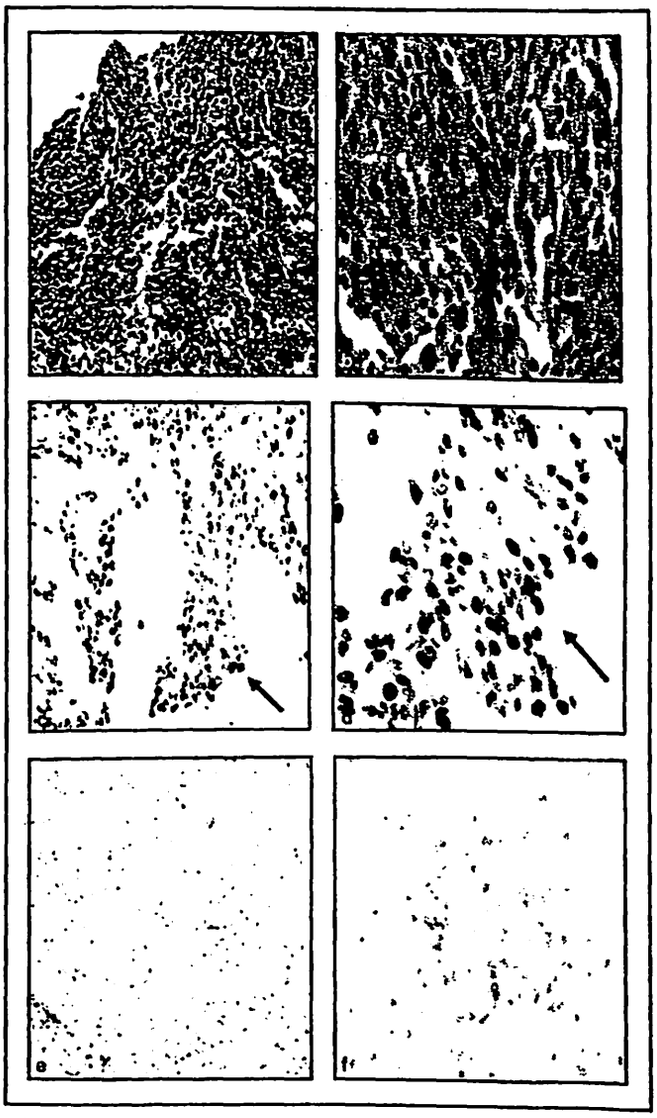

Fig. 2. *In situ* hybridization detection of Epstein-Barr virus (EBV)/EBV-encoded RNA (EBER) in positive control nasopharyngeal carcinoma (NPC) tissue samples. **a & b** illustrate NPC tissue sample stained with hematoxylin and eosin (original magnifications: a = × 200; b = × 400). **c & d** illustrate the corresponding serial NPC section to which the EBV/EBER probe was applied, showing EBV/EBER staining as blue/purple color (arrow) in the carcinoma cells (original magnifications: c = × 200, d = × 400). **e & f** are serial sections to which a negative control probe was applied with no staining present (original magnifications: e = × 200; f = × 400).

In this study, we investigated EBER transcripts by ISH because it is well established that two EBV-encoded RNAs (EBER1 and EBER2) are expressed at high levels in latently infected cells (approximately $10^7$ copies per cell).[13-18]

In the first EBV/EBER-positive case reported here, EBV was detected in NHL of the small intestine. Only a few studies have previously shown an association between EBV/EBER and NHL of the small intestine.[9-11] Borisch et al.[9] detected the EBV genome in lymphoma tissue by PCR, which was then localized via EBER ISH to some of the transformed lymphocytes.[9] Kersten et al.[11] also reported the presence of EBV DNA in NHL of small intestine tissue from a kidney transplant patient. Yang et al.[10] reported one EBV/EBER-positive signal in 12 cases of NHL of the small intestine in a group of Korean patients. Another study[19] that examined the prevalence of EBV in intestinal NHL in European and Mexican populations showed that the prevalence was higher in the Mexican population than in the European population.

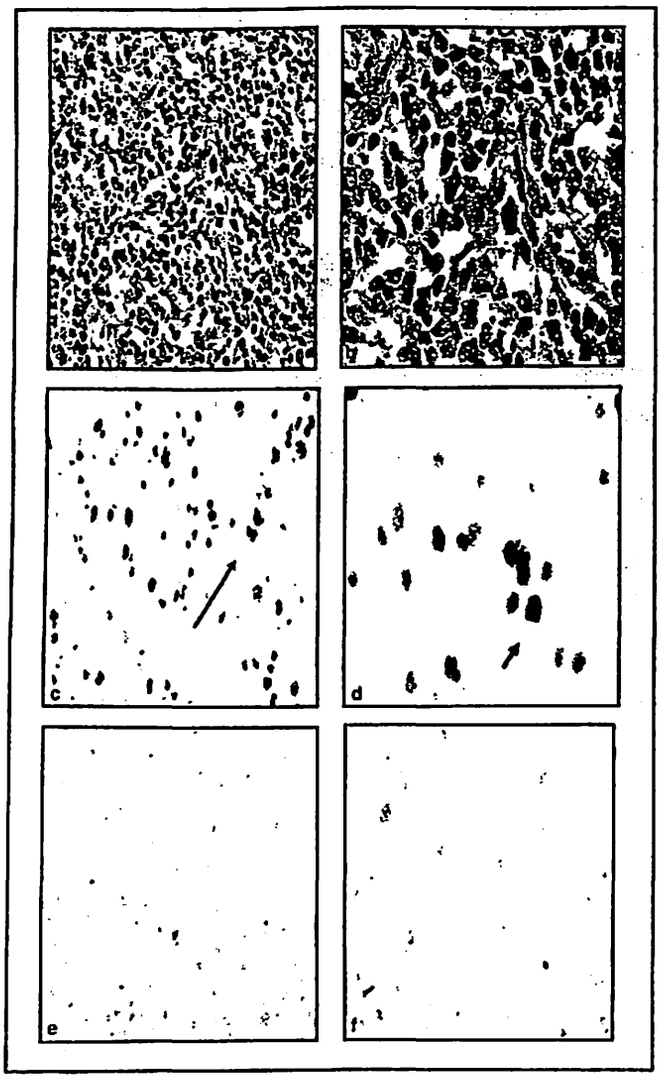


Fig. 3. *In situ* hybridization detection of Epstein-Barr virus (EBV)/EBV-encoded RNA (EBER). Example of the results for the analysis of non-Hodgkin lymphoma (NHL) of the ileocecal tissue samples. a & b illustrate NHL of the ileocecal tissue samples stained with hematoxylin and eosin (original magnifications: a = × 200; b = × 400). c & d illustrate the corresponding serial section to which the EBV/EBER probe was applied, depicting EBV/EBER staining as blue/purple color (arrow) in the diffuse B-cell lymphoma cells (original magnifications: c = × 200; d = × 400). e & f are serial sections to which a negative control probe was applied with no staining present (original magnifications: e = × 200; f: = × 400).

In the second EBV/EBER-positive case reported in our study, positive EBV signals were seen in the lymphoma cells of the ileocecal specimen. To our knowledge, there has been only one case report study that looked at the presence of EBV/EBER in NHL of the ileocecum, which was in a western patient.[8] This report described the case of an 8-year-old boy who developed an ileocecal B-cell lymphoma after liver transplantation. EBV/EBER was demonstrated to be present in lymphoma cells and hyperplasic follicular germinal center cells in various ileocecal tissue samples.[8]

Our study is the first to investigate the presence of EBV/EBER in NHL of the rectum.

It is not known what role latent EBV infection played in the development of the two cases of NHL of the lower GI tract reported to be EBV positive in this study. However, it has been suggested[20] that the reason for detecting EBV/EBER in a small percentage of gastric carcinomas could be the presence of EBV reservoir lymphocytes that may randomly reach the GI tract mucosa, as do other inflammatory cells. Epithelial cells may therefore become exposed to EBV derived from these reservoir lymphocytes.

## Conclusions

This study demonstrates a rare association between EBV and NHL of the lower GI tract in our study population. In addition, our study may indicate that anti-herpesvirus drug therapy may prove to be effective in the future for similar cases. In order to confirm and implement this therapeutic approach using anti-herpesvirus drugs, more studies on GI tract lymphoma tissue samples will be necessary.

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The authors have no conflicts of interest that are directly relevant to the content of this study.

## References

1. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004; 4 Suppl. 10: 757-68
2. Rickinson AB, Kieff E. In: Fields BN, Knipe E, Howley PM, editors. Epstein-Barr virus, fields virology. 3rd ed. Vol. 2. Philadelphia (PA): Lippincott-Raven, 1996: 2397-446
3. Peh SC. Host ethnicity influences non-Hodgkin's lymphoma subtype frequency and Epstein-Barr virus association rate the experience of a multi-ethnic patient population in Malaysia. Histopathology 2001; 38 Suppl. 5: 458-65
4. Muller AM, Ihorst G, Mertelsmann R, et al. Epidemiology of non-Hodgkin's lymphoma (NHL): trends, geographic distribution, and etiology. Ann Hematol 2005; 84 Suppl. 1: 1-12
5. Fahraeus R, Fu HL, Ernberg I, et al. Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. Int J Cancer 1988; 42 Suppl. 3: 329-38
6. Lee SS, Jang JJ, Cho KJ, et al. Epstein-Barr virus-associated primary gastrointestinal lymphoma in non-immunocompromised patients in Korea. Histopathology 1997; 30 Suppl. 3: 234-42
7. Wong NA, Herbst H, Herrmann K, et al. Epstein-Barr virus infection in colorectal neoplasms associated with inflammatory bowel disease: detection of the virus in lymphomas but not in adenocarcinomas. J Pathol 2003; 201 Suppl. 2: 312-8

8. Sadahira Y, Kumori K, Mikami Y, et al. Post-transplant malignant lymphoma with monoclonal immunoglobulin gene rearrangement and polyclonal Epstein-Barr virus episomes. J Clin Pathol 2001; 54 Suppl. 11: 887-9
9. Borisch B, Hennig I, Horber F, et al. Enteropathy-associated T-cell lymphoma in a renal transplant patient with evidence of Epstein-Barr virus involvement. Virchows Arch A Pathol Anat Histopathol 1992; 421 Suppl. 5: 443-7
10. Yang WI, Cho MS, Tomita Y, et al. Epstein-Barr virus and gastrointestinal lymphomas in Korea. Yonsei Med J 1998; 39 Suppl. 3: 268-76
11. Kersten MJ, Surachno S, Koopman MG, et al. Epstein-Barr virus in a donor kidney as a cause of non-Hodgkin lymphoma. Ned Tijdschr Geneeskd 1999; 143 Suppl. 7: 360-4
12. Gulley ML. Molecular diagnosis of Epstein-Barr virus-related diseases. J Mol Diagn 2001; 3 Suppl. 1: 1-10
13. Leong IT, Fernandes BJ, Mock D. Epstein-Barr virus detection in non-Hodgkin's lymphoma of the oral cavity: an immunocytochemical and in situ hybridization study. Oral Surg Oral Med Oral Pathol 2001; 92 Suppl. 2: 184-93
14. Glickman JN, Howe JG, Steitz JA. Structural analyses of EBER1 and EBER2 ribonucleoprotein particles present in Epstein-Barr virus-infected cells. J Virol 1988; 62 Suppl. 3: 902-11
15. Gilligan K, Rajadurai P, Resnick L, et al. Epstein-Barr virus small nuclear RNAs are not expressed in permissively infected cells in AIDS-associated leukoplakia. Proc Natl Acad Sci U S A 1990 Nov; 87 (22): 8790-4
16. Ambinder RF, Mann RB. Detection and characterization of Epstein-Barr virus in clinical specimens. Am J Pathol 1994 Aug; 145 (2): 239-52
17. Harris NL, Jaffe ES, Stein H, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood 1994 Sep 1; 84 (5): 1361-92
18. Peh SC, Nadarajah VS, Tai YC, et al. Pattern of Epstein-Barr virus association in childhood non-Hodgkin's lymphoma: experience of university of Malaya medical center. Pathol Int 2004; 54 Suppl. 3: 151-7
19. Quintanilla-Martinez L, Lome-Maldonado C, Ott G, et al. Primary non-Hodgkin's lymphoma of the intestine: high prevalence of Epstein-Barr virus in Mexican lymphomas as compared with European cases. Blood 1997 Jan 15; 89 (2): 644-51
20. Cho YJ, Chang MS, Park SH, et al. In situ hybridization of Epstein-Barr virus in tumor cells and tumor-infiltrating lymphocytes of gastrointestinal tract. Hum Pathol 2001; 32 Suppl. 3: 297-301

Correspondence and offprints: Professor *Mohamed J.E.M.F. Mabruk*, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Komplex Eureka, Penang, 11800 USM, Malaysia.
E-mail: mmabruk03@yahoo.co.uk

BERGMANN-LEITNER ES, DUNCAN EH, MULLEN GE, BURGE JR, **KHAN F**, LONG CA, ANGOV E & LYON JA, 2006. Critical evaluation of different methods for measuring the functional activity of antibodies against malaria blood stage antigens. *American journal of tropical medicine and hygiene*, 75 (3), 437-442.

*Am. J. Trop. Med. Hyg.*, 75(3), 2006, pp. 437–442


# CRITICAL EVALUATION OF DIFFERENT METHODS FOR MEASURING THE FUNCTIONAL ACTIVITY OF ANTIBODIES AGAINST MALARIA BLOOD STAGE ANTIGENS

ELKE S. BERGMANN-LEITNER,* ELIZABETH H. DUNCAN, GREGORY E. MULLEN, JOHN ROBERT BURGE, FARHAT KHAN, CAROLE A. LONG, EVELINA ANGOV, AND JEFFREY A. LYON

*Department of Immunology, CD&I, Walter Reed Army Institute of Research, Silver Spring, Maryland; Malaria Vaccine Development Branch, NIAID/NIH, Rockville, Maryland*

*Abstract.* Antibodies are thought to be the primary immune effectors in the defense against erythrocytic stage *Plasmodium falciparum*. Thus, malaria vaccines directed to blood stages of infection are evaluated based on their ability to induce antibodies with anti-parasite activity. Such antibodies may have different effector functions (e.g., inhibition of invasion or inhibition of parasite growth/development) depending on the target antigen. We evaluated four methods with regards to their ability to differentiate between invasion and/or growth inhibitory activities of antibodies specific for two distinct blood stage antigens: AMA1 and $MSP1_{42}$. We conclude that antibodies induced by these vaccine candidates have different modes of action that vary not only by the antigen, but also by the strain of parasite being tested. Analysis based on parasitemia and viability was essential for defining the full range of anti-parasite activities in immune sera.

## INTRODUCTION

Antibodies directed against bloodstage malaria have been shown to be efficacious in the prevention of disease as shown in passive transfer experiments in humans.[1–3] The mechanisms by which the antibodies neutralize parasites *in vitro* differ greatly depending on the target antigen. Modalities include merozoite opsonization, targeting them toward phagocytic cells of the host,[4] prevention of invasion,[5] inhibition of parasite development within the erythrocyte,[6,7] and interference with merozoite dispersal by agglutination.[8,9] Most methods for analyzing functional antibodies against bloodstage parasites *in vitro* are based on microscopic evaluation of blood smears or detection of DNA in erythrocytes and do not assess parasite viability.[8,10–12] In contrast, the measurement of enzymatic activity of the parasite-derived lactate dehydrogenase (pLDH),[13,14] the conversion of dihydroethidine to ethidium,[15,16] and the quantification of $^3$H-hypoxanthine incorporation into newly synthesized DNA[17,18] all assess parasite viability and thus can measure both invasion and growth inhibition.

The objective of this study was to compare parasite inhibition results obtained with four methods that measure either *in vitro* invasion inhibition or growth inhibition (viability) using a model system comprised of antigen-specific immune sera and 3D7 as well as FVO parasite cultures. Antisera were raised against the 3D7 and FVO alleles of AMA1[19] and $MSP1_{42}$,[20,21] antigens that are candidates for bloodstage malaria vaccines. Invasion inhibition was measured by quantifying parasites in Giemsa-stained blood smears and by flow cytometric analysis of parasites whose DNA was stained with Syto16.[22] Whereas staining with Giemsa can reveal antibody-induced, morphologic changes in parasite development, concluding that these changes also affect parasite viability is subjective. Syto16 readily permeates membranes of both viable and non-viable cells and is therefore not useful for measuring cell viability. Growth inhibition (viability) was measured by flow cytometric analysis of parasites whose DNA was stained by using hydroethidine (HE) and by measuring the enzymatic activity of pLDH.[13,14] HE staining depends on the intracellular conversion of HE into ethidium by NADPH oxidase and has been described in various protozoan systems including malaria to be a reliable metabolic indicator of parasite viability.[15,16]

Comparing the four techniques in our model system allowed us to determine that these Abs function either by invasion inhibition or by growth inhibition and that the mechanism of inhibition depended on the parasite test strain. In cases where Abs acted by invasion inhibition, all four methods gave similar results. As observed previously, AMA1-specific Abs were invasion inhibitory,[23,24] whereas antibodies directed against $MSP1_{42}$ preferentially inhibited invasion or inhibited parasite growth and development, depending on the parasite test strain. This study clearly shows that, when analyzing bloodstage malaria parasite-specific antibodies, methods that can distinguish between invasion inhibition and viability of the intraerythrocytic parasite must be used to more fully define the Ab mechanism of action.

## MATERIALS AND METHODS

**Parasite cultures.** Complete medium was prepared with RPMI 1640 (Invitrogen, Carlsbad, CA) containing 25 mmol/L HEPES, 7.5% wt/vol $NaHCO_3$, and 10% human pooled serum (blood type O+). *Plasmodium falciparum* strains 3D7 and FVO were maintained and synchronized by the temperature cycling method.[25]

For the evaluation of immune sera, triplicate cultures were set up in replicate culture plates in the presence or absence of 20 vol% immune serum ~6 hours before rupture occurred (starting parasitemia, 0.3%; 1% hematocrit uninfected erythrocytes) in 96-well plates under static conditions. Replicate culture plates were set up to preclude repeated sampling at different time-points from the same plate, ruling out sampling effect on the growth of the parasites. Time-points indicated refer to time after schizont rupture in every experiment. Thus, each time-point was collected from its own plate and analyzed by the various methods in triplicate. All experiments were repeated independently at least three times.

* Address correspondence to Elke S. Bergmann-Leitner, Department of Immunology, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910. E-mail: elke.bergmannleitner@us.army.mil

**Antisera.** New Zealand White rabbits (Spring Valley Laboratories, Woodbine, MD) were immunized subcutaneously four times with either recombinant $MSP1_{42}$ of the FVO strain (36/36/36/36 μg, six rabbits)[21] or the 3D7 strain (200/50/50/50 μg, five rabbits)[20] emulsified in complete/incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO), and serum pools were prepared. Antisera against recombinant AMA1 of the FVO and the 3D7 strains were raised in individual rabbits after immunizing intramuscularly three times with 50 μg PpAMA1 using Montanide ISA720 (SEPPIC, Fairfield, NJ) as adjuvant.[19] Pooled control sera were prepared from sera of four rabbits immunized three times with 50 μg of reduced/alkylated $MSP1_{42}$ (3D7) and $MSP1_{42}$ (FVO) in complete/incomplete Freund's adjuvant.

**Microscopic analysis.** Cultures were harvested at various time-points as indicated, and blood smears were made from each well. Blood smears were fixed in methanol and stained in a 10% Giemsa solution (Sigma) for 10 minutes. Slides were washed in water and allowed to air dry before analysis. Evaluation was performed at ×100 magnification (oil immersion) using a Nikon (Nikon, Tokyo, Japan) E400 Eclipse. Three slides per group were evaluated by counting 2,000 red blood cells (RBCs) or 100 parasitized RBCs/slide. Growth inhibition was calculated using the following formula: percent growth inhibition = (1 − [parasitemia of culture/parasitemia of control culture]) × 100.

**Flow cytometry.** Cells were recovered at different time-points, and a 50-μL aliquot was transferred into polystyrene tubes (Becton Dickinson, Mountain View, CA) for subsequent staining with hydroethidine (Polysciences, Warrington, PA) or Syto16 (Molecular Probes, Eugene, OR). Stock solutions of HE were prepared at 10 mg/ml dissolved in dimethylsulfoxide (DMSO) (Sigma) and stored at −30°C. Samples were stained by adding 500 μL of freshly diluted HE (diluted 1:200 in 37°C phosphate buffered saline [PBS]; BioWhittaker, Walkersville, MD) to the parasite suspensions and incubated for 20 minutes at 37°C. Syto16, purchased as a 1 mmol/L solution, was diluted to 200 nmol/L with PBS; a 500-μL aliquot was added to the parasite suspensions and incubated for 30 minutes at 37°C. To stop the staining for both methods, samples were transferred to ice (which allowed stabilization of the staining for up to 2 hours) and were diluted with 1 mL PBS before analysis. The data were acquired by a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software for acquisition and analysis. Growth inhibition was calculated using the following formula: percent growth inhibition = (1 − [parasitemia of culture/parasitemia of control culture]) × 100.

**LDH assay.** Cultures for growth inhibition assays were set up at the schizont stage in 96-well plates and cultured for one cycle (i.e., 40 hours for 3D7, 48 hours for FVO). Cells were harvested, washed, and frozen at −30°C until analysis. pLDH was detected and measured as described elsewhere.[26] Growth inhibition was calculated using the following formula: % growth inhibition = $\{1 - ([OD_{sample} - OD_{RBC}]/[OD_{control\ serum} - OD_{RBC}])\} \times 100$.

**Statistical methods.** Results from detection of growth/invasion inhibition obtained using Syto16 and HE in three independent experiments were compared with the Student *t* test (two-tailed). For comparing the results of all four assays, the Box-Cox power transformation was used to stabilize variance in the data. Differences in growth inhibitory activity among these factors were evaluated by using an analysis of variance technique for a 3 × 4 factorial experiment of a randomized block design and adjusted for multiple comparisons with Tukey's simultaneous test (family error rate = 5%). Results from the two parasite test strains were evaluated separately.

## RESULTS

**Microscopic analysis of invasion/growth inhibitory effects of bloodstage-specific antisera.** Parasite cultures were established at the early schizont stage in the presence of immune or control sera. The AMA1-specific sera were only tested against the homologous strain, whereas anti-$MSP1_{42}$ was tested against both homologous and heterologous strains. Figure 1 summarizes the microscopic evaluation of blood smears stained with Giemsa. The graphs show the mean percentage of reduction in parasitemia and the 95% CI of 3D7 (Figure 1A) and FVO (Figure 1B) cultures at various time-points during the bloodstage cycle. For this analysis, every parasitized cell was counted as one invasion event including cells infected with multiple parasites. We counted all parasites associated with RBCs, i.e., rings as well as residual schizonts. We did not exclude these residual schizonts from the parasitemia analysis because of the possibility that they may rupture at a later time-point and produce a delayed new burst of young rings. In three independent experiments conducted with 3D7 parasite cultures, treatment with $MSP1_{42}$ (3D7)-specific, $MSP1_{42}$ (FVO)-specific, and AMA1 (3D7)-specific antisera caused an overall reduction of parasitemia by 13%, 25%, and 46%, respectively. Similarly, with FVO parasite cultures, treatment with $MSP1_{42}$ (3D7)-specific, $MSP1_{42}$ (FVO)-specific, and AMA1 (FVO)-specific antisera caused an overall reduction of parasitemia by 10%, 52%, and 42%, respectively. To determine if any of the antibodies merely delay development rather than inhibit growth, we extended the analysis by 6 hours after the second round of invasion was completed. Typically for the *in vitro* culture conditions, the 3D7 and FVO strains, have cycle lengths of 40 and 48 hours, respectively. Thus, for the extended analysis, 3D7 and FVO cultures were collected 48 and 58 hours, respectively, after the initial invasion event. No delays were observed (data not shown).

**Comparison of DNA-binding dyes Syto16 and HE for their ability to measure the effect of bloodstage-specific antisera on parasite viability.** We next explored flow cytometric analysis as a means for automated measurement of the anti-parasitic effects of immune sera, because analysis by Giemsa-stained blood typically is time consuming and high throughput screening is difficult. Syto16 staining is useful for detecting the presence of DNA but is not able to distinguish between live and dead cells,[22] whereas HE has been described in various protozoan systems including malaria as a vital stain for viable parasites,[15,16] owing to the requirement that parasite-derived enzymes convert hydroethidine to ethidium.

Results from Syto16 and HE staining (Figure 2), in a single cycle assay, for homologous strain pRBC cultured with AMA1-specific Ab (FVO strain *P. falciparum* cultured with anti-AMA1 [FVO] or 3D7 strain-cultured with AMA-1 [3D7]) were essentially identical, indicating that all inhibition by AMA1-specific Abs is by invasion inhibition.

In the case of $MSP1_{42}$-specific Abs, the situation was more

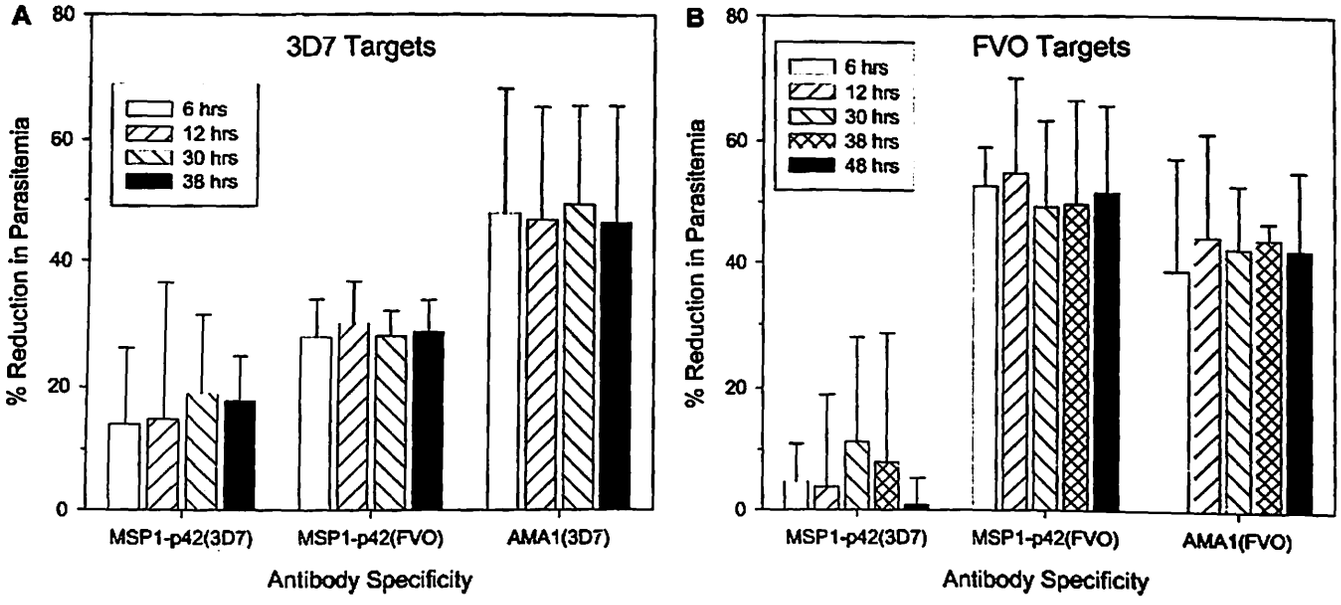


FIGURE 1. Culturing parasites in the presence of antisera specific for either AMA1 or $MSP1_{42}$ reduces parasitemia as determined by Giemsa-stained blood smears: 3D7 (A) and FVO strain *P. falciparum* cultures (B). Graphs show results of culturing schizont-infected erythrocytes with pooled anti-$MSP1_{42}$ (3D7) or pooled anti-$MSP1_{42}$ (FVO), or anti-AMA1 (3D7) (A) or anti-AMA1 (FVO) (B). Anti-AMA1–specific sera were only tested against their respective homologous strains. Data are expressed as mean percentage of reduction in parasitemia (with respect to control serum) and 95% CIs of three independent experiments. In each experiment three slides/time-point were evaluated.

complex. By 6 hours after invasion, detection with Syto16 or HE showed that anti-$MSP1_{42}$ (3D7)-specific antiserum suppressed homologous 3D7 strain parasite growth only marginally compared with control serum (11% inhibition). By the end of the experiment, there was no change in the amount of inhibition observed by staining with Syto16, but the amount of inhibition measured by staining with HE increased significantly to 22.6% (two-sample $t$ test, $P = 0.02$, two-tailed). This was also the case when 3D7 parasites were treated with heterologous anti-$MSP1_{42}$ (FVO) antiserum, although the overall levels of inhibition were higher (Syto16, 25% inhibition; HE, 44% inhibition, two-sample $t$ test, $P = 0.038$, two-tailed). These results indicate that the primary effect of these antisera on 3D7 strain parasites was inhibition of development rather than inhibition of invasion.

In contrast, when FVO strain parasites were treated with either homologous anti-$MSP1_{42}$ (FVO) or heterologous anti-$MSP1_{42}$ (3D7) antisera, Syto16 and HE staining detected similar levels of inhibition throughout the experiment. By the end of the experiment, the inhibition by anti-$MSP1_{42}$ (FVO) antisera, as detected by Syto16 and HE, was 51% and 53%, respectively ($P = 0.87$) and was substantially greater than the inhibition with the anti-$MSP1_{42}$ (3D7) antisera (5% and 9%, respectively, $P = 0.31$). These results indicate that the primary effect of antisera against the two alleles of $MSP1_{42}$ when tested against FVO strain *P. falciparum* is invasion inhibition. Thus, we were able to discern the different effects of the antisera on the various test strains by comparing results from DNA staining using Syto16 and the vital stain HE.

**Measurement of parasite metabolic activity.** Last, we sought to confirm the parasite growth inhibition detected with HE staining by quantifying pLDH levels. Figure 3 is representative of three experiments and shows that the parasites must reach early trophozoite stage to produce enough pLDH to meet the threshold of detection. It also shows that the measurement of pLDH activity is optimal at the end of schizogony because the OD values are maximal at this time. pLDH levels were not different in 3D7 strain cultures that were either not treated or treated with pooled control sera, whereas pLDH levels in FVO strain cultures that were treated with pooled control sera were slightly higher than in untreated cultures. Both of the anti-$MSP1_{42}$ (3D7) and (FVO)-specific sera caused growth inhibition when tested against the homologous parasite strain (21% and 60%, respectively) as well as the heterologous strain (20% and 41%, respectively). Considering the kinetics of the pLDH measurement and the trends observed for the various treatments, we conclude that measuring pLDH is a sensitive method for detecting viable parasites that were able to invade and continue to develop into trophozoites and schizonts in the presence of immune serum.

**Comparison of the methods for measuring the growth inhibitory activity of immune sera.** Figure 4 summarizes the mean levels of growth inhibition induced by three specific anti-sera (anti-$MSP1_{42}$ [3D7], anti-$MSP1_{42}$ [FVO], and anti-AMA1), each evaluated using four different methods (Giemsa, Syto16, HE, and pLDH) against the two parasite strains. In each test strain, the antisera produced similar patterns of inhibition among the four methods. Testing for the interaction between factors, antisera and method, was not significant. In addition, analysis of variance revealed significant main effects among methods and among antisera. The differences in growth inhibition among the four methods were stronger for the 3D7 test strain [$F(3,18) = 13.9$, $P < 0.001$] but still reached significance in the FVO strain [$F(3,18) = 3.2$, $P = 0.048$]. To further evaluate differences among the four methods, Tukey's post hoc test procedure was used. For the 3D7 test strain, there was no difference in detection of inhibition between the pair Giemsa and Syto16 or between the pair HE and pLDH, but detection of inhibition by either

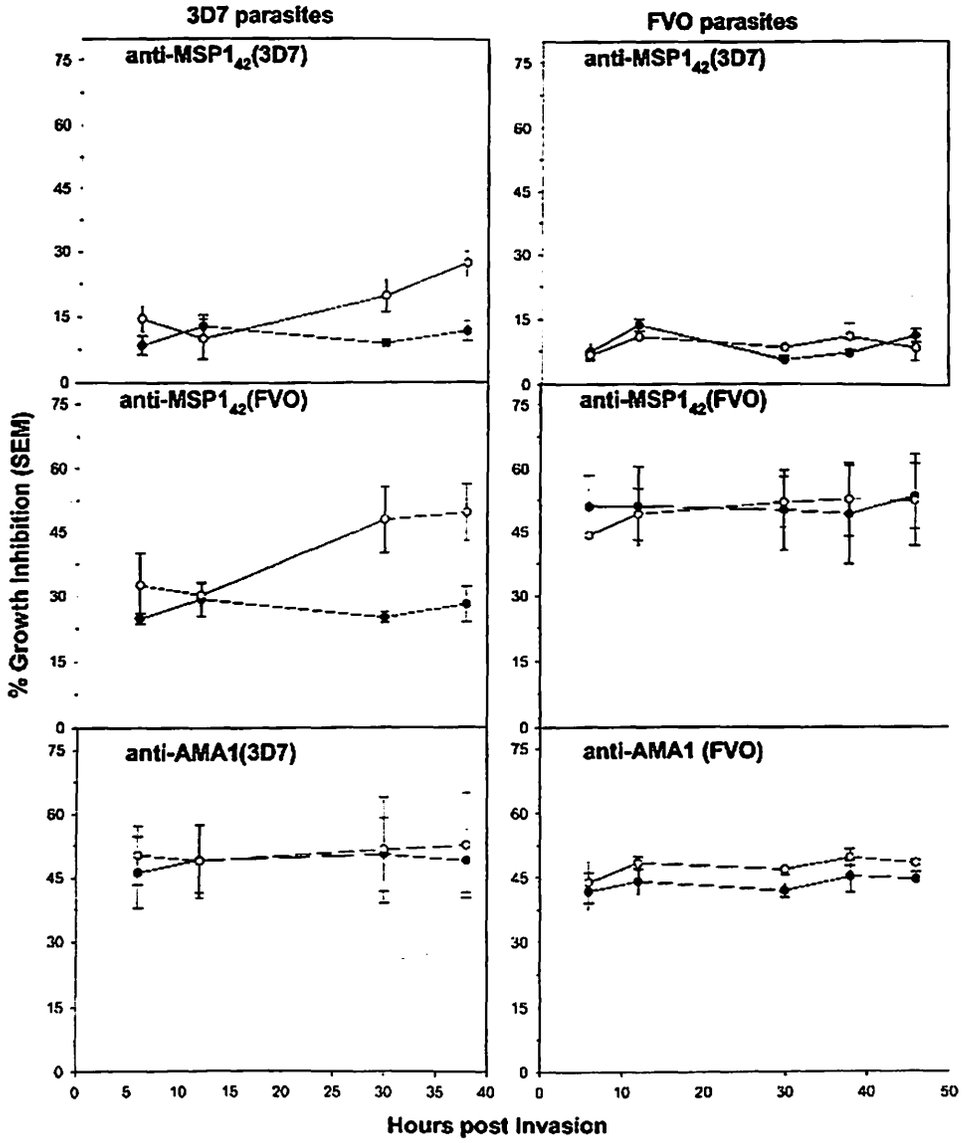


FIGURE 2. Syto16 (●) detects invasion inhibition and hydroethidine (○) detects both invasion and growth inhibitory effects of immune sera. Parallel cultures were established for each time-point at early schizont stage in the presence of 20% serum and harvested at the times indicated on the *x*-axis. The responses of 3D7 parasite cultures in the presence of indicated antisera are shown in the left column; the responses of the FVO parasites are shown in the right column. Data are expressed as mean percentage growth inhibition ± SEM of three independent experiments.

Giemsa or Syto16 was significantly less sensitive than detection by HE or pLDH. For the FVO test strain, pLDH consistently showed the strongest detection of inhibition across all three antisera; however, the Tukey procedure failed to reject any pairwise differences between methods.

## DISCUSSION

The evaluation of vaccines directed against the bloodstages of *P. falciparum* often consists of measuring vaccine induced antibody titers (e.g., by ELISA) in preclinical models and correlating these titers with some biologically relevant functional activity. It is widely considered that measuring the growth inhibitory capacity of an immune serum or antibody preparation will be one of the prime components for such an immune correlate. When selecting a technique for the evaluation of parasite inhibitory activities within immune sera, several aspects should be considered: 1) what does the selected technique actually measure, i.e., parasitemia or metabolic activity, 2) how sensitive and reproducible is the assay, and 3) how feasible is the sample preparation for large scale screening of sera. This study focuses on the first two points and compares the results obtained from methods that are based on either measurement of parasitemia and/or viability/metabolic activity of parasites. Figure 4 summarizes the inhibitory effect of the anti-MSP1$_{42}$ and anti-AMA1 antisera, as measured by microscopic analysis of blood smears, and flow cytometric analysis using Syto16, both of which measure parasitemia, as well as by flow cytometric analysis with HE and measuring pLDH, both of which measure parasite viability. Comparison of the results from the four methods by use of a general linear model shows that 3D7 strain parasites treated with anti-MSP1$_{42}$ antisera were more susceptible to killing by mechanisms that affect parasite viability than by inhibition of

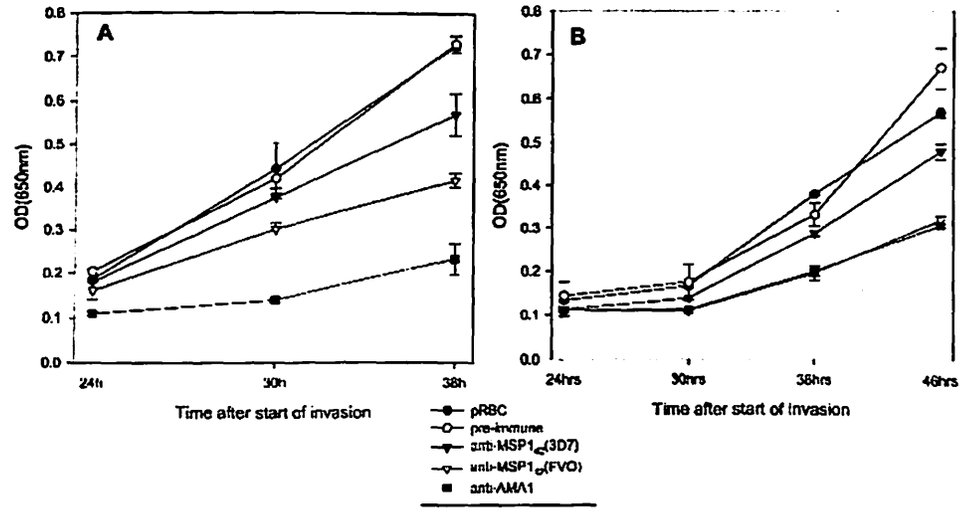


FIGURE 3. pLDH activity is diminished in parasite cultures incubated with immune serum. Parallel cultures of 3D7 parasites (A) and FVO parasites (B) were set up at the schizont stage (~6 hours before rupture begins) and incubated for various lengths of time with 20% of control serum, anti-MSP1$_{42}$ (3D7), anti-MSP1$_{42}$ (FVO), or anti-AMA1–specific serum (tested against the homologous strain only). Experiment is representative of three separate experiments; data shown are mean OD ± SD of triplicate cultures.

invasion, whereas FVO strain parasites treated with the same antisera seemed to be most susceptible to invasion inhibition. When treated with homologous antisera specific for AMA1, both strains were neutralized by invasion inhibition.

The idea that parasite viability can be affected by anti-MSP1 Ab after invasion is plausible owing to the fact that MSP1-specific Abs coat merozoites and can be found on the surface of ring stage parasites after invasion.[27] Our observation that MSP1$_{42}$-specific antisera can affect intracellular parasite viability is consistent with the results of another recent study, in which MSP1-specific antisera affected the progression of intracellular parasite development.[7] In that study, Giemsa-stained parasites were evaluated for their morphologies, and flow cytometry was used to differentiate and quantify parasite populations based on DNA content by staining with propidium iodide. We prefer to measure viability by either of the methods presented above over measurement of DNA content with propidium iodide because gate settings are subjective and vary between experiments, and it is not possible to distinguish between retarded trophozoite stage and newly developing ring stage parasites.

Our data showed that invasion inhibitory activities of Ab can be measured by any of the methods used in this study, whereas growth inhibitory activities are best measured by using methods that determine viability. We also show that some Ab work by more than one modality, which can vary with the test strain. We propose that the evaluation of bloodstage-specific antisera for preclinical or clinical evaluations include techniques that are based on vital stains such as HE and/or measurement of parasite metabolic activity such as the pLDH assay. These methods measure both invasion and growth inhibition and therefore reveal a greater portion of the spec-

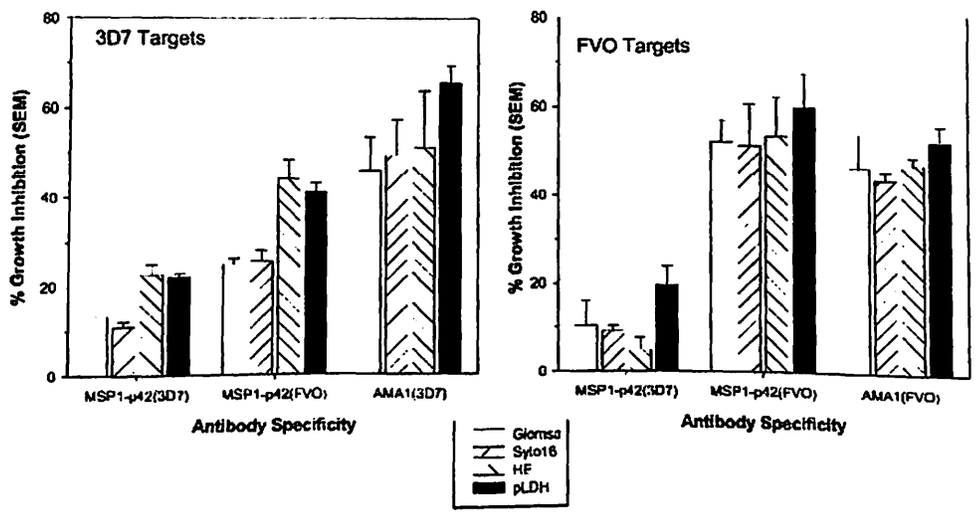


FIGURE 4. Methods evaluating the viability or metabolic activity of the parasite to measure a greater range of anti-parasite activity in immune sera. Growth inhibition of 3D7 parasites (left) and FVO parasites (right) induced by homologous or heterologous anti-MSP1$_{42}$ or homologous anti-AMA1 as measured by the various methods. Data are expressed as mean percentage inhibition ± SEM of three independent experiments.

trum of anti-parasite activities associated with the Ab being tested. We further propose that other unidentified parasite phenotypes might also be affected by Ab and that learning to measure these may also be useful for developing immune correlates.

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Disclaimer: Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition. The authors' views are private and are not to be construed as official policy of the Department of Defense or the US Army.

Authors' addresses: Elke S. Bergmann-Leitner, Elizabeth H. Duncan, John Robert Burge, Farhat Khan, Evelina Angov, and Jeffrey A. Lyon, Department of Immunology, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD. Gregory E. Mullen and Carole A. Long, Malaria Vaccine Development Branch, NIAID/NIH, 12441 Parklawn Drive, Twinbrook 2, Rockville, MD 20852.

## REFERENCES

1. Cohen S, Mc GI, Carrington S, 1961. Gamma-globulin and acquired immunity to human malaria. *Nature 192:* 733–737.
2. Butcher GA, Cohen S, Garnham PC, 1970. Passive immunity in *Plasmodium knowlesi* malaria. *Trans R Soc Trop Med Hyg 64:* 850–856.
3. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphajaisiddhi T, Druilhe P, 1990. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. *J Exp Med 172:* 1633–1641.
4. Groux H, Gysin J, 1990. Opsonization as an effector mechanism in human protection against asexual blood stages of *Plasmodium falciparum*: Functional role of IgG subclasses. *Res Immunol 141:* 529–542.
5. Perkins M, 1991. Approaches to study merozoite invasion of erythrocytes. *Res Immunol 141:* 662–665.
6. Ahlborg N, Iqbal J, Bjork L, Stahl S, Perlmann P, Berzins K, 1996. *Plasmodium falciparum*: Differential parasite growth inhibition mediated by antibodies to the antigen Pf332 and Pf155/RESA. *Exp Parasitol 82:* 155–163.
7. Wochlbier U, Epp C, Kauth CW, Lutz R, Long CA, Coulibaly B, Kouyate B, Arevalo-Herrera M, Herrera S, Bujard H, 2006. Analysis of antibodies directed against merozoite surface protein 1 of the human malaria parasite *Plasmodium falciparum*. *Infect Immun 74:* 1313–1322.
8. Green TJ, Morhardt M, Brackett RG, Jacobs RL, 1981. Serum inhibition of merozoite dispersal from *Plasmodium falciparum* schizonts: indicator of immune status. *Infect Immun 31:* 1203–1208.
9. Chulay JD, Aikawa M, Diggs C, Haynes JD, 1981. Inhibitory effects of immune monkey serum on synchronized *Plasmodium falciparum* cultures. *Am J Trop Med Hyg 30:* 12–19.
10. Pang X-L, Mitamura T, Horii T, 1999. Antibodies reactive with the N-terminal domain of *Plasmodium falciparum* serine repeat antigen inhibit cell proliferation by agglutinating merozoites and schizonts. *Infect Immun 67:* 1821–1827.
11. Tebo AE, Kremsner PG, Luty AJ, 2001. *Plasmodium falciparum*: A major role for IgG3 in antibody-dependent monocyte-mediated cellular inhibition of parasite growth in vitro. *Exp Parasitol 98:* 20–28.
12. Haynes JD, Moch JK, Smoot DS, 2002. Erythrocytic malaria growth or invasion inhibition assays with emphasis on suspension culture GIA. *Methods Mol Med 72:* 535–554.
13. Prudhomme JG, Sherman IW, 1999. A high capacity in vitro assay for measuring the cytoadherence of *Plasmodium falciparum*-infected erythrocytes. *J Immunol Meth 229:* 169–176.
14. Makler MT, Hinrichs DJ, 1993. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *Am J Trop Med Hyg 48:* 205–210.
15. Wyatt CR, Goff W, Davis WC, 1991. A flow cytometric method for assessing viability of intraerythrocytic hemoparasites. *J Immunol Meth 140:* 117–122.
16. van der Heyde HC, Elloso MM, vande Waa J, Schell K, Weidanz WP, 1995. Use of hydroethidine and flow cytometry to assess the effects of leukocytes on the malarial parasite *Plasmodium falciparum*. *Clin Diagn Lab Immunol 2:* 417–425.
17. Rahman NN, 1997. Evaluation of the sensitivity in vitro of *Plasmodium falciparum* and in vivo of *Plasmodium chabaudi* Malaria to various drugs and their combinations. *Med J Malaysia 52:* 390–398.
18. Bungener W, Nielsen G, 1968. Nucleic acid metabolism in experimental malaria. 2. Incorporation of adenosine and hypoxanthine into the nucleic acids of malaria parasites (*Plasmodium berghei* and *Plasmodium vinckei*). *Z Tropenmed Parasitol 19:* 185–197.
19. Kennedy MC, Wang J, Zhang Y, Miles AP, Chitsaz F, Saul A, Long CA, Miller LH, Stowers AW, 2002. In vitro studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. *Infect Immun 70:* 6948–6960.
20. Angov E, Aufiero BM, Turgeon AM, Van Handenhove M, Ockenhouse CF, Kester KE, Walsh DS, McBride JS, Dubois MC, Cohen J, Haynes JD, Eckels KH, Heppner DG, Ballou WR, Diggs CL, Lyon JA, 2003. Development and pre-clinical analysis of a *Plasmodium falciparum* merozoite surface protein-1(42) malaria vaccine. *Mol Biochem Parasitol 128:* 195–204.
21. Darko CA, Angov E, Collins WE, Bergmann-Leitner ES, Girouard AS, Hitt SL, McBride JS, Diggs CL, Holder AA, Long CA, Barnwell JW, Lyon JA, 2005. The clinical-grade 42-kilodalton fragment of merozoite surface protein 1 of *Plasmodium falciparum* strain FVO expressed in *Escherichia coli* protects *Aotus nancymai* against challenge with homologous erythrocytic-stage parasites. *Infect Immun 73:* 287–297.
22. Brand V, Sandu CD, Duranton C, Tanneur V, Lang KS, Huber SM, Lang F, 2003. Dependence of *Plasmodium falciparum in vitro* growth on the cation permeability of the human host erythrocyte. *Cell Physiol Biochem 13:* 347–356.
23. Healer J, Murphy V, Hodder AN, Masciantonio R, Gemmill AW, Anders RF, Cowman AF, Batchelor A, 2004. Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in *Plasmodium falciparum*. *Mol Microbiol 52:* 159–168.
24. Triglia T, Healer J, Caruana SR, Hodder AN, Anders RF, Crabb BS, Cowman AF, 2000. Apical membrane antigen 1 plays a central role in erythrocyte invasion by *Plasmodium* species. *Mol Microbiol 38:* 706–718.
25. Haynes JD, Moch JK, 2002. Automated synchronization of *Plasmodium falciparum* parasites by culture in a temperature-cycling incubator. *Methods Mol Med 72:* 489–497.
26. Miura K, Zhou H, Muratova OV, Miles A, Miller LH, Saul A, Long CA, 2006. Development and standardization of an in vitro *Plasmodium falciparum*, Growth inhibition assay utilizing measurements of lactate dehydrogenase (LDH) activity. *Mol Biochem Parasitol:* in press.
27. Blackman MJ, Scott-Finnigan TJ, Shai S, Holder AA, 1994. Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein. *J Exp Med 180:* 389–393.

**GURJEET KAUR**, SHARIFAH EMILIA TUAN SHARIFF, SYED HASSAN SYED ABD AZIZ, AMRI A RAHIM & RAHIMAH ABDULLAH, 2006. Concordance between endoscopic and histological gastroesophageal reflux disease. *Indian journal of gastroenterology*, 26 (1), 46-47.

## Concordance between endoscopic and histological gastroesophageal reflux disease

The gold standard for diagnosis of erosive Gastroesophageal reflux disease (GERD) is upper gastrointestinal endoscopy while there is presently no gold standard for the diagnosis for non-erosive GERD (NERD).[1] Though 24-hour esophageal pH monitoring can confirm the diagnosis of GERD, it is not widely available. Patients without obvious esophageal erosions are treated with a two-week course of proton pump inhibitor and their symptom response evaluated (PPI test). Histology does not appear to play a significant role in the diagnosis of GERD.[2,3]

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Eighty-one patients (median age 49 years, range 13-80; 42 male) who had reflux symptoms and were referred for upper GI endoscopy were recruited into the study. History of treatment with acid-suppressive drugs was noted and consent taken for esophageal biopsy. At endoscopy esophageal mucosa was assessed by trained endoscopists, and mucosal breaks classified using the LA classification.[4] Biopsies taken at 3 cm above the gastroesophageal junction were evaluated by a single pathologist, blind to the clinical findings. A histological diagnosis of GERD was made when there was coexistence of basal cell hyperplasia greater than 15% of mucosal thickness and papillary height greater than 50% of mucosal thickness.[5] Alcian blue/periodic acid Schiff (AB-PAS) stain was used to delineate the basal layer of the squamous epithelium (which is glycogen-depleted) and to demonstrate the presence of intestinal metaplasia in Barrett's esophagus.

The predominant symptom was retrosternal pain (66 subjects), followed by indigestion (54). Classical reflux symptoms of heartburn and regurgitation were elicited in 43.2% (35) and 38.3% (31) of subjects, respectively. Regurgitation was the only symptom that correlated significantly with endoscopy-positive GERD (Fisher's exact test, p=0.006).

Thirty six of the 81 subjects (44.4%) had erosive GERD with a majority having mild grades, i.e., LA grade A – 25 subjects, grade B – 7 subjects, grade C – 1 subject and grade D – 3 subjects. The esophageal mucosa was endoscopically normal in 39 (48.1%) subjects. Other findings included Barrett's esophagus (1 patient), white patches on esophageal mucosa (2), benign stricture (1), irregular Z line (1) and esophageal web (1). Hiatus hernia was present in 4 cases.

Histological GERD was diagnosed in 33.3% (27/81) of subjects. Less than half (15/36) the number of patients with erosive esophagitis showed histological evidence of GERD. In LA grade A, 9 of 25 subjects had histological GERD; grade B – 3 of 7 subjects; grade C – 1 of 1 subject and grade C – 2 of 3 subjects. In contrast, 12/45 subjects (26.7%) with non-erosive esophageal mucosa had evidence of histological GERD. Sixteen of 44 patients with classical reflux symptoms had evidence of histological GERD. The present study reports a 4.9% (4/81) prevalence of Barrett's esophagus by histology, which is slightly higher compared to other studies.[6] One of the 2 patients who had white patches on endoscopy had Candida infection. Two of the four cases with hiatus hernia had histological evidence of GERD. There was no significant difference in the presence of histological GERD between patients who had previous treatment with acid-suppressive agents and those who did not (Fisher's exact test, p=0.06).

One of the major findings in this study is the poor concordance between erosive GERD and histological evidence of GERD, throughout all the grades of the LA classification ($k$ value of 0.04 to 0.07). When all LA grades were grouped together as 'endoscopic GERD' and normal endoscopy was classified as 'no GERD', the agreement between endoscopy and histology remained poor ($k$ value 0.16). The concordance between endoscopy and histology was poor ($k$ value 0.025) even taking into consideration only patients with classical symptoms of GERD.

In conclusion, most patients in the present study suffered from NERD or mild erosive esophagitis. The macroscopic appearance of esophageal erosions did not correlate with classical histological features of reflux esophagitis. Conversely, a normal-looking esophagus was not proof of histologically normal mucosa.

Gurjeet Kaur, Sharifah Emilia Tuan Shariff, Syed Hassan Syed Abd Aziz, Amry A Rahim, Sarimah Abdullah

Universiti Sains Malaysia, Penang, Malaysia

## References

1. Fock KM, Talley N, Hunt R, Fass R, Nandurkar S, Lam SK, *et al.* Report of the Asia-Pacific consensus on the management of gastroesophageal reflux disease. *J Gastroenterol Hepatol* 2004;19:357-67.
2. Nandurkar S, Talley NJ, Martin CJ, Ng T, Adams S. Esophageal histology does not provide additional useful information over clinical assessment in identifying reflux patients presenting for esophagogastroduodenoscopy. *Dig Dis Sci* 2000;45:217-24.
3. Schindlbeck NE, Wiebecke B, Klauser AG, Voderholzer WA, Muller-Lissner SA. Diagnostic value of histology in non-erosive gastro-oesophageal reflux disease. *Gut* 1996;39:151-4.
4. Lundell LR, Dent J, Bennett JR, Blum AL, Armstrong D, Galmiche JP, *et al.* Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. *Gut* 1999;45:172-80.
5. Behar J, Sheahan D. Histologic abnormalities in reflux esophagitis. *Arch Pathol* 1975;99:387-91.
6. Goh KL, Chang CS, Fock KM, Ke M, Park HJ, Lam SK. Gastro-esophageal reflux disease in Asia. *J Gastroenterol Hepatol* 2000;15:230-8.

**Correspondence to: Dr Kaur, Advanced Medical and Dental Institute (Clinical Center), Universiti Sains Malaysia, No. 29 Lorong Bertam Indah 4/9, Taman Bertam Indah, 13200 Kepala Batas, Penang, Malaysia. E-mail: gurjeet@notes.usm.my**

HOGE EA, TAMRAKAR SM, CHRISTIAN KM, MAHARA N, **NEPAL MK**, POLLACK MH, SIMON NM, 2006. Cross-cultural differences in somatic presentation in patients with generalized anxiety disorder. *The journal of nervous and mental disease*, 194 (12), 962-966.

# Cross-Cultural Differences in Somatic Presentation in Patients With Generalized Anxiety Disorder

*Elizabeth A. Hoge, MD,* Sharad M. Tamrakar, MD,† Kelly M. Christian, BS,* Namrata Mahara, MD,† Mahendra K. Nepal, MD,† Mark H. Pollack, MD,* and Naomi M. Simon, MD, MSc**

**Abstract:** Little is known about cultural differences in the expression of distress in anxiety disorders. Previous cross-cultural studies of depression have found a greater somatic focus in Asian populations. We examined anxiety symptoms in patients with generalized anxiety disorder (GAD) in urban mental health settings in Nepal ($N = 30$) and in the United States ($N = 23$). Participants completed the Beck Anxiety Inventory (BAI). The overall BAI score and somatic and psychological subscales were compared. While there was no difference in total BAI scores, the Nepali group scored higher on the somatic subscale (i.e. "dizziness" and "indigestion," $t[df] = -2.63[50]$, $p < 0.05$), while the American group scored higher on the psychological subscale (i.e. "scared" and "nervous," $t[df] = 3.27[50]$, $p < 0.01$). Nepali patients with GAD had higher levels of somatic symptoms and lower levels of psychological symptoms than American patients with GAD. Possible explanations include differences in cultural traditions of describing distress and the mind-body dichotomy.

**Key Words:** Cross-cultural comparison, anxiety disorders, transcultural studies.

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Mood and anxiety disorders generally include symptoms comprised of somatic experiences (e.g., fatigue or palpitations) as well as psychological experiences (e.g., feeling sad or afraid). "Somatization" generally refers to the presentation of medically unexplained physical symptoms without reference to possible psychological origins (Kellner, 1990). In this article, to avoid confusion about the term "somatization," which may suggest somatoform disorders, we will use the term "somatic presentation," which instead refers to the patient's reported physical symptoms, separate from the issue of the patients' perceived cause of these symptoms.

*Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts; and †Tribhuvan University Teaching Hospital, Kathmandu, Nepal.

Send reprint requests to Elizabeth A. Hoge, MD, Department of Psychiatry, Massachusetts General Hospital, Simches Research Bldg., 185 Cambridge St., Suite 2200, Boston, MA 02114. E-mail: ehoge@partners.org.



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Many studies have reported a higher rate of somatic symptom presentation in Asian versus Western patients with depression. For example, Kleinman (1977) examined patients with depressive disorders in Taiwan and in America, and found that 88% of the Taiwanese patients first presented with somatic complaints, compared with 16% of the American patients. Similarly, Kleinman and Kleinman (1985) interviewed 100 Chinese patients with depressive disorders, and although all of the patients experienced dysphoria, somatic complaints were the predominant type of symptom presentation: 90% reported headaches and 73% reported dizziness as their chief complaint. Further, Lin et al. (1985) found that of 92 Vietnamese patients seeking medical care at an international community health center in Washington state, 95% of those who later met criteria for a diagnosis of depression presented with only physical symptoms such as headache, musculoskeletal pain, or shortness of breath. A similar emphasis on somatic symptoms has been found in studies carried out in India, the Philippines, Taiwan, and Hong Kong (for review see Cheng, 1989). Other cross-cultural studies have not found this difference (Hollifield et al., 2003; Simon et al., 1999). While this research suggests that some types of symptoms are preferentially reported, the patient's causal attribution (physical or emotional) is not known.

Previous research on somatic symptoms has focused on depression, and there is a relative lack of comparable data examining anxiety disorders. However, anxiety disorders such as panic disorder and generalized anxiety disorder (GAD) include somatic symptoms such as restlessness, muscle tension, sleep disturbance, palpitations, shortness of breath and dizziness prominently in their diagnostic criteria, so it remains unclear whether the differences seen in depression also apply to these anxiety disorders.

In addition, a criticism of previous research is that the data was collected in different health settings: Asian patients presented to primary care providers, and American patients often to mental health providers (Kirmayer, 2001), potentially biasing the findings. For example, the presentation in a medical or mental health setting may reflect the patient's causal attribution of the symptoms as due to a medical or psychological problem, respectively. In this study, we attempt to control for this potential confound by comparing subjects who present to mental health providers with a "chief complaint" of anxiety. The groups were both studied in a

psychiatry department in an urban general hospital, one in Boston, Massachusetts, the other in Kathmandu, Nepal. We hypothesized that Nepali individuals with GAD would have a greater somatic focus when asked about symptoms than American participants with GAD.

## METHODS

### Subjects

Subjects were male and female outpatients age 18 to 75 with GAD by DSM-IV criteria as assessed by the Structured Clinical Interview for DSM-IV (SCID-IV; First et al., 1997). Thirty subjects (33% female) were recruited from the general psychiatry clinic at the Tribhuvan University Teaching Hospital in Kathmandu, Nepal, and 23 (39% female) from the Center for Anxiety And Traumatic Stress Disorders Research Program at the Massachusetts General Hospital in Boston, Massachusetts.

### Interview and Diagnosis

Thirty consecutive patients presenting with a chief complaint of anxiety were assessed at the Tribhuvan University Teaching Hospital in Kathmandu, Nepal, with the translated version of the anxiety disorders section of the SCID-IV (First et al., 1997). Translation and back-translation of this instrument had been performed by Nepali study staff (S. M. T. and N. R.). Individuals with evidence of psychotic disorder were excluded. Patients who met criteria for GAD completed the Beck Anxiety Inventory (BAI), a 21-item questionnaire previously validated in Nepali (Beck et al., 1988; Kohrt et al., 2001). These instruments were used as part of standard clinical monitoring in this clinic, and were approved by the director of the Tribhuvan University Teaching Hospital.

American subjects were recruited from those with a primary diagnosis of GAD who had participated in a treatment study in 2003 and 2004. Subjects completed the BAI at baseline assessment, prior to treatment initiation. The Institutional Review Board at the Massachusetts General Hospital approved all studies, and all participants received and signed informed consent prior to study entry. At the initial interview, subjects were diagnosed with GAD using the SCID-IV; patients with a history of bipolar disorder or schizophrenia were excluded.

### Instruments

The BAI is a 21-item Likert self-report questionnaire measuring common symptoms of anxiety, such as "nervous" and "unable to relax." Each symptom is rated on a 4-point scale ranging from 0 (not at all) to 3 (severely, I could barely stand it), with possible total scores ranging from 0 to 63.

Kabacoff et al. (1997) performed a factor analysis of the BAI item pool, and found a two-factor structure, with one factor describing somatic aspects of anxiety and the other describing subjective aspects of anxiety comprised of psychological symptoms. We used these factors to examine separately the somatic items as a "somatic subscale" and the psychological items as a "psychological subscale" (Table 1).

**TABLE 1.** Beck Anxiety Inventory Subscale Items

| Somatic Subscale Items |
|---|
| Numbness or tingling |
| Feeling hot |
| Wobbliness in legs |
| Dizzy or lightheaded |
| Heart pounding or racing |
| Unsteady |
| Feeling of choking |
| Hands trembling |
| Shaky |
| Difficulty breathing |
| Indigestion or discomfort in abdomen |
| Faint |
| Face flushed |
| Sweating (not due to heat) |
| Psychological subscale items |
| Unable to relax |
| Fear of worst happening |
| Terrified |
| Nervous |
| Fear of losing control |
| Fear of dying |
| Scared |

Although DSM-IV diagnostic information was not available for the Nepali group, we felt it important to examine whether any differences seen might be explained by differences in depression comorbidity. Thus, we used a Beck Depression Inventory (BDI) score, completed concurrently with the BAI, to detect the possible presence of a major depressive episode. Prior research has found a score of 15 or higher on the BDI to have high sensitivity and specificity as an indicator of a major depressive episode (areas under the receiver operating characteristic curves of 0.81 and 0.93; Viinamaki et al., 2004). For the American group, the comorbidity of current major depressive disorder was determined by the SCID-IV.

### Data Analysis

As there was no evidence that the total BAI scores were not normal on tests of skewness and kurtosis, two-sided $t$ tests were used to analyze group differences in overall mean scores, subscale scores, and individual item scores of the BAI. Linear regression analyses were used to adjust for age and gender. We used a $p$ value of 0.05 for statistical significance, without adjustment for multiple testing.

## RESULTS

There was no significant difference in gender between the two groups, but the American sample was significantly older (mean = 45 ± 15 years) versus (mean = 31 ± 7 years; $t[df] = 4.44[58]$, $p < 0.001$). We compared the overall BAI score, somatic and psychological subscales, as well as single symptom item ratings for the Nepali and American GAD groups. The total BAI score was not significantly different between the Nepali (mean total score = 15 ± 4.0) and the

American (mean total score = 14.7 ± 7.4) groups ($t[df]$ = −0.19[49], $p$ = 0.8). Nepali subjects had higher scores on the somatic subscale (Nepal group = 10.4 ± 3.1, American group = 7.5 ± 4.6; $t[df]$ = −2.63[50], $p < 0.05$), while American subjects had higher scores on the psychological subscale (Nepal group = 4.6 ± 1.9, American group = 7.3 ± 4.0, $t[df]$ = 3.27[50], $p < 0.01$). Single items that were significantly higher in the Nepal group were "numbness or tingling" ($p < 0.001$), "wobbliness in legs" ($p < 0.001$), "dizzy or lightheaded" ($p < 0.001$), "feeling of choking" ($p < 0.001$), "indigestion or discomfort in abdomen" ($p < 0.001$), "feeling hot" ($p < 0.05$), "hands trembling" ($p < 0.05$), and "faint" ($p < 0.05$). Items significantly higher in the American group were "unable to relax" ($p < 0.001$), "nervous" ($p < 0.01$), and "fear of dying" ($p < 0.05$; see Figures 1 to 3). The number of subjects in the analyses fluctuates because of single missing items for two subjects (American), who were not included in the total score analyses or relevant subscale analyses.

Although there was no significant difference in rates of current depression comorbidity in the two samples (FET $p$ = NS), 33.3% (10/30) of the Nepali sample and 13.0% (3/23) of the US sample were found to have a current major depressive episode; this lack of statistical significance may be due to the relatively small sample size. Thus, to control for the potential contribution of age, gender, and depression comorbidity, we performed a regression analysis of each BAI subscale score with these covariates. For the psychological subscale, scores remained lower for the Nepali group compared with the US group (group $\beta$ = −2.44, $t$ = −2.31, $p$ = 0.03), and somatic subscale scores were higher for the Nepali compared with the US group (group $\beta$ = 2.88, $t$ = 2.18, $p$ = 0.03).

## DISCUSSION

We found that Nepali patients with GAD had higher rates of somatic complaints than the American patients with GAD. This study of GAD is consistent with prior studies of depression showing that individuals from Asian cultures, relative to those from Western cultures, tend to emphasize somatic symptoms in depression. Various explanations have been given for the tendency to focus on somatic symptoms. Some researchers have hypothesized that stigmatization of mental illness in some cultures leads patients to minimize emotional distress and emphasize somatic symptoms. However, our data suggest that even patients seeking care in a mental health setting report more somatic symptoms, suggesting the need to explore explanations beyond stigma. For example, some researchers have emphasized the importance of the cultural conceptualization of health: traditional medicine in many parts of Asia does not distinguish between mind and body, making distinctions in symptom type irrelevant and increasing the likelihood that individuals will manifest psychological distress with somatic symptomatology (Hsu, 1999). Another theory suggests that Asian cultures do not have the same semantic framework for conceptualizing or expressing affect (Tseng, 1973) and are less inclined toward intense introspection of personal affective states than are those from Western cultures. In this way, some emotions may be difficult to articulate. Cultural factors may also influence acceptable patterns of expression, leading to increased discomfort in discussing emotional difficulties. A focus on somatic presentation of psychological distress is observed in a variety of cultures, and some researchers have pointed out that since it is more widely found, it may represent the norm, and that the Western "psychologization" is the pattern that merits explanation (Hsu and Folstein, 1997).

Regardless, it is important to note that symptom presentation style does not necessarily indicate the patient's explanatory model of their symptoms. For example, in his review on the subject of somatization, Kirmayer (2001) suggested that somatic presentation may serve as a "ticket

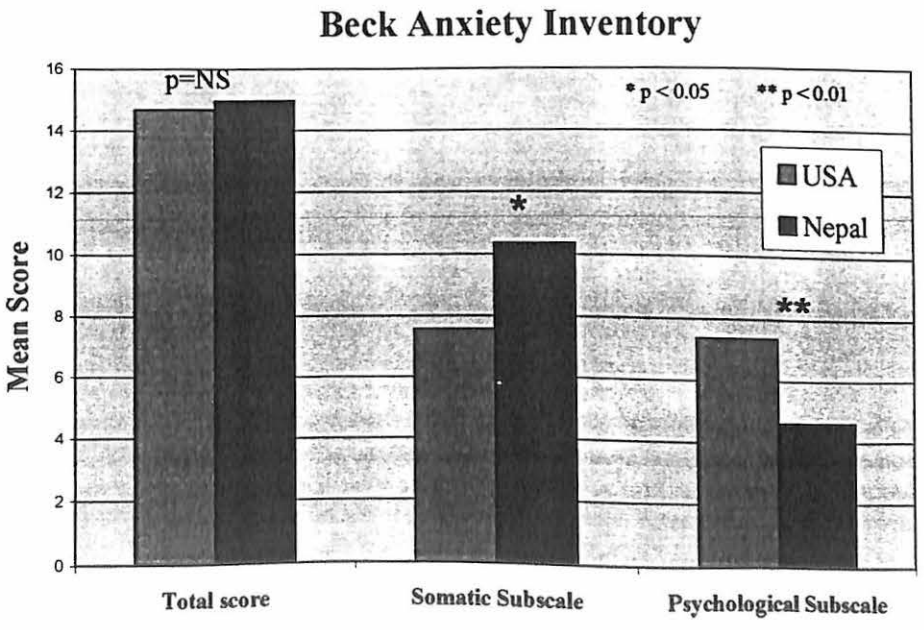


Figure 1. Comparison of total scores and subscale scores for the BAI in Nepali and American GAD subjects.

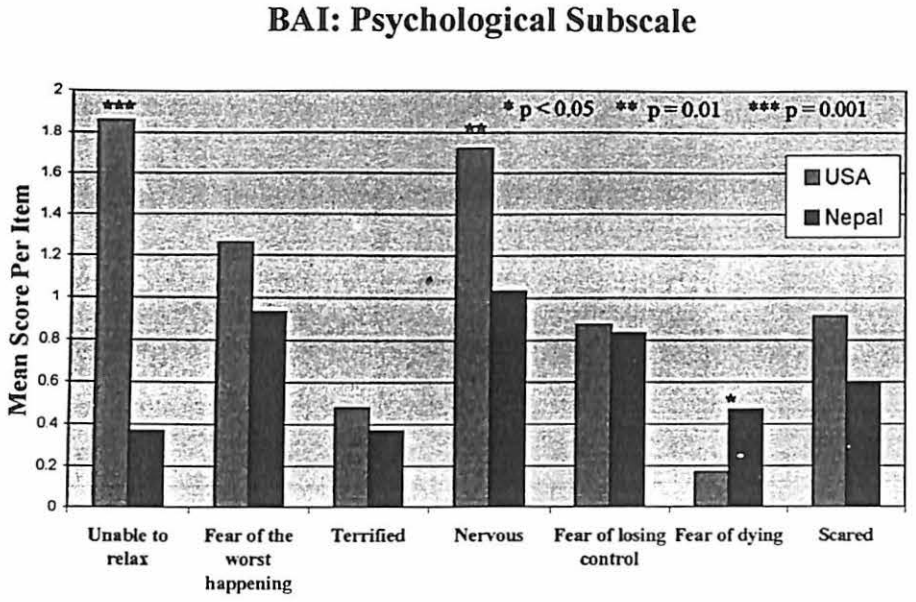


Figure 2. Comparison of individual items of the BAI psychological subscale in Nepali and American GAD subjects.

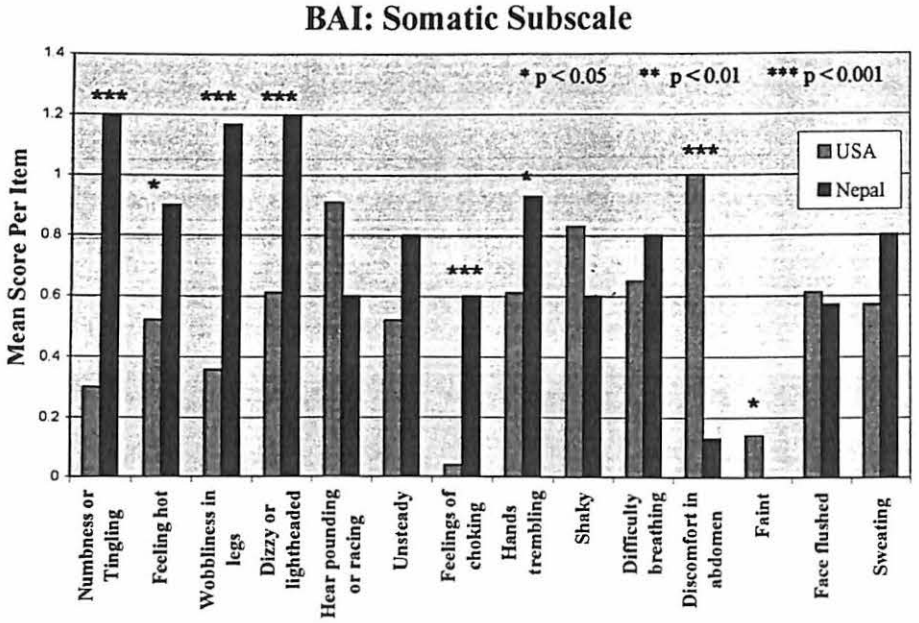


Figure 3. Comparison of individual items of the BAI somatic subscale in Nepali and American GAD subjects.

behavior" with the somatic symptom serving as "an appropriate and nonstigmatized reason to seek help from a biomedical practitioner." In this way, the patient can access help even if they see the cause of their symptoms as psychological or a result of stress. In exploring the patient's perceived cause of somatic symptoms, some researchers have reported that Asian patients are more likely to persist in attributing somatic complaints to physical causes, even when psychosocial causes are suggested (Hsu and Folstein, 1997). Others have found this pattern to exist equally in Western and non-Western cultures (Kirmayer, 2001). However, our data do not allow dissection of these possibilities.

Limitations of this study include a relatively small sample size and lack of adjustment for multiple testing. Further research in this area should use larger sample sizes from more than one site per country, and should measure the effect of comorbid depression. In addition, it is possible differences between a clinical versus research patient population may be relevant. Some researchers have noted that patients participating in a research trial may not represent patients in a general clinical practice, because research exclusion criteria may affect comorbidity patterns and illness severity (Zimmerman et al., 2002). However, in the present study, BAI total scores, reflecting severity of GAD, were the same for the research and clinical populations. In addition, similar exclusion criteria were employed in both groups: psychotic disorders were excluded but depression was allowed as long as GAD was the primary disorder (i.e., causing

the most distress). Finally, while our finding remained after controlling for current comorbid depression, our determination of current depression differed for the two groups.

General medical practitioners should be aware of this somatic focus when evaluating patients from different cultures, since the focus on somatic symptoms may obscure psychological or emotional distress. Even when the patient's chief complaints are of a purely somatic nature, careful assessment may uncover an extant mental disorder that may respond to appropriate treatment and reduce the overall level of distress. Further research is needed to understand culturally based explanatory models for a somatic focus in mood and anxiety disorders, and their impact on compliance with and response to treatment interventions.

## REFERENCES


Beck AT, Epstein N, Brown G, Steer RA (1988) Related articles, properties. *J Consult Clin Psychol.* 56:893–897.

Cheng TA (1989) Symptomatology of minor psychiatric morbidity: A crosscultural comparison. *Psychol Med.* 19:697–708.

First MB, Spitzer RL, Gibbon M, Williams JBW (1997) Structured Clinical Interview for DSM-IV Axis I Disorders. Washington (DC): American Psychiatric Press.

Hollifield M, Finley R, Skipper B (2003) Panic disorder phenomenology in urban self-identified Caucasian-non-Hispanics and Caucasian-Hispanics. *Depress Anxiety.* 18:7–17.

Hsu LK, Folstein MF (1997) Somatoform disorders in Caucasian and Chinese Americans. *J Nerv Ment Dis.* 185:382–387.

Hsu SI (1999) Somatisation among Asian refugees and immigrants as a culturally-shaped illness behaviour. *Ann Acad Med Singapore.* 28:841–845.

Kabacoff RI, Segal DL, Hersen M, Van Hasselt VB (1997) Psychometric properties and diagnostic utility of the Beck Anxiety Inventory and the State-Trait Anxiety Inventory with older adult psychiatric outpatients. *J Anxiety Disord.* 11:33–47.

Kellner R (1990) Somatization: Theories and research. *J Nerv Ment Dis.* 178:150–160.

Kirmayer LJ (1984) Cultural affect and somatization. *Transcultural Psychiatr Res Rev.* 21:159–188.

Kirmayer LJ (2001) Cultural variations in the clinical presentation of depression and anxiety: Implications for diagnosis and treatment. *J Clin Psychiatry.* 62:22–28.

Kleinman A, Kleinman J (1985) Somatization: The interconnections in Chinese society among culture, depressive experiences and the meaning of pain. In A Kleinman, B Good (Eds), *Culture and Depression* (pp 429–490). Berkeley: University of California Press.

Kleinman A (1977) Depression, somatization and the "new cross-cultural psychiatry." *Soc Sci Med.* 11:3–10.

Kohrt BA, Kunz RD, Koirala NR, Sharma VD, Nepal MK (2001) Validation of the Nepali version of Beck Depression Inventory. *Nepalese J Psychiatry.* 2:123–130.

Lin EH, Ihle LJ, Tazuma L (1985) Depression among Vietnamese refugees in a primary care clinic. *Am J Med.* 78:41–44.

Simon GE, VonKorff M, Piccinelli M, Fullerton C, Ormel J (1999) An international study of the relation between somatic symptoms and depression. *N Engl J Med.* 341:1329–1335.

Tseng WS (1973) The development of psychiatric concepts in traditional Chinese medicine. *Arch Gen Psychiatry.* 29:569–575.

Viinamaki H, Tanskanen A, Honkalampi K, Koivumaa-Honkanen H, Haatainen K, Kaustio O, Hintikka J (2004) Is the Beck Depression Inventory suitable for screening major depression in different phases of the disease? *Nord J Psychiatry.* 58:49–53.

Zimmerman M, Mattia JI, Posternak MA (2002) Are subjects in pharmacological treatment trials of depression representative of patients in routine clinical practice? *Am J Psychiatry.* 159:469–473.

**KAMIL SM**, MOHAMAD NH, **NARAZAH MY**, **KHAN FA**, 2006. Dengue haemorrhagic fever with unusual prolonged thrombocytopaenia. *Singapore Medical Journal*, 47 (4), 332-334.

# Dengue haemorrhagic fever with unusual prolonged thrombocytopaenia

Kamil SM, Mohamad N H, Narazah M Y, Khan F A


## ABSTRACT

**We describe a case of dengue haemorrhagic fever with prolonged thrombocytopaenia. A 22-year-old Malay man with no prior illness presented with a history of fever and generalised macular rash of four days duration. Initial work-up suggested the diagnosis of dengue haemorrhagic fever based on thrombocytopaenia and positive dengue serology. Patient recovered from acute illness by day ten, and was discharged from the hospital with improving platelet count. He was then noted to have declining platelet count on follow-up and required another hospital admission on day 19 of his illness because of declining platelet count. The patient remained hospitalised till day 44 of his illness and managed with repeated platelet transfusion and supportive care till he recovered spontaneously.**


**Keywords: dengue haemorrhagic fever, macular rash, mosquito, platelet transfusion, prolonged thrombocytopaenia, thrombocytopaenia**

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Advanced Medical and Dental Institute University Sains Malaysia
29 Lorong Taman Bertam Indah 4/9 Taman Bertam Indah 13200 Kepala Batas Pulau Pinang Malaysia

Kamil SM, MBBS, FCPS Consultant Physician and Clinical Oncologist

Narazah M Y, MBBS, MMed, PhD Consultant

Khan F A, MBBS, MD Consultant

Hospital Kepala Batas 13200 Kepala Batas Pulau Pinang Malaysia

Mohamad N H, MBBS Medical Officer

Correspondence to: Dr Muhammad Kamil Sheikh Tel: (60) 4 579 2006/1990 Fax: (60) 579 1570 Email: kamilpk2001@yahoo.com

## INTRODUCTION

Dengue fever is an acute viral illness. It has a spectrum ranging from mild illness to serious shock-like state with significant mortality. Dengue fever is usually a self-limited mild illness if detected early and managed properly. It is transmitted to humans by the mosquito *Aedes aegypti*. Annually, there are an estimated 50 to 100 million cases of dengue fever and 250,000 to 500,000 cases of dengue haemorrhagic fever (DHF) in the world, and over half of the world's population live in areas at risk of dengue fever[1,2]. The case fatality rate in patients with dengue shock syndrome can be as high as 44%. The epidemic of dengue fever originated in Southeast Asia (Manila) in 1953 but now the disease has spread to India, Pakistan, Sri Lanka and China[1]. Dengue fever needs to be recognised early and managed properly to decrease its mortality.

## CASE REPORT

A 22-year-old Malay man with no previous past medical history, presented with fever and generalised macular rash of four days duration. He was residing in an army camp and had no history of recent travel abroad. He was admitted to hospital with a provisional diagnosis of dengue fever, based on his low platelet count at presentation. The diagnosis of dengue fever was confirmed later by positive dengue serology. The patient's platelet count was 7,000/$\mu$L (normal range: 150,000-400,000/$\mu$L) at presentation but no active bleeding was noted. He was discharged from hospital on day 12 of his illness. He required 12 units of platelets to maintain his platelet count in the range of 30,000 to 40,000/$\mu$L. At the time of discharge from hospital, his platelet count was 61,000/$\mu$L and he was asymptomatic. He was advised regular follow-up at a local clinic with regular checks on platelet count and the platelet count improved to 130,000/$\mu$L.

On day 19 of his illness, his platelet count again dropped to 89,000/$\mu$L with a high haematocrit. He was again admitted to hospital and worked-up for concurrent infection but work-up for sepsis was negative. His platelet count dropped again and remained low till day 40 of his illness, and the lowest platelet count of 7,000/$\mu$L was noted on day 38. During this hospital admission, 35 units of platelets were transfused. At this point of time, possibilities of underlying platelet disorder or development of anti-platelet antibodies were considered but the patients' platelet count started improving spontaneously by day 39 and reached 163,000/$\mu$L on day 44 of his illness. The pattern of prolonged thrombocytopaenia with initial recovery was unusual in our case.

## DISCUSSION

Dengue is the most important human viral disease transmitted by arthropod vectors. Dengue is a homonym for the African Ki denga pepo, which first appeared in English literature during an 1827-28 Caribbean outbreak. Benjamin Rush described the first case of dengue in 1789. DHF and dengue shock syndrome are now leading causes of hospital admissions and deaths among children in Asia[3-4].

Dengue fever is caused by four dengue viruses labelled as types 1, 2, 3 and 4. Clinical features of dengue virus infection range from mild illness to shock leading to death. Dengue fever is an acute febrile illness lasting five to six days. Headache, retroocular pain, muscle and joint pain, vomiting and rash are common manifestations[5,6]. The virus disappears from the blood after an average of five days[7,8]. DHF is characterised by a bleeding tendency, secondary to thrombocytopaenia and evidence of plasma leakage as determined by a rising haematocrit.

Dengue shock syndrome is defined as dengue fever with signs of circulatory failure. The prognosis depends on prevention or early recognition and treatment. Case fatality rate is as high as 12% to 14% once shock has set in. Other severe manifestations described with dengue syndrome include hepatic damage, cardiomyopathy and encephalopathy[9,10]. In a typical case of dengue fever, thrombocytopaenia is seen on days five to six, and the mean duration of thrombocytopaenia is few days[11]. In our case, thrombocytopaenia lasted more than a month after initial recovery. This pattern of thrombocytopaenia is unusual for dengue.

Laboratory diagnosis requires serum for virological or serological studies. The virus can be isolated during the febrile period, which is usually around five days. The virus may be isolated utilising cell culture and detected rapidly by using PCR but this is not available freely and only is experimental[12]. Serological diagnosis requires either a presence of IgM antibody or a rise in IgG antibody in paired acute and convalescent phase serum. IgM antibody may be detected in 90% of patients by day six, and will remain positive for 60 days.

Currently, the most common IgM assay is a capture ELISA (enzyme linked immunosorbent assay)[13]. IgG can be measured by haemagglutination inhibition test or ELISA. Confirmed diagnosis of dengue requires isolation of the virus but a probable diagnosis can be made on positive serology. Paired acute and convalescent samples are more suggestive of current infection rather than single IgM titre which may reflect a recent infection as long as 30 days ago.

Management of dengue fever requires rest, oral fluids to compensate for losses via diarrhoea or vomiting, antipyretics and analgesics. Intravenous fluid may be required for few days since the period of vasculopathy causing plasma leakage may be short, lasting only a few days. Plasma leakage is evidenced by a rising haematocrit. Patients who present with shock may require central venous pressure monitoring. An arterial line may be required in unstable patients for the assessment of blood gases, electrolytes and coagulation profile to help identify patients needing ventilatory support. Insertions of vascular lines should be done under blood products support in view of the thrombocytopaenia and possible coagulopathies.

Patients should remain in hospital till at least day three of recovery from shock. A decision for discharge from hospital may be made once the patient is stable and platelet counts are greater than 50,000/$\mu$L. In our case, the patient suffered prolonged thrombocytopaenia following DHF, which is unusual for this infection. The possibility of the patient acquiring bacterial infection during recovery phase leading to sepsis was considered, but work-up for sepsis was negative. The possibility of underlying platelet disorder was also considered, however, the patient recovered spontaneously. We postulate that this could be an unusual or mutated strain of the dengue virus. A further consideration was that the patient acquired another infection of dengue virus with a different strain during the recovery phase. However, the patient was not febrile at the time of second presentation to the hospital and the isolation of the virus is only possible during the short febrile period, which corresponds to the period of viraemia.

## REFERENCES


1. Gubler DJ. Dengue and dengue hemorrhagic fever; its history and resurgence as a global public problem. In: Gubler DJ, Kuno G, eds. Dengue and Dengue Hemorrhagic Fever. Wallingford, UK: CAB International, 1997.
2. Pinheiro FP, Corber SJ. Global situation of dengue and dengue haemorrhagic fever, and its emergence in the Americas. World Health Stat Q 1997; 50:161-9.
3. World Health Organization. In: Dengue haemorrhagic fever: diagnosis, treatment and control. WHO, Geneva, 1986.
4. Kalayanarooj S, Vaughn DW, Nimmannitya S, et al. Early clinical and laboratory indicators of acute dengue illness. J Infect Dis 1997; 176:313-21.
5. Cobra C, Rigau-perez JG, Kuno G, et al. Symptoms of dengue fever in relation to host immunologic responses and virus serotype, Puerto Rico, 1990-1991. Am J Epidemiol 1995; 142:1204-11.