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Review

STRATEGY TOWARDS MEASLES ELIMINATION —PROGRESS IN THE ENGLISH-SPEAKING CARIBBEAN COUNTRIES—

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Abstract: The strategy currently used to control and eliminate measles was reviewed on the basis of the achievement in Americas, especially in the English-speaking Caribbean countries. The author participated in one phase of the strategy; measles follow-up immunization campaign in Belize, and reviewed the achievement of the campaign. Several factors were identified as important factors for the success of mass immunization campaign. According to the review and experience of the strategy and its practice for measles elimination in the Americas, the author discussed the importance of political commitment, enthusiastic community's participation and establishment of effective surveillance system for the success of measles elimination program. Although there are still several obstacles for the development of the measles elimination program to other regions, the most important components are the strong political commitment in each country and international cooperation financially and technically. On this point of view, Japan is able to play an important roll towards measles elimination in Asia.

Key words: measles, immunization, elimination, Pan American Health Organization, English-speaking Caribbean countries

INTRODUCTION

There is a new, large scale measles control program of Pan American Health Organization (PAHO)/World Health Organization (WHO), which is intended to eliminate the circulation of measles virus in the Americas. Although the program has been functioning successfully since 1991, there is much to learn about the program through evaluation and assessment. The author participated in one of the ongoing programs, and had the opportunity to review and evaluate one phase of it; the measles follow-up immunization campaign in Belize. In this paper, the author reviewed the strategy and its practice, and considered its development in other regions.

1. Public health importance of measles

Although measles is a ubiquitous and highly infectious compared with other infectious diseases such as varicella and mumps, there is a strong preventive measure against measles; vaccine. The vaccines currently in use are attenuated live measles virus, and many countries prefer the combined MMR (measles-mumps-rubella) vaccine to ensure that immunity is obtained for all three viruses (Cutts and Markowitz, 1994).

Despite the availability of measles vaccine for more than thirty years, WHO estimates that more than one million children worldwide die each year due to measles and its complications (EPI/WHO, 1994). Moreover, sequelae from measles, possibly related to nutritional effects and altered immune status, may be expressed for many months after infection. Thus, the true number dying annually as a result of measles may be twice the estimated one million if the delayed effects of the disease are taken into account (Gellin and Katz, 1994). Therefore, measles ranks as a leading cause of childhood mortality in the world, and the impact of measles on the lives of millions of young children every year, particularly in developing countries, indicates the urgent need for further reductions in measles incidence (Clements *et al.*, 1992). In September 1994, the Pan American Sanitary Conference decided that measles virus should

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no longer be a threat to the health of infants and children of the Western Hemisphere and voted unanimously to establish the goal of measles elimination from the Americas by the year 2000 (PAHO/WHO, 1994).

2. Strategies towards measles elimination in Americas

The program for measles elimination relied on experiences gained during two successful eradication programs which were conducted worldwide during the past thirty years; smallpox and poliomyelitis. These two target diseases as well as measles have common characteristics which imply that eradication may be feasible; 1) man is the only host of the virus, 2) inexpensive vaccine provides long-lasting immunity among vaccinees. In the smallpox eradication campaign, the major strategies were surveillance and containment, which included surveillance for the detection of smallpox cases and tracing of the source of infection and chains of transmission, with vaccination targeted to all contacts in the infected areas. These strategies were effective and led to the ultimate worldwide eradication of smallpox, in part because smallpox virus spreads more slowly than measles virus. In the poliomyelitis eradication program, country-wide immunization was achieved by national immunization days in order to quickly increase population immunity levels and to interrupt chains of poliovirus transmission. This strategy was supplemented by house-to-house vaccination in areas with low immunization coverage (mop-up immunization activity). Since more than 95% of polio virus infections are inapparent or induce nonspecific symptoms, a containment of outbreaks was unable to be conducted as was possible with smallpox. Nevertheless, a surveillance system for the detection of suspected polio cases and a laboratory network were created to confirm the presence of wild virus. These were important in order to monitor progress of the program (de Quadros *et al.*, 1996).

The strategy for measles elimination which is currently being implemented in the Americas has several stages. First, the transmission of measles virus is interrupted by vaccinating all children between nine months and fourteen years of age in a short period (**catch-up immunization campaign**). Second, a special vaccination activity (**mop-up immunization**) is then carried out in low coverage areas. Third, efforts are made to sustain high immunization coverage by routine immunization activities, which are incorporated in the public health services, through improved access to vaccination services including mobile house-to-house vaccination services. Fourth, periodic immunization campaigns for children from one to five years of age (**follow-up immunization campaigns**) are conducted in order to decrease the accumulated susceptibles to measles. They are conducted whenever estimates of the number of susceptibles from one to five years of age approaches the number of an average birth cohort. This arbitrary interval between campaigns is empirically best and will be evaluated as the program progresses. A surveillance system for measles (**rash and fever illness surveillance**) is being established and investigation and control measures for each reported outbreak are being conducted (**outbreak response and prevention**) (de Quadros *et al.*, 1996). These strategies have been implemented successfully in the English-speaking Caribbean countries since 1991, and, so far, they have achieved a measles-free status, which means there is no indigenous measles in this area, for the first time in this region's history.

3. Achievement in the English-speaking Caribbean countries

The English-speaking Caribbean countries initiated the first stage of measles elimination in May 1991. May was "Measles Immunization Month" during which the measles catch-up immunization campaign was conducted in the eighteen countries in order to vaccinate all children between nine months and fourteen years of age, regardless of their previous history of disease and immunization. The target population for this campaign was about 1,775,000 children. The achievement was remarkably successful with 91.4% overall coverage. Mop-up measles immunization activities were continued through June and July of 1991, and considerable numbers of children who were missed during May were immunized.

The measles catch-up immunization campaign to interrupt measles virus transmission was an important first step in the strategy of measles elimination. Very high levels of immunization coverage have been maintained by scheduling periodically an "immunization month", during which mop-up immunization activities are conducted in low immunization coverage areas by improving access to vaccination services, including the use of mobile immunization teams for hard-to-reach areas. As a result, an overall immunization coverage level of over 80% has been maintained in almost all countries since the measles catch-up immunization campaign in 1991.

After the campaign, surveillance systems were implemented which called for a weekly report of suspected measles cases (rash and fever illness). This system was intended to detect all suspected measles cases, which

were defined as either 1) any case which a trained health care worker suspects to be measles, or 2) any case with macropapular rash, fever, and one of the following: cough, coryza, or conjunctivitis. Each suspected measles case is supposed to be investigated using serological diagnosis, and either classified as "confirmed" or discarded if another diagnosis is made. If confirmed, appropriate control measures are carried out. Over five hundred reporting sites are incorporated in this surveillance system. The sites include persons and institutions which are likely to see cases of measles. These comprise health centers, hospitals, private physicians, *etc.* About 85% of them report every week. Between January and May of 1991, prior to the campaign, 301 cases of measles were reported from the English-speaking Caribbean countries. From June onwards, reports of measles cases rapidly declined. During September and October of 1991, 105 suspected measles cases were reported, but only two cases were confirmed as measles; one was an imported case, the other was lost to follow-up. Many of the suspected measles cases were diagnosed as dengue fever or rubella (Hospedales, 1992). However, from 1992 until the end of 1995, there were no confirmed measles cases (PAHO/WHO, 1995). Thus, measles transmission appears to have been interrupted in these countries.

Measles Elimination in Belize

The author had a chance to participate in the measles follow-up immunization campaign in Belize, and evaluated it using questionnaires and interviews to public health nurses in each district. Through this process, several factors were identified as critical points for the success of mass immunization campaign.

1. Background of Belize

Belize, one of the member countries of the English-speaking Caribbean nations, is a small country, located on the eastern seaboard of the Central American isthmus. It is bordered in the north by Mexico and in the south and west by Guatemala. It has a sparse but extremely diverse population. The estimated total population in 1993 was 205,000; almost 45% of the population is under the age of fifteen years (Central Statistic Office, Ministry of Finance, Belize, 1994). There are six administrative districts: Corozal, Orange Walk, Belize, Cayo, Stann Creek, and Toledo. In recent years, Belize has experienced high levels of immigration with approximately 30,000 people each year from neighboring Central American countries. One of the most unique characteristics of Belize is its variety of ethnic groups: Mestizo, Creole, Maya, Carifuna, Ketchi, Mennonite, and so on (UNICEF, 1995). As a result, there are several difficulties in conducting public health activities, because some of them use their own language and are geographically isolated from others.

2. Measles control in Belize

Belize had been trying to intensify its immunization programme in order to achieve an immunization coverage of at least 80% among children under one year of age against the WHO's Expanded Programme on Immunization (EPI) target diseases (diphtheria, pertussis, tetanus, tuberculosis, poliomyelitis, and measles). Although immunization coverage with Bacille Calmette-Guerin vaccine (BCG) had reached over 80%, the coverage with diphtheria-tetanus-pertussis vaccine (DTP), oral polio vaccine (OPV) and measles vaccine continued to be unsatisfactory (40-60%). In 1986, Belize, in collaboration with PAHO, United Nations Children's Fund (UNICEF), and Rotary International, decided to initiate a national EPI campaign to increase coverage with BCG, DTP, OPV and measles vaccine. As a result, Belize achieved a coverage of over 80% immunization for all the EPI target diseases among children under one year of age (UNICEF, 1995). In May 1991, the measles catch-up immunization campaign, which covered all children from nine months through fourteen years of age, was implemented in the effort to eliminate measles from the Americas. During the campaign, 67,200 children (84% of the target group) were vaccinated with measles vaccine. Since then, the immunization coverage with BCG, DTP, OPV and measles vaccine have been sustained at a high level (over 80%), and there have been no reported measles cases in Belize since 1992 (Ministry of Health, Belize, 1991-1995).

3. Measles follow-up immunization campaign in Belize

The measles catch-up immunization campaign dramatically decreased the number of susceptibles. However, during routine immunization activities after the 1991 campaign, the proportion of susceptibles began to rise. Yearly immunization coverage remained at 80 to 90% overall and some who were vaccinated were not protected because the vaccine is only 90 to 95% effective under field conditions. Additionally, some vaccine may have been impotent due to cold chain failures or inappropriate vaccine administration. As a result, the number of susceptibles to measles steadily accumulated.

Since measles immunization is given in Belize at twelve months of age, there are in any year, approxi-

mately 7,500 infants (all infants under one year of age) unimmunzed in Belize. If it is assumed that 30% of these infants are unprotected either by lack or loss of maternal antibody during their first year of life, this would provide about 2,000 susceptibles in the under one year age group. In addition, if it is assumed that measles vaccination coverage was 80% each year and that vaccine efficacy was 90%, approximately 2,000 susceptibles would accumulate each year. Thus, it was estimated that, as of 1995, approximately 10,000 susceptibles had accumulated in Belize since the measles catch-up immunization campaign in 1991. It is believed that such a number of susceptibles would be sufficient to support an epidemic if measles virus were imported from endemic countries. In order to sustain measles-free status in Belize and to prevent measles outbreaks by imported measles cases from endemic countries, it was decided to reduce the number of accumulated susceptibles by implementing a special measles follow-up immunization campaign.

The measles follow-up immunization campaign in Belize was conducted in October 1995. The target population for this campaign was all children from twelve through fifty nine months of age (approximately 34,000 children). All were to be given one dose of measles vaccine, regardless of the past history of immunization or disease. Unfortunately, a severe hurricane attacked the northern area of Belize, especially Orange Walk District, at the end of September. Following the hurricane, a flood occurred in Orange Walk District, and a number of villages had to be evacuated because of the damage from the hurricane and flood. Thus, in Orange Walk District, the immunization activities were not fully performed because transportation and communication system had been devastated, and immunization teams had difficulty in tracing the evacuated people. Since other districts had been damaged very little by the hurricane and flood, the campaign was conducted as scheduled in Corozal, Belize, Cayo, Stann Creek, and Toledo Districts.

One of the most unique characteristics in the campaign was the close collaboration with other United Nations (UN) agencies such as UNICEF, United Nations High Commissioner for Refugees (UNHCR), and United Nations Development Programme (UNDP) in celebration of the fiftieth anniversary of the UN; October 24, 1995. Their participation in the campaign generated much publicity among people in Belize. In addition, they provided vehicles and other resources for the campaign during the third week of October. As a result, in Corozal District, the immunization teams were provided vehicles by UNHCR, and they performed immunization activities much better than would have been possible with the one vehicle which they had to share with other public health activities in the district. The campaign was also supported by a great deal of community participation. A number of school teachers and community leaders participated in health education activities distributing promotional materials provided by the Ministry of Health, providing parents information about specific times and places for immunization activities, and encouraging them to bring their children to the immunization units. Some teachers helped public health nurse and community health workers in estimating target populations, and in recording names, addresses, and ages of vaccinated children.

According to the reports from EPI manager and public health nurses in each district, overall immunization coverage of this campaign was 74%, the highest being 98% in Corozal District, and the lowest 46% in Orange Walk District.

4. Lessons from measles follow-up immunization campaign in Belize

According to reports and questionnaires from public health nurses who were engaged in the campaign, the unexpectedly low coverage, especially in some districts, could be attributed to three factors. First, the hurricane and flood following it hampered the implementation of the immunization campaign, especially in Orange Walk District. Second, many public health nurses committed to the campaign complained of the shortage of transportation, especially vehicles for mobile immunization teams. In commemoration of the fiftieth anniversary of the United Nations, UN agencies provided vehicles and other resources, but only for the third week of October. Immunization teams were unable to implement enough immunization activities for this campaign on the other weeks because of the lack of transportation. Third, the promotional activities and materials such as posters and leaflets were too late to support the immunization activities, because the promotional activities began and materials reached health centers in the middle of October, while the campaign was initiated in the beginning of October.

Several points have been identified to be of special relevance to future immunization campaigns.

1) The season of the campaign

The major obstacles for the campaign in Belize were the hurricane and flood, which destroyed transportation routes and community health systems in the northern areas, especially in Orange Walk District.

Although the occurrence of hurricanes is unpredictable, it would be better to implement immunization campaigns in months when few or no hurricanes are expected.

2) The degree of collaboration with other organizations

The principal UN agencies (UNICEF, UNHCR, and UNDP) participated in the campaign in commemoration of the fiftieth anniversary of the United Nations, and provided several vehicles and other resources. This collaboration greatly enhanced the publicity and efficiency of the campaign, and facilitated the work of the mobile immunization teams. Such collaborations with other agencies are clearly beneficial and supportive in facilitating immunization activities. In addition, involving NGOs, which have close relations with communities, can enhance community participation and contribute to the sustainability of mass immunization campaigns.

3) The extent of target-specific and timely promotion

In this campaign, Belize had a special promotion targeting hard-to-reach communities, especially the Mennonites, using their language and photographs of the Mennonite community. This group used to refuse modern scientific medicine including immunization, and some still now reject immunization. This effort was intended to enhance their community's interest in immunization and to decrease difficulty in implementing the campaign. In general, it is important to target such hard-to-reach communities because of the risk of measles outbreaks among them in both developing and developed countries. (Novotny *et al.*, 1988)

Many public health nurses complained of the delay in providing promotional materials and, in some cases, of their unavailability. Obviously the promotional activities must be timely and should be made before the campaign begins in order to enhance public knowledge of the campaign and to facilitate immunization activities for the campaign. If there are high level of access to the media, and sufficient expertise and funds available to develop and produce radio and television advertisements, promotion for immunization campaigns using mass communications can significantly improve immunization coverage (Zimicki *et al.*, 1994).

4) The capability of District Health Teams

During visits in Belize, the author was impressed by the well-organized District Health Teams and the close and friendly communication between central level and district levels. At each health center, public health nurses were responsible for the campaign activities and several community health workers, who were familiar with the unique situations of the area, supported immunization activities during the campaign. These teams were given great flexibility in scheduling campaign activities according to each unique situation such as weather conditions and other routine community activities. As a result, very few health centers complained that the campaign had hampered their routine public health activities at the health center level. Such a partially decentralized system and a flexible schedule in a mass immunization campaign can be very effective in facilitating immunization activities, although strict overall supervision is still necessary.

In addition, the District Health Teams were able to encourage the voluntary support of parents because each team had close and friendly communication with their communities. The importance of close relationships with the community and the success of community participation have been reported in mass immunization campaign in Cameroun (McBean *et al.*, 1976), Chile (Borgono *et al.*, 1978), and Ghana (Belcher *et al.*, 1978).

5) The importance of transportation

The principal complaints of the public health nurses were shortages of transportation for mobile immunization teams. With enough transportation, they could conduct satisfactory immunization activities including mobile immunization activities as seen in Corozal District, which achieved the highest immunization coverage (98%) due to vehicles provided by UNHCR. A shortage of vehicles is not unique to the campaign in Belize. In Senegal, for example, the mobile vaccination activities were one of the main strategies of their immunization campaign, but these activities were limited by the shortage of vehicles, and both the provision of vehicles and the effective performance of mobile teams were recommended for successful achievement of immunization campaigns (Desgrees and Pison, 1994). Thus, in planning immunization campaigns, we have to take this issue into consideration, and try to provide effective transportation as much as possible.

6) Missed opportunities to provide vaccination

Two unique factors were identified as important reasons for failures to immunize during the campaign in Belize.

Migration: Almost all the public health nurses complained that the migrating population was a major reason for failures to immunize. They explained that it is very difficult even for community health workers, who are quite familiar with their health center areas, to estimate the size of the migrating population and to immunize them during the campaign.

Private physicians: Some public health nurses complained about interference with campaign activities by

private physicians who had provided parents inconsistent information with the plan of the campaign. Such confusion and misunderstanding of parents were counter-productive for the immunization activities.

These two factors were not "missed opportunities", according to the usual definition, which defines missed opportunities as "circumstances when a child, who is eligible for immunization and who has no contraindication to immunization, visits a health service and does not receive the needed vaccines" (Hutchins *et al.*, 1993; EPI/WHO, 1993; EPI/WHO, 1994). Although many public health nurses understood "missed opportunities" to mean failures of parents to bring their children to immunization units, these factors were very important as well as true missed opportunities in order to attain a high performance for the campaign.

In other countries, reasons for poor vaccine coverage are diverse. In Mozambique and Conakry in Guinea, management and logistics problems were largely at fault along with poor knowledge and attitudes on the part of health workers and mothers (Cutts *et al.*, 1991). In Puerto Rico, the major problems were non-availability of vaccines, lack of integration of services, provider misconceptions about contraindications, and failure to administer vaccines simultaneously (Ginder *et al.*, 1993). Missed opportunities to vaccinate children are major barriers for immunization program not only in developing countries, but also in developed countries as was revealed in United States of America during the measles epidemic of 1989 and 1990. The major reason for that epidemic was failure to provide vaccine to vulnerable children on schedule, primarily due to two types of missed opportunities: 1) observing inappropriate contraindications and 2) failure to vaccinate children at each visit for health care (The National Vaccine Advisory Committee, 1991; Peter, 1992; Cutts *et al.*, 1992; Wood and Brunell, 1995).

Major barriers to adequate vaccination coverage are the failures to vaccinate children when they are seen by health care practioners either during an immunization campaign or routine immunization services. Two major strategies can help to decrease missed opportunities: 1) encouraging health care providers such as public health nurses and private physicians to involve themselves in immunization activities and to take advantage of every chance to vaccinate children in need of immunization, 2) providing appropriate health information to parents in order to stimulate their interest in immunization and to get them to participate. These two strategies have proved effective under different circumstances (Hutchins *et al.*, 1993; EPI/WHO, 1993; EPI/WHO, 1994).

7) The influence of behavioral issues on immunization

In the campaign in Belize, a number of public health nurses complained that some parents exhibited little interest in measles immunization because of the absence of measles over the past four years, fear of side effects of vaccine, and negligence of parents. A study in Togo reported that the major factor in the acceptability of childhood immunization was interest in immunization on the part of parents rather than logistic and management problems of the program (Eng *et al.*, 1991). Pillsbury (1991) reviewed qualitative researches about the behavioral perspective of immunization and summarized the reasons why children did not get immunized as follows:

Reasons related to characteristics of the mother and other caretakers.
- —Time constraints and competing priorities
- —Other socioeconomic constraints
- —Lack of knowledge about immunization
- —Low motivation for immunization
- —Fears
- —Community opinion

Reasons related to characteristics of the vaccines.
- —Side-effects of the vaccines
- —Belief that vaccination is not effective

Reasons related to characteristics of the delivery of immunization services.
- —Accessibility
- —Availability
- —Acceptability
- —Affordability

Reasons related to communication to the public about immunization.
- —Inadequate communication

Based on the factors mentioned above, it is essential for persons who plan and implement immunization programs to understand local perceptions and behavior which influence parental willingness to have their children vaccinated. A better understanding of local perceptions and behavior related to immunization is especially beneficial for extending coverage to hard-to-reach communities, enhancing community participation, and building sustainable programs.

DISCUSSION

Based on the progress of measles elimination program in the Americas and the evaluation of the measles follow-up immunization campaign in Belize, the author depicted the important components for the strategy, and

considered the development of measles elimination program to other regions.

1. Important components for measles elimination strategy

Since 1991, the strategy for measles elimination has been functioning effectively in the Americas, and achieved great success in the English-speaking Caribbean countries. This success can be attributed to the strong government commitment of the member countries in the English-speaking Caribbean countries. The government of each country has supported the international goal on measles elimination of this region, and collaborated technically and administratively through the international organization (PAHO/WHO, UNICEF, World Bank, *etc.*) and other non-governmental organizations (Rotary International, *etc.*).

As discussed previously in the measles follow-up immunization campaign in Belize, strong political commitment and community's participation are the major factors not only for the mass immunization campaign but also the ongoing measles elimination program. Without those factors, the program cannot obtain administrative and logistic support such as money, personnel, and equipment. Moreover, if there were no community's support on the basis of strong political advocacy, the program could not be carried out effectively due to low compliance of mass immunization program.

In addition to the commitment by government and community, the international collaboration is indispensable for the success of the measles elimination program. Since measles is ubiquitous all over the world, there is always a risk of importation of measles virus, even if one country or region has achieved measles elimination. In order to reduce the possibility of measles virus importation or exportation, each country in some region has to implement the measles elimination program simultaneously. Under such condition, international organization (WHO, UNICEF, *etc.*) and non-governmental organization (Rotary International *etc.*) are able to play an important roll in order to organize and integrate each national program into international program, as seen in the measles catch-up immunization campaign in the English-speaking Caribbean countries in 1991 (Hospedales, 1992).

In general, the health information system about the target diseases and the technical support system to confirm the information are indispensable for success of the disease control program. The former is one of the most critical components for measles elimination program, which is called "rash and fever surveillance". In this system, all cases which have rash and fever as explicit symptoms should be reported and investigated both serologically and epidemiologically. Then, such information should be returned to the primary reporting level as feedback of information. For example, in order to provide information to public health workers throughout the Americas, PAHO has published a weekly measles surveillance bulletin since 1994. This bulletin summarizes the number of current outbreaks by country, total cases under investigation, and cumulative annual number of confirmed measles cases by country (de Quadros *et al.*, 1996). For the technical support system, it is necessary to establish the international laboratory network, on which there are several reference laboratories that can perform advanced laboratory investigation in order to confirm or rule out measles virus circulation in one region. Moreover, laboratory sequencing of the measles virus genome from isolates can help to determine geographic sources of outbreaks and to identify pathways of transmission of measles virus.

2. Development of measles elimination program to other regions

Although the new program and strategy have been proceeding successfully in the Americas and measles elimination has been achieved in the English-speaking Caribbean countries, there may be still several obstacles for global measles elimination to be developed to other regions.

First, the predecessor of measles elimination program; polio eradication program, has not been achieved in the rest of the world, while there have been no poliomyelitis since 1991 in the Americas, and since then the program had entered the observation stage. Eventually, the eradication of polio in Western Hemisphere was announced in 1994. Although the number of the cases of polio has decreased drastically in the rest of the world, the polio eradication program is now at its very important and critical stage; mass immunization campaign and strengthening surveillance. Under such condition, all countries except the Americas are now engaged in the polio eradication program enthusiastically as the priority program of public health. There may be very little resources for the new measles elimination program.

Second, in developing countries, the health information system such as disease notification or surveillance system has not been developed enough to support public health program in general. Therefore, it is quite diffi-

cult financially and administratively to introduce a new disease surveillance system such as "rash and fever illness surveillance" for measles elimination program in developing countries.

Third, in developed countries including Japan, measles is no more a major concern as priority of public health importance, because the mortality from measles is very low. Therefore, they are not enthusiastic enough to support global measles elimination program financially and technically. Moreover, basic researches about measles, such as vaccines, are not conducted enough to provide new scientific information for the measles elimination program, because of low interest in measles in developed countries.

Although they are several obstacles for implementing the measles elimination in other regions, as mentioned previously, measles has been a leading killer for children. Moreover, the American region is able to initiate the program almost five years ago, and in English-speaking Caribbean countries, they attained a marvelous success story through this strategy. Therefore, these experiences indicated feasibility of the measles elimination program. Towards the global elimination of measles, it is indispensable for all countries not only developing countries, but also developed countries to participate in this program simultaneously. If each country has its strong political commitment and enthusiastic community's participation, this measles elimination program will be able to function effectively and we can expect the glorious achievement which we had experienced in the smallpox eradication and poliomyelitis eradication. The measles elimination program cannot wait until the polio eradication program will achieve the worldwide success.

On the view of international cooperation and regional political commitment in Asia, Japan is able to play an important roll towards the measles elimination not only in this region but also in the world. Actually, in polio eradication program, Japan has supported this program financially, dispatched expert personnel, and donated vaccines and other equipment for immunization program to several countries in Asia in collaboration with WHO and UNICEF. For the future perspective of health development in Asia, Japan has to take the initiative in the new measles elimination program in order to support health development in Asian region.

As mentioned previously, there are several obstacles for implementation of measles elimination program. When Japan will support this program financially and administratively, these obstacles such as priority conflict of health problems and under-developed health system, it may be much more easier for such developing countries in Asia to promote political commitment and community's participation enough to implement measles elimination program enthusiastically.

In addition to financial and administrative point of view, basic researches for measles and its epidemiology are indispensable for the measles elimination program. On this field, Japan can contribute its potential competence to applied technologies for measles elimination. For example, development of high-titer measles vaccine, which enable vaccine administration in earlier ages such as six months or earlier, or the innovative vaccine delivery system.

In summary, measles elimination program is feasible, and has already achieved its goal in the English-speaking Caribbean countries. Although there are still several obstacles for development of the measles elimination program to other regions, the most important components are the strong political commitment in each country and international cooperation financially and technically. On this point of view, Japan is able to play an important roll towards the measles elimination in Asia.

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CYTOKINE PRODUCTION ASSAYED BY RT-PCR IN PREGNANT MICE INFECTED BY *TOXOPLASMA GONDII* AS A MODEL OF CONGENITAL TOXOPLASMOSIS

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Abstract: To explore the mechanisms of immune responses of host to *Toxoplasma gondii* (*T. gondii*) infection in pregnant mice, we evaluated roles of cytokines [interferon gamma (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 4 (IL-4)] by measuring mRNAs of these cytokines in placentas, lungs and spleens. The pathogenic effects of time and duration of the Fukaya infection on cytokine mRNA levels in pregnant mice were analyzed. The abundance of mRNAs encoding these cytokines was measured by reverse transcriptase (RT)-PCR at early and late stages of pregnancy in various organs of both susceptible C57BL/6 and resistant BALB/c pregnant mice infected with *T. gondii*. IFN-γ and TNF-α but not IL-6 or IL-4, were predominant in the immune responses of placentas, lungs and spleens of BALB/c and C57BL/6 mice during *T. gondii* infection. Levels of IFN-γ and TNF-α mRNA in placentas of early stage pregnant BALB/c mice (infected at one-week pregnancy and examined on day 4 after infection; 1W4D) were higher than those in corresponding C57BL/6 pregnant mice, which might correlate with the fact that higher parasite numbers in placentas and lungs of C57BL/6 mice (infected at one-week pregnancy and examined on day 11 after the Fukaya infection; 1W11D) were observed than those in placentas and lungs of corresponding BALB/c mice, but not correlate with the result of parasite numbers (*T. gondii* No./mg tissue) in spleens of C57BL/6 (0) and BALB/c (120 ± 56) pregnant mice. In the late stage of pregnancy, levels of IFN-γ and TNF-α did not show definite correlations with *T. gondii* loads in placentas, lungs and spleens. These results indicate that endogenous IFN-γ and TNF-α of early stage pregnancy may be essential for inhibition of *T. gondii* growth in some organs (placentas and lungs), but not in spleens, and the mechanisms of genetic influence involved in the susceptibility and resistance to acute *T. gondii* infection may include several immune responses acting together.

Key words: *Toxoplasma gondii*, cytokine mRNA, congenital toxoplasmosis

INTRODUCTION

Human congenital toxoplasmosis is caused by maternal transplacental transmission of *T. gondii* parasites to the fetus, mainly by the acute initial maternal infection during pregnancy (Yokota, 1995). Any route of *T. gondii* infection leading to a maternal parasitemia during pregnancy may result in toxoplasmosis of the placenta and transmission of the protozoa to the offspring before birth (Cowen and Wolf, 1950). In human congenital infection of *T. gondii*, severity of disease appears to be strongly correlated with trimester of maternal acquisition (Luft and Remington, 1982). The depressed immune response during pregnancy would be expected to have a bearing on the severity of *T. gondii* infection and hence provides a greater opportunity for transplacental spread to occur (McLeod *et al.*, 1989).

Pathological changes are much more common and more severe in the placenta than in the fetus, and placental damage is probaly the primary cause of fetal death (Loke, 1982). These facts clearly demonstrate that immune responsiveness of the placenta is critically important for resistance to the parasite entering the fetus and study of the pathology of the placenta is of great practical importance.

There have been several reports on maintenance of

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pregnancy, and recording the placental expression and/or synthesizes of both harmful and protective cytokines, including interleukin 3 (IL-3), IL-4, IL-6, IL-10, IFN-γ, TNF, colony stimulating factor-1 (CSF-1), granulocyte macrophage-CSF (GM-CSF), and transforming growth factor β (TGF-β) (Chen *et al.*, 1993; Clark *et al.*, 1995; De *et al.*, 1992; Hunter *et al.*, 1993; Yelavarthi *et al.*, 1991). These two patterns of harmful and protective cytokines overlap substantially with the Th1 and Th2 cytokine patterns that were originally identified among a panel of mouse Th clones (Mosmann *et al.*, 1986), and mutually inhibit the differentiation and/or activation of the other (Fiorentino *et al.*, 1989). Thus, the inflammatory/anti-inflammatory balance of cytokines in the placenta can alternatively be viewed as a Th1/Th2 balance. The protective Th2 cytokine pattern (together with TGF-β) may be functionally dominant over potentially harmful Th1 cytokines during normal, successful pregnancy (Krishnan *et al.*, 1996). Among Th2 cytokines, IL-4 has the ability to drive precursor T cells into a subpopulation of T helper effectors known as Th2 cells, which are important regulations of humoral immunity, eosinophilia, and mastocytosis. In some infectious disease models, administration of anti-IL-4 at the time of infection will divert the ensuing response away from Th2 cells toward the Th1 subpopulation of T helper effectors (Sadick *et al.*, 1990). We reported that the Th1-like human CD4$^+$ cytotoxic T lymphocytes specific for *T. gondii*-infected melanoma cells were existent in peripheral blood lymphocytes of patients with toxoplasmosis and were secreting IFN-γ and IL-6 (Yang *et al.*, 1995). Both IFN-γ and IL-6 enhanced HLA-DR molecules of *T. gondii*-infected melanoma cells, and these cytokines may play a role in pathogenicity of toxoplasmosis. IL-6 enhances intracellular replication of *T. gondii* and reverses IFN-γ-mediated activation of murine peritoneal Mϕ, and that certain of the interactions between these two cytokines may be at the level of TNF-α triggering (Beaman *et al.*, 1994). Conversely, an acute *T. gondii* infection during pregnancy may disrupt the maternal-fetal immunological balance in favor of antiparasitic proinflammatory abortogenic cytokines such as IFN-γ and TNF-α (Roberts *et al.*, 1995). Since IFN-γ and TNF-α may be necessary for resistance to *T. gondii* infection, it is perhaps more realistic to view the situation in pregnancy as an altered ballance between the two types of cytokines, which can shift in either direction for a variety of reasons (Clark *et al.*, 1995).

Our present work studied the immune responses of the placenta to *T. gondii* infection as compared to other organs of resistant (H-2^d) or susceptible (H-2^b) strains of pregnant mice. The cytokine (IFN-γ, TNF-α, IL-6 and IL-4) mRNA levels in placentas, lungs and spleens were analyzed by RT-PCR.

Materials and Methods

Mice and matings. Inbred female and male BALB/c (H-2^d) and C57BL/6 (H-2^b) mice (Charles River, Yokohama, Japan) were housed at the Laboratory Animal Center for Biomedical Research in Nagasaki University School of Medicine under conventional conditions. Eight- to ten-wk-old mice were used for mating. One male and two females were housed per cage overnight for mating and the presence of the vaginal plugs in the females was checked on the following morning. The day on which the plug was observed was considered to be day 0 of pregnancy. Pregnant females thus identified were removed from the mating cages and housed randomly with other female mice.

***T. gondii* strains and infections of mice.** A virulent strain RH and an avirulent cyst-forming strain Fukaya (Asai and Suzuki, 1990; Watanabe *et al.*, 1993) of *T. gondii* were used for infection experiments and prepared as previously described (Luo *et al.*, 1995).

For the experiment with the RH strain, tachyzoites were adjusted to 10^7 viable organisms/0.25 ml in phosphate-buffered saline (PBS) for inoculation. One- or two-week pregnant mice were infected intravenously via their retro-ocular venular plexus with 0.25 ml RH suspension. All pregnant mice died on day 6 after infection, therfore the infected mice were sacrificed on day 4 after infection. The organ samples, placentas, lungs and spleens, were collected to analyze the abundances of certain cytokines.

For the experiment with the Fukaya strain, the brains from mice infected 2 months earlier with the Fukaya strain of *T. gondii* were removed and homogenized in 5 ml of PBS with a mortar and pestle. Then 50 μl of brain suspension was placed on a glass microscope slide and mounted with a cover-slip and the number of cysts determined microscopically by scanning the entire preparation under 100× magnification. One- or two-week pregnant mice were infected by gavage with 0.5 ml of a brain tissue homogenate in PBS suspension, containing 20 cysts. The mice which were infected during the first week of pregnancy were sacrificed on day 4 after infection. Mice which were infected at two weeks of pregnancy were sacrificed and examined on day 4 after infection, just before delivery. Each experimental group consisted of three animals. The number of

Table 1 Summary of experimental groups

Mouse strain	*T. gondii* strain	Pregnant stage when infected	Analysis time post infection	Abbreviation
BALB/c, C57BL/6	RH, Fukaya	one week	day 4	1W4D
			day 11	1W11D
		two-week	day 4	2W4D

examined placentas were 9 to 20 in each group. Every experiment was performed two or three times and the results were shown to be essentially identical. The mice used in this present study were sacrificed by anaesthetic overdose.

Experimental groups. Experimental groups of pregnant BALB/c and C57BL/6 mice infected with the RH or the Fukaya strain are summarized in Table 1. One- or two-week pregnant mice were infected with *T. gondii* and the infectivities in these mice were examined on day 4 or 11 after infection. These experimental groups are abbreviated as 1W4D, 1W11D and 2W4D respectively. We tried to establish an experimental group in which the mice were infected on day 0 of pregnancy however, these mice did not become pregnant.

Detection of cytokine mRNA by reverse transcriptase (RT)-PCR. Total cellular RNA was extracted with Trizol (Gibco BRL, Grand Island, NY, U.S.A.) from placentas, lungs and spleens of *T. gondii*-infected or non-infected pregnant mice according to the instructions of the manufacture. After extraction, the RNA concentration was determined spectrophotometrically using GeneQuant (Pharmacia LKB Biochrom Ltd, Science Park, Cambridge CB4 4FJ, England). One μg of total RNA was reversed transcribed in a final volume of 20 μl using the RNA PCR kit (R019A, Takara, Shiga, Japan) according to the manufacturer's instructions. The quantity of RNA used for RT and RT-PCR was decided as manufacturer's recommendations. The reaction mixture contained 10 mM Tris•HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate (dATP, dUTP, dCTP and dTTP), 20 U of RNase inhibitor, 5 U of avian myeloblastosis virus reverse transcriptase XL, and 2.5 mM random nine-nucleotide oligomers and was incubated at 30°C for 10 min, 50°C for 30 min, 99°C for 5 min, and 4°C for 5 min. The RT product mixture (cDNA) was then diluted to 100 μl with double-distilled water, and 5 μl of the diluted solution was used for each PCR reaction. The PCR reaction conditions were optimized for each set of primers (Table 2). Contaminations of total RNA with genomic DNA were examined by attempting to amplify cytokine genes from total RNA by conventional PCR and no cytokine genomic DNA was detected in 1 μg of total RNA. Each of the above-mentioned groups of pregnant mice were accompanied by a group of age-matched pregnant but uninfected control mice.

Ten microliter of each PCR product was electrophoresed in a 1.2% agarose gel with ethidium bromide

Table 2 PCR conditions (sequences of the oligonucleotide primers used for PCR amplification of cytokine mRNA, number of PCR cycles and product size predicated)

Cytokine		Primer sequences	Annealing temperature (°C)	Cycles	Product size (bp)
GAPDH	3′	TCCACCACCCJGTTGCTGTA	55	37	452
	5′	ACCACAGTCCATGCCATCAC			
IFN-γ	3′	CGACTCCTTTTCCGCTTCCTGAG	60	37	460
	5′	TGAACGCTACACACTGCATCTTGG			
TNF-α	3′	CCAAAGTAGACCTGCCCGGACTC	60	37	691
	5′	ATGAGCACAGAAAGCATGATCCGC			
IL-6	3′	CACTAGGTTTGCCGAGTAGATCTC	56	37	638
	5′	ATGAAGTTCCTCTCTGCAAGAGACT			
IL-4	3′	GCTCTTTAGGCTTTCCAGGAAGTC	54	37	399
	5′	ATGGGTCTCAACCCCCAGCTAGT			

Primer sequences were obtained from CLONTECH Laboratories, Inc., 4030 Fabian Way, Palo Alto CA 94303 USA.

staining for 25 min at 100 V and quantification of RNA was performed with an IPLab Gel Densitometer (Sigal Analytical Corp., Vienna, VA). The relative intensity of bands for each cytokine mRNA was related to the intensity of the autoradiogram band used as the internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are expressed as the ratio of the optical density (OD) value of the PCR product of each cytokine to the OD value of products of GAPDH according to the following formula: (OD of cytokine/OD of GAPDH) $\times 100$. The OD value for the blank control (distilled water) was set at zero.

Statistical analysis. Student's t-test was used to analyse differences in parasite loads or levels of cytokines between the various groups of mice. $P<0.05$ was accepted as significant.

RESULTS

The abundances of cytokine mRNA transcripts in BALB/c and C57BL/6 pregnant mice infected with a virulent RH *T. gondii* strain. High levels of *T. gondii* loads were detected in placentas, lungs and spleens from BALB/c and C57BL/6 pregnant mice in the early (1W4D) and late (2W4D) pregnant stages (Fig. 1A). To examine the correlation between parasite loads and cytokine responsiveness, mRNA transcripts encoding IFN-γ, TNF-α, IL-6 and IL-4 were tested in 1W4D and 2W4D BALB/c and C57BL/6 pregnant mice (Fig. 1).

High levels of IFN-γ mRNA were observed after infection in placentas, lungs and spleens of 1W4D BALB/c and C57BL/6 pregnant mice. Also high levels of IFN-γ were observed in lungs of 2W4D BALB/c mice and in spleens of 2W4D BALB/c and C57BL/6 mice after infection. In placentas of the RH-infected BALB/c and C57BL/6 pregnant mice, IFN-γ mRNA was detected at a higher level in the early stage (1W4D) than in the late stage (2W4D) of pregnancy ($P<0.05$). However, there was no significant difference in the infectivity of placentas between 1W4D and 2W4D pregnant BALB/c and C57BL/6 mice (Fig. 1A).

Significant levels of TNF-α mRNA were detected in placentas, lungs and spleens from 1W4D and 2W4D BALB/c and C57BL/6 pregnant mice after infection. IL-6 mRNA was induced at low levels in placentas, lungs and spleens of the RH-infected BALB/c and C57BL/6 pregnant mice. In the late stage (2W4D) of pregnancy, levels of IL-6 mRNA in placentas, lungs and spleens of BALB/c and C57BL/6 mice were much lower than those in the early stage (1W4D). 1W4D C57BL/6 pregnant mice had higher IL-6 levels in placentas and lungs than 1W4D BALB/c pregnant mice ($P<0.05$).

In the RH-infected BALB/c and C57BL/6 pregnant mice, no detectable level of IL-4 was observed in placentas and lungs of the early and the late stages of pregnancy. Significant levels of IL-4 were only observed in spleens from 1W4D BALB/c and C57BL/6 pregnant mice and 2W4D BALB/c pregnant mice.

At the late stage infection with the virulent RH strain, IFN-γ and TNF-α mRNA but not either IL-6 or IL-4 mRNA were dominant cytokines detected in placentas of both BALB/c and C57BL/6 mice (Fig. 1B).

The abundances of cytokine mRNA transcripts in BALB/c and C57BL/6 pregnant mice infected with an avirulent Fukaya *T. gondii* strain. A marked difference in susceptibility to the avirulent strain Fukaya infection between 1W11D BALB/c and 1W11D C57BL/6 pregnant mice was observed (Fig. 2). To examine the involvement of cytokines in the genetic basis of *T. gondii* infectivity in BALB/c and C57BL/6 pregnant mice, mRNA levels of IFN-γ, TNF-α, IL-6 and IL-4 in placentas, lungs and spleens were assayed (Fig. 2).

Levels of IFN-γ mRNA in placentas, lungs and spleens were higher in 1W4D BALB/c pregnant mice than in 1W4D C57BL/6 pregnant mice ($P<0.05$). This correlates with susceptibility to infection in 1W11D pregnant BALB/c and C57BL/6 mice (Fig. 2). The infection rate in placentas and lungs of 1W11D C57BL/6 pregnant mice was higher than that in 1W11D BALB/c pregnant mice ($P<0.05$) (Fig. 2). However, parasite loads in the spleen did not differ significantly between 1W11D BALB/c and C57BL/6 mice. Significant or low level of IFN-γ was not observed in placentas of 1W11D and 2W4D BALB/c and C57BL/6 pregnant mice, indicating that cytokines produced in the placenta of 1W4D BALB/c and C57BL/6 pregnant mice are transient and are not sufficient to protect placentas of C57BL/6 mice from Fukaya infection at a late stage of pregnancy. Levels of IFN-γ in lungs and spleens did not differ significantly between 1W11D BALB/c and C57BL/6 pregnant mice, and levels of IFN-γ were higher in lungs than in spleens in the two strains of mice. Thus the markedly lower *T. gondii* loads in 1W11D BALB/c pregnant mice than in 1W11D C57BL/6 pregnant mice can be attributed to other genetic differences but not to protection by IFN-γ. The fact that the IFN-γ level in the placenta of the Fukaya-infected BALB/c and C57BL/6 mice was higher in the early stage (1W4D) than in the late stage (1W11D or 2W4D) of pregnancy aggrees with the observations of the RH infection as shown in Fig. 1.

Levels of TNF-α in placentas, lungs and spleens

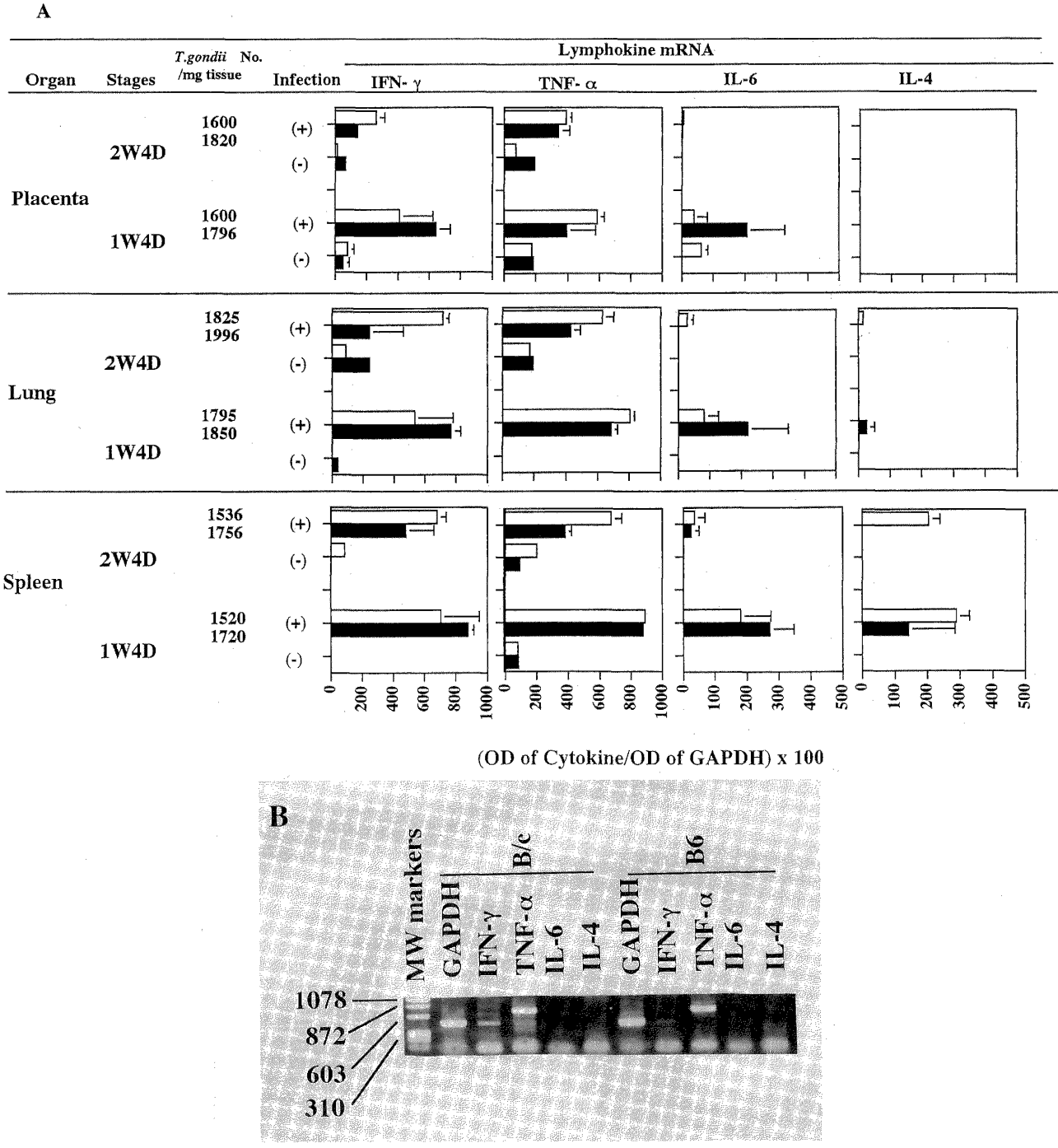

Figure 1 (A) Cytokine mRNA levels in virulent *T. gondii*-infected pregnant mice. Time courses of change in the abundance of mRNAs encoding IFN-γ, TNF-α, IL-6 and IL-4 in the placenta, lung and spleen of BALB/c (□) and C57BL/6 (■) pregnant mice after infection with RH strain. At each time point, experimentally-infected group of pregnant mice (+) was accompanied by a group of age-matched uninfected pregnant mice (−). Lymphokine mRNAs were assayed by RT-PCR and the data is expressed as described in Materials and Methods. (B). Cytokine mRNA levels in placentas of virulent *T. gondii*-infected pregnant mice. Abundance of IFN-γ, TNF-α, IL-6 and IL-4 mRNA in the placenta of BALB/c (abbreviated as B/c) and C57BL/6 (abbreviated as B6) pregnant mice infected at two-week after pregnancy with RH strain and tested on day 4 (2W4D) after infection. MW markers are Hae III digest of ϕx174.

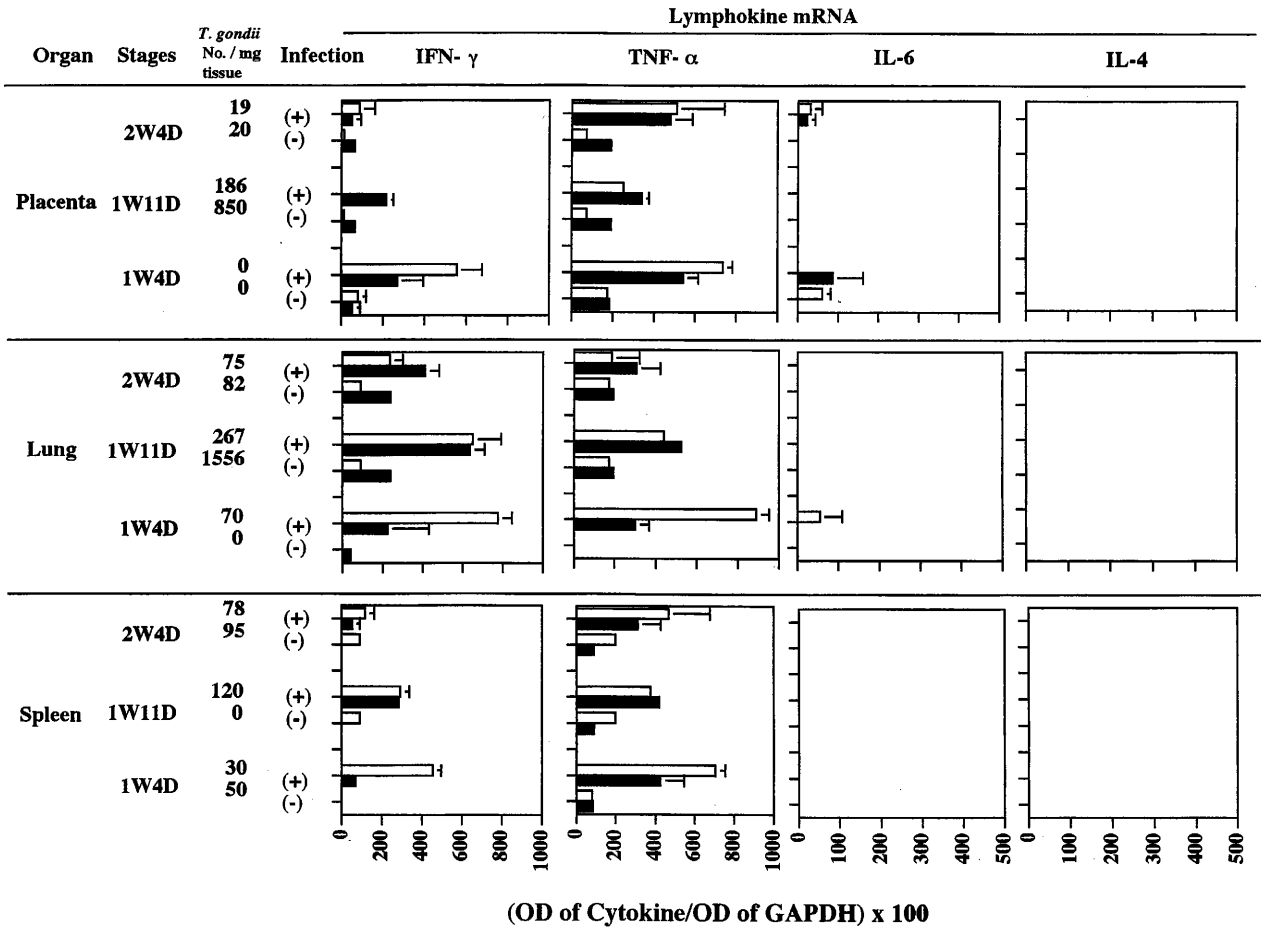

Figure 2 Cytokine mRNA levels in avirulent *T. gondii*-infected pregnant mice. Time courses of change in the abundance of mRNAs encoding IFN-γ, TNF-α, IL-6 and IL-4 in the placenta, lung and spleen of BALB/c (□) and C57BL/6 (■) pregnant mice after infection with Fukaya strain. At each time point, the experimentally-infected group of pregnant mice (+) was accompanied by a group of age-matched uninfected pregnant mice (−). Lymphokine mRNAs were assayed by RT-PCR and the data is expressed as described in Materials and Methods.

were higher in 1W4D (BALB/c pregnant mice than in 1W4D) C57BL/6 pregnant mice ($P<0.05$). TNF-α production paralleled the production of IFN-γ. However, in placentas, lungs and spleens of 2W4D and 1W11D pregnant mice, no significant difference in the TNF-α level was observed between infected BALB/c and C57BL/6 pregnant mice.

No significant or very low levels of IL-6 mRNA were detected in placentas, lungs and spleens of infected or uninfected BALB/c and C57BL/6 pregnant mice. No significant IL-4 mRNA level was detected in any of placentas, lungs or spleens from either strain of pregnant mice (Fig. 2).

Discussion

An acute *T. gondii* infection during pregnancy may disrupt the delicate hormone-influenced maternal-fetal immunological balance in favor of antiparasitic proinflammatory cytokines such as IFN-γ and TNF-α (Roberts *et al.*, 1995). As shown in Figs. 1 and 2, at the mRNA level, using lungs and spleens as references, IFN-γ and TNF-α, but not IL-6 or IL-4, were predominant in the immune response of the placenta, lung and spleen of BALB/c and C57BL/6 pregnant mice during *T. gondii* infection. On the other hand, IFN-γ and TNF-α have deleterious abortogenic effects, leading to fetal demise in otherwise normal pregnancy (Krishnan *et al.*, 1996). We observed lower pregnancy ratio and higher

abortion ratio of fetuses in the Fukaya-infected pregnant mice than in non-infected pregnant mice (data not shown), suggesting that a beneficial Th1 antiparasite response can prove deleterious to successful gestation as it can influence both placental maintenance and preimplantation events (Krishnan *et al.*, 1996). It has been shown that the Th1 response to *Leishmania major* increases the risk of pre- and post-implantation pregnancy loss (Krishnan *et al.*, 1996). Positive correlations have also been reported between abortions and the expression of IFN-γ, IL-2 and TNF (Tangri and Raghupathy, 1993). One of the mechanisms of fetal damage in response to cytokine production may be the induction of natural killer activity by IL-2 and IFN-γ (Wegmann *et al.*, 1993). Placenta cells from mice undergoing fetal abortion were less able to suppress natural killer reactivity as compared to normal placental cells (Chaouat *et al.*, 1985).

We previously analyzed the mRNA levels of cytokines (IFN-γ, TNF-α, IL-6 and IL-4) of brains from BALB/c and C57BL/6 non-pregnant mice infected by the avirulent strain Fukaya, and the results indicate that endogenous IFN-γ and TNF-α are not sufficient for inhibition of *T. gondii* growth *in vivo* (Luo and Yano, 1996). As with IFN-γ, many studies have demonstrated a protective role for TNF-α in toxoplasmosis (Gerard *et al.*, 1993; Marchant *et al.*, 1994), although there also exists some evidence that overproduction of TNF-α may be detrimental by increasing mortality (Black *et al.*, 1989). From our data shown in Figs. 1 and 2 (1W4D), TNF-α production occurs in parallel with IFN-γ production, which may be consistent with the previous report that TNF-α functions as an endogenous modulator of macrophage activation by triggering IFN-γ-primed cells to express antitoxoplasmal activity (Sibley *et al.*, 1991). In Fig. 2, IFN-γ and TNF-α levels in placentas and lungs of 1W4D BALB/c and C57BL/6 inversely correlated well with the infectivity of Fukaya strain in 1W11D pregnant mice, however, no difference in the level of IFN-γ or TNF-α in the 1W11D mice was observed between BALB/c and C57BL/6 pregnant mice. These results suggest strongly that IFN-γ and TNF-α production in the early stage of pregnancy (1W4D) may play a very important role and have protective immunity against parasites in the placenta and other organs in *T. gondii*-infected BALB/c pregnant mice. These speculations seem reasonable when parasite loads and cytokine production in 1W4D pregnant mice were compared to those in 2W4D pregnant mice infected with the Fukaya *T. gondii* stain.

It has been demonstrated that IL-6 enhances intracellular replication of *T. gondii* and reverses IFN-γ mediated activation of murine peritoneal macrophages, and that certain interactions between these two cytokines may be at the level of TNF-α triggering (Beaman *et al.*, 1994). Previous studies have detected the presence of high circulating levels of IL-6 in a lethal *T. gondii* infection in mice (Beaman *et al.*, 1991) as well as induction of IL-6 mRNA in the brains of mice infected with *T. gondii* (Hunter *et al.*, 1993). We have reported that Th1 cytotoxic T lymphocytes specific for *T. gondii*-infected melanoma cells derived from a patient with toxoplasmic chorioretinitis secreted high levels of IL-6 (Yang *et al.*, 1995). However, we observed no significant (2W4D) or very low levels (1W4D) of productions of IL-6 in placentas, lungs and spleens of *T. gondii*-infected BALB/c and C57BL/6 pregnant mice (Figs. 1 and 2). Like IL-6, IL-4 has been suggested to be involved in disease exacerbation. IL-4 impedes the induction and effector function of the subpopulation of Th1 cells which regulate cell-mediated immunity (Le *et al.*, 1990). In our experiment, Th1 dominancy in the *T. gondii*-infected pregnant mice was more impressive when IL-4 mRNA levels were examined (Figs. 1 and 2). The differences in the levels of IL-6 and IL-4 production between infections with the RH and the Fukaya strains may be explained by the differences between *T. gondii* strain properties, size of inoculum and/or infection route.

Further study, including pathological investigations into the transplacental spread of *T. gondii* and study of the immunological status of infected and control placentas of animals and the characteristics of the T lymphocytes involved in the process of placental destruction may provide further insight into the pathogenesis of congenital toxoplasmosis.

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THREE NEW BLACK FLY SPECIES OF *SIMULIUM* (DIPTERA: SIMULIIDAE) FROM SUMATRA, INDONESIA

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Abstract: Three new black fly species, i.e., *Simulium* (*Gomphostilbia*) *padangense* sp. nov., *S.* (*Simulium*) *sumatraense* sp. nov. and *S.* (*S.*) *minangkabaum* sp. nov. are described based on females, males, pupae and mature larvae collected from West Sumatra, Indonesia. *S.* (*G.*) *padangense* is unique within the subgenus *Gomphostilbia* in having the pupal gill with one much inflated and seven slender filaments. *S.* (*S.*) *sumatraense* and *S.* (*S.*) *minangkabaum* are most closely related to *S.* (*S.*) *celsum* from Java and *S.* (*S.*) *argentipes* from Peninsular Malaysia, respectively.

Key words: black fly, Simuliidae, *Simulium, Gomphostilbia,* Sumatra

The simuliid fauna of Sumatra has been little investigated since Edwards (1934) recorded eight simuliid species from Sumatra. Later, only one new species was added (Takaoka and Davies, 1995).

Recently we made surveys on black flies at various localities of Sumatra, and collected about 20 species including several new taxa. In this paper we describe three new simuliid species.

The classification follows that of Crosskey (1969). Collecting and rearing methods, as well as dissection of anatomical parts for description, were mentioned in Takaoka (1983). Type specimens will be deposited at the Natural History Museum, London, U.K. (BMNH), the Department of Parasitology and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, Indonesia (BAU), and Bogor Zoological Museum, Bogor, Indonesia (BZM).

Simulium (*Gomphostilbia*) *padangense* sp. nov.

DESCRIPTION. **Female**. Body length 2.3 mm. ***Head***. Slightly narrower than thorax. Frons dark brown, whitish gray pruinose, shiny, densely covered with whitish, recumbent, scale-like pubescence interspersed with a few, whitish, simple hairs; frontal ratio 1.6: 1.0: 3.3. Frons-head ratio 1.0: 6.4. Fronto-ocular area (Fig. 1) well developed. Clypeus dark brown, whitish gray pruinose, densely covered with whitish, recumbent, scale-like pubescence interspersed with several dark hairs. Antenna composed of 2+9 segments, brown except scape, pedicel and base of 1st flagellar segment yellowish when viewed from above; 1st flagellomere somewhat elongate, ca. 1.6× as long as 2nd one (right antenna abnormal, with flagellar segments 1-3 partially connected on dorsal surface). Maxillary palp with 5 segments; proportional lengths of 3rd, 4th and 5th segments 1.0:1.0:2.1; 3rd segment a little enlarged; sensory vesicle (Fig. 2) of moderate size, ca. 0.33× as long as 3rd segment, with medium opening distally. Maxillary lacinia with 12 inner and 13 or 14 outer teeth. Mandible with ca. 24 small inner teeth and lacking outer teeth (though apical part of outer margin very weakly indented). Cibarium (Fig. 4) with pair of submedial round projections on posterior margin, and with median longitudinal dark band on floor. ***Thorax***. Scutum dark brown in ground color, whitish gray pruinose (shining in light), with 3 dark, rather broad, longitudinal vittae distinct when viewed anterodorsally; scutum densely covered with bright, whitish, recumbent pubescence. Scutellum light brown with whitish pubescence and long, upright, dark hairs along posterior margin. Postscutellum dark brown, whitish gray pruinose (shining in light), bare. Pleural membrane bare. Katepisternum dark brown, whitish gray pruinose (shining in light), with numerous pale and dark hairs, longer than deep; sulcus distinct. ***Legs***. Foreleg: coxa and trochanter

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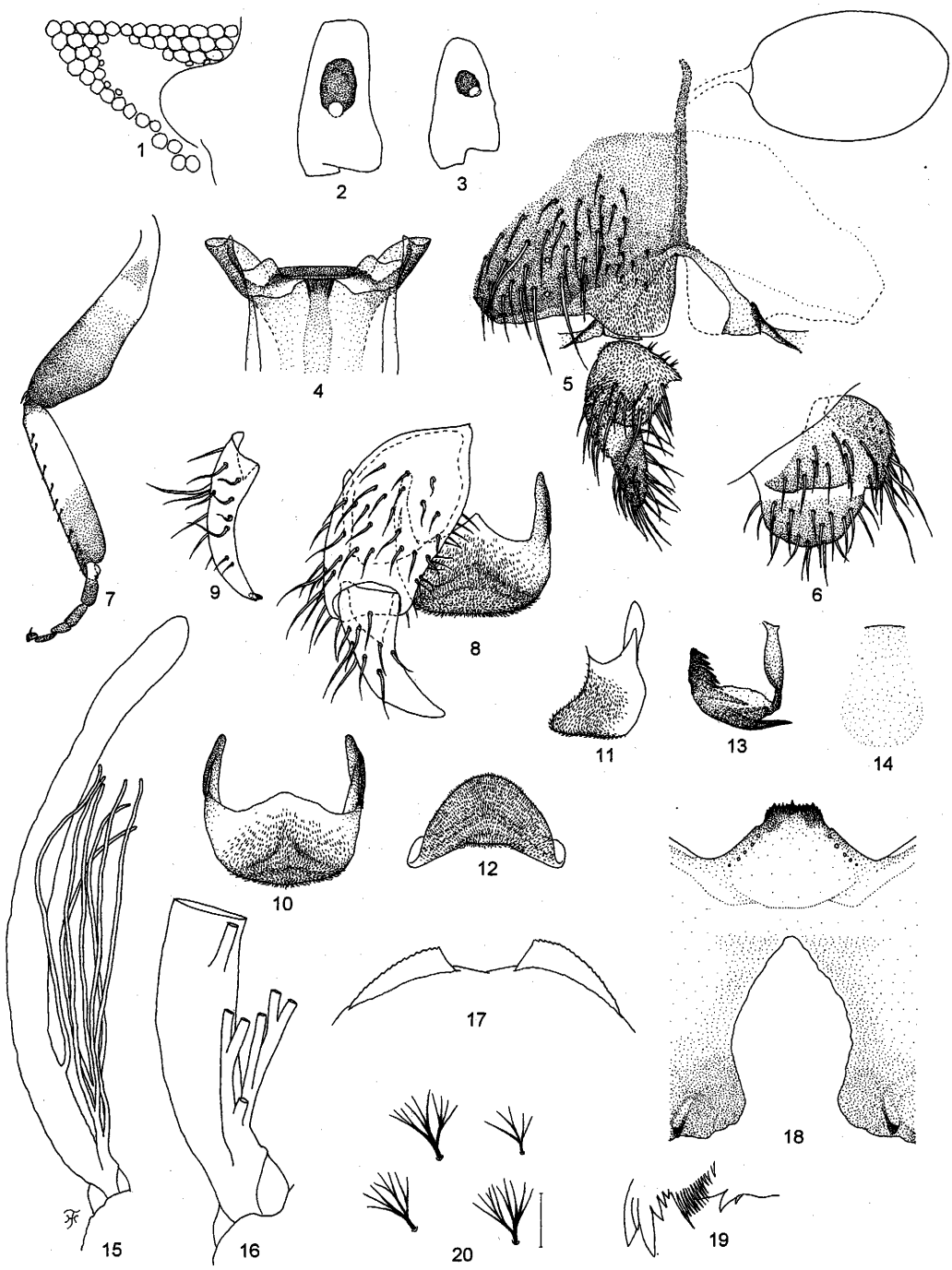
yellow; femur dark yellow or light brown with apical cap brown on outer surface; tibia largely whitish yellow on outer surface, somewhat dark along inner margin and brown on distal 1/4; tarsi dark brown; basitarsus slender, ca. 7.1× as long as its greatest width. Midleg: coxa light brown; trochanter yellow; femur dark yellow or light brown with base yellow and apical cap brown; tibia yellow on basal 1/3 with somewhat dark subbasal ring, light to dark brown on apical 2/3; tarsi brown with basal 1/2 of basitarsus yellow. Hind leg: coxa somewhat dark yellow; trochanter yellow; femur dark yellow or light brown with apical cap brown; tibia yellow on basal 1/2 with somewhat dark subbasal ring, gradually darkened toward apex, brown on apical 1/4; tarsi brown with basal 3/5 of basitarsus and basal 1/2 of 2nd tarsomere white; basitarsus slender, parallel-sided, ca. 7.6× as long as wide. Calcipala well developed, ca. 1.4× as long as wide. Pedisulcus well developed. Hind femur and tibia ca. 2.3× and 2.1× as wide as hind basitarsus, respectively. All tibiae densely covered with white, shiny, scale-like hairs on outer or posterior surface of basal 2/3 or 3/4; fore and mid femora, on outer surface, each clustered with numerous, dark, scale-like hairs from middle to just before apical dark tip, appearing as a dark broad band. Claws each with large basal tooth 0.5× as long as claw. ***Wing***. Length 2.1 mm. Costa with spinules as well as hairs. Subcosta haired. Hair tuft of stem vein yellow. Basal portion of radius fully haired. Basal cell absent. ***Abdomen***. Basal scale dull yellow with fringe of whitish hairs. Dorsal surface of abdomen dark yellowish brown or grayish brown on segment 2, somewhat darker on segments 3 and 4, and dark brown on segments 5-9, with dark hairs; tergite of 2nd segment dark yellowish and whitish pruinose, tergites of other segments brown to dark brown, those of segments 6-9 shiny. ***Genitalia*** (Figs. 5 & 6). Sternite 8 bare medially, with 30-35 dark macrosetae on each side. Anterior gonapophysis thin, membraneous, rounded posterointernally, covered densely with microsetae with a few short setae near anterior border; inner margin well sclerotized. Genital fork of usual inverted-Y form, with arms folded medially, lacking any projection directed forward. Paraproct moderately produced ventrally, ca. 0.7× as long as wide in lateral view. Cercus ca. 0.46× as long as wide, with posterior border rounded when viewed laterally. Spermatheca ellipsoidal, well sclerotized except small area around tubal juncture; internal setae absent.

Male. Body length 2.6 mm. ***Head***. Slightly wider than thorax. Upper eye consisting of large facets in 11 or 12 vertical columns and in 12 or 13 horizontal rows. Clypeus brownish black, thickly whitish pruinose (shining in light), covered densely with whitish yellow pubescence interspersed with several dark hairs. Antenna composed of 2+9 segments, pale yellow with a few apical segments somewhat darkened; 1st flagellomere somewhat elongate, ca. 1.6× as long as 2nd one. Maxillary palp with 5 segments; proportional lengths of 3rd, 4th and 5th segments 1.0:1.1:3.0; sensory vesicle (Fig. 3) small, 0.21× as long as 3rd segment, with medium opening distally. ***Thorax***. Scutum brown, faintly with 3 narrow, dark longitudinal vittae, densely covered with bright, yellow, recumbent pubescence; scutum entirely whitish pruinose on anterodorsal surface when illuminated dorsally and viewed anteriorly; also largely white pruinose on prescutellar area in certain angle of light. Scutellum brown, with bright, yellow pubescence and several dark, long hairs along posterior margin. Postscutellum brown, whitish pruinose (shining in light), bare. Pleural membrane and katepisternum as in ♀. ***Legs***. Coloration nearly as in ♀ except following features: hind tibia whitish yellow on basal 2/5, light and dark brown on rest; hind basitarsus whitish on basal 1/2, dark brown on distal 1/2. Fore basitarsus slender, ca. 8.4× as long as its greatest width. Hind basitarsus (Fig. 7) somewhat inflated, almost parallel-sided, ca. 4.7× as long as wide. Hind femur and tibia ca. 1.4× and 1.5× as wide as hind basitarsus, respectively. Calcipala well developed, ca. 1.1× as long as wide. Pedisulcus well developed. ***Wing***. Length 2.0 mm. Other features as in ♀ except subcosta bare. ***Abdomen***. Basal scale light brown, with fringe of yellow hairs. Dorsal surface of abdominal segments brownish black except that of segment 2 pale, and those of segments 3 and 4 light brown, and covered with yellow and dark hairs; segments 2, 5-7 each with dorsolateral pair of shiny, whitish pruinose patches; those on segment 2 widely connected in middle. ***Genitalia*** (Figs. 8-14). Coxite nearly rectangular in ventral view, ca. 1.7× as long as wide. Style ca. 0.85× length of coxite, tapered toward apical tip, gently curved inward, with apical spine (though 1 of 3 ♂ examined lacked apical spine). Ventral plate transverse, with posterior margin nearly straight or slightly convex medially in ventral view; ventral plate widely produced ventrally along posterior margin, with microsetae almost entirely on ventral, posterior and dorsal surface; basal arms slightly converging. Paramere with 3 long parameral hooks and several small, indistinct ones. Median sclerite plate-like, widened toward tip, with round and transparent tip.

Pupa. Body length (excluding gill filaments) ca. 3.0 mm. ***Head*** and ***thorax***. Integument pale yellow, moderately



Figs. 1-20 *Simulium* (*Gomphostilbia*) *padangense* sp. nov. 1, ♀ fronto-ocular area; 2 & 3, 3rd maxillary palpal segment with sensory vesicle (2, ♀; 3, ♂); 4, ♀ cibarium; 5 & 6, ♀ genitalia —— 5, 8th sternite, anterior gonapophyses, genital fork, paraproct, cercus and spermatheca *in situ* (ventral view); 6, paraproct and cercus (lateral view); 7, ♂ hind tibia and tarsi; 8-14, ♂ genitalia —— 8, coxite, style and ventral plate *in situ* (left coxite and style omitted, ventral view); 9, style with apical spine (ventromedial view); 10, ventral plate with posterior border somewhat rounded (ventral view); 11 & 12, ventral plate (11, lateral view; 12, end view); 13, paramere (ventral view); 14, median sclerite; 15, left pupal gill (dorsal view); 16, basal 1/4 of pupal gill showing arrangement of 7 slender filaments (dorsomedial view); 17, terminal hooks of pupal abdomen (central view); 18, larval head capsule showing hypostomium and postgenal cleft (ventral view); 19, apex of larval mandible; 20, dark, branched spinules on dorsal surface of larval abdominal segments 5-9 (scale bar 0.02 mm).

covered with tubercles. Head trichomes 4 pairs, all long and simple. Thoracic trichomes 6 pairs (3 anterodorsally, 2 anterolaterally, 1 posterolaterally), all long and simple. Antennal sheath with smooth surface. Gill (Figs. 15 & 16) composed of 1 long, inflated, tubular filament (ca. 1.8 mm long by 0.07-0.11 mm wide) and 7 slender filaments (ca. 1.1 mm long by 0.01-0.016 mm wide); inflated filament pale brown, gently curved inwards, with round distal tip, densely covered with minute tubercles on its surface; 7 slender filaments arranged in 3 groups, i.e., 2 triplets with short common stalk arising dorsomedially near base of inflated filament, and 1 isolated filament arising a little far from base of inflated filament; all slender filaments pale brown, directing forward, subequal in thickness to one another, with numerous transverse ridges, moderately covered with minute tubercles. ***Abdomen***. Terga 1 and 2 almost transparent, without tubercles; tergum 1 with 2 long setae on each side, tergum 2 with 6 simple setae on each side, 1 seta much longer than others. Terga 3 and 4 each with 4 hooked spines directed forward on each side. Terga 6-9 each with transverse row of spine-combs directed caudad on each side. Tergum 9 with pair of flat, widened terminal hooks with outer margin weakly serrate (Fig. 17). Sternum 4 with 1 distinct simple hook and a few minute setae on each side; sternum 5 with pair of bifid hooks on each side; sterna 6 and 7 each with pair of inner bifid (or trifid) and outer simple hooks on each side; last segment with a few grapnel-like hooklets ventrolaterally on each side. ***Cocoon***. Simple, wall-pocket-shaped, moderately woven.

Mature larva. Body length 4.6-5.4 mm. Body color light yellowish gray. Cephalic apotome yellowish with faint or moderate positive head spots, moderately covered with minute, uncolored setae; ventral surface of head capsule (Fig. 18) markedly darkened along lateral border of postgenal cleft. Antenna with 3 segments and apical sensillum, longer than stem of labral fan; proportional lengths of 3 segments from base to tip 1.00:0.89:0.78. Labral fan with ca. 39 main rays. Mandible (Fig. 19) with comb-teeth decreasing in size from 1st to 3rd teeth; mandibular serration composed of 1 large and 1 small tooth, without supernumerary serration. Hypostomium (Fig. 18) with row of 9 apical teeth; median tooth somewhat longer than each corner tooth which is slightly longer than 3 intermediate teeth on each side; lateral margin smooth; hypostomal setae 4 or 5 in number lying subparallel to lateral margins. Postgenal cleft (Fig. 18) lanceolate, deep, 6.0-7.5× as long as postgenal bridge, constricted basally. Thorax and abdominal segments 1-4 moderately covered with minute, dark spinules with 4-8 branches dorsally; remaining posterior segments of abdomen densely covered with minute, dark spinules with 2-11 (mostly 7-10) branches dorsally (Fig. 20); last segment densely covered with numerous uncolored setae on each side of anal sclerite. Rectal papilla compound, each of 3 lobes with 7-9 finger-like secondary lobules. Anal sclerite of usual X-form, with anterior arms 0.8× as long as posterior ones, broadly sclerotized at base. Ventral papillae well developed. Posterior circlet with ca. 86 rows of up to 14 hooklets per row.

TYPE SPECIMENS. WEST SUMATRA: Holotype ♀ (BMNH), slide-mounted with pupal exuvia, collected from a stream, crossing a road, nearly midpoint from Padang to Lubukslasih, near a restaurant just before the mountain pass, 1,060 m in altitude, 9. VIII. 1994, by H. Takaoka. Paratypes: 1 ♂ and 2 mature larvae (BMNH), slide-mounted, 2 ♂ together with pupal exuvia and cocoon, and 6 mature larvae (BAU), in alcohol, same data as holotype.

ECOLOGICAL NOTES. Pupae and larvae of this species were collected from fallen pine leaves in a small, shaded, forest stream with a bedrock and a width of 0.3-0.5 m. Water temperature was 19°C.

REMARKS. This new species is characterized by its pupal gills consisting of one inflated tubular and seven slender filaments. There is no report on such unique pupal gills among species of *Gomphostilbia*.

The female and male of this species are similar in leg coloration to *S.* (*G.*) *sundaicum* from Java and Sumatra and *S.* (*G.*) *whartoni* and *S.* (*G.*) *cheongi* from Peninsular Malaysia (Takaoka and Davies, 1995; 1996) but are distinguished by the shape of the cibarium and the mandible in the female and somewhat inflated hind basitarsus in the male.

The mature larva of this species is easily separated by its unique shape of gill histoblast developing on each side of the thorax.

Simulium (*Simulium*) *sumatraense* sp. nov.

DESCRIPTION. **Female**. Body length 2.5 mm. ***Head***. Narrower than width of thorax. Frons black, shiny, with several dark stout hairs along lateral margins; frontal ratio 1.3:1.0:1.2; frons-head ratio 1.0:4.0. Fronto-ocular area (Fig. 21) well developed. Clypeus black, shiny, thickly whitish gray pruinose, with scattered,

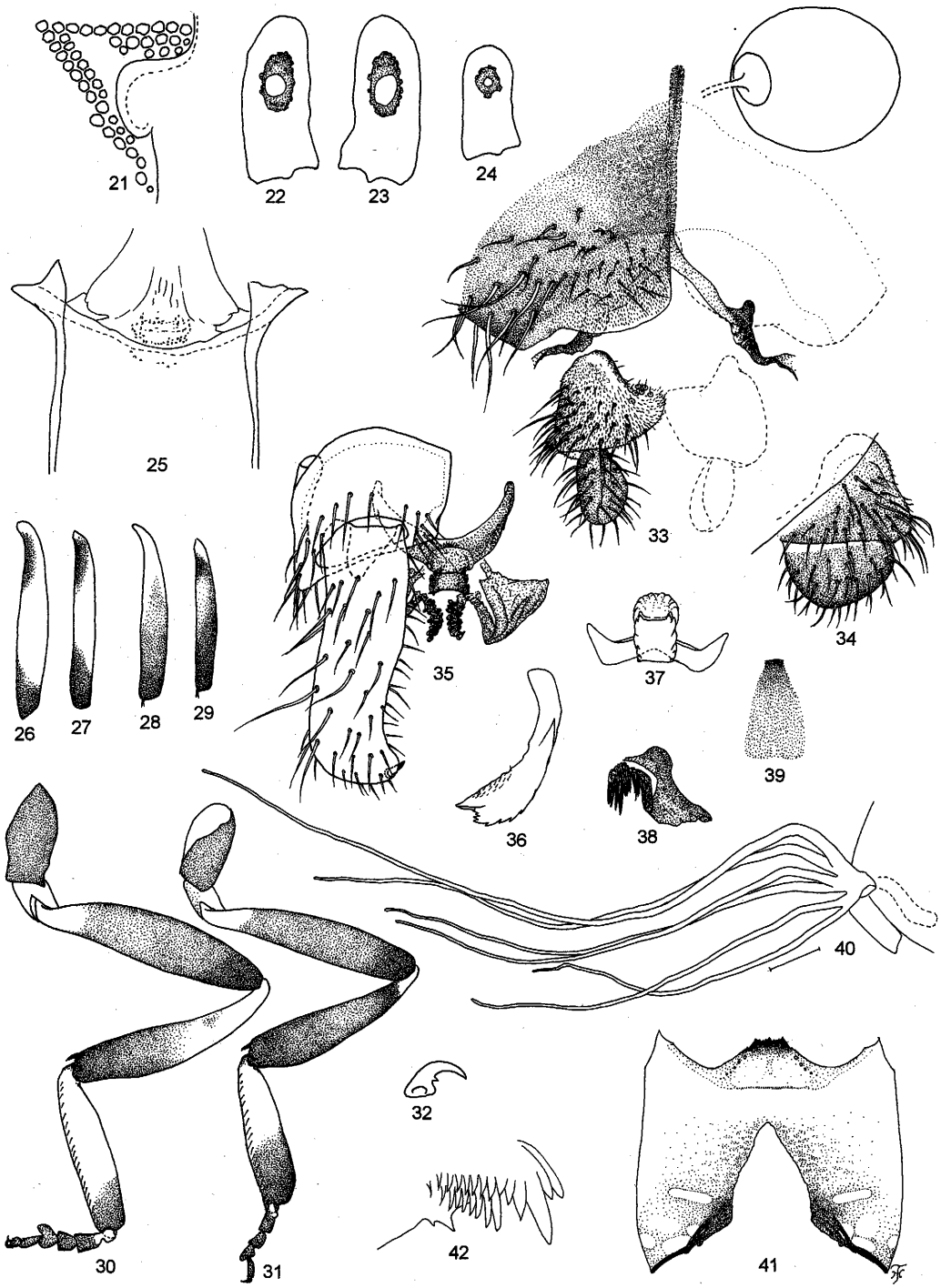
dark, stout hairs; when illuminated, silvery iridescent. Antenna composed of 2+9 segments, dark brown, scape and pedicel yellow. Maxillary palp composed of 5 segments, proportional lengths of 3rd, 4th and 5th segments 1.0:1.0:2.3; 3rd segment of moderate size; sensory vesicle (Figs. 22 & 23) of medium size, elliptical, with rugged surface, 0.37-0.40× length of 3rd segment, with large, round opening a little distad to center. Maxillary lacinia with 13 inner and 16 outer teeth. Mandible with ca. 32 inner and 13-15 outer teeth. Cibarium (Fig. 25) with numerous minute tubercles. ***Thorax***. Scutum brownish black, shiny, moderately covered with recumbent, dark brown pubescence, interspersed with long, upstanding, dark hairs on prescutellar area; scutum with bright iridescent spot on each shoulder extending posteriorly along lateral border to wing base in certain angles of lights. Scutellum brownish black, with long, dark hairs. Postscutellum brownish black, shiny, silvery iridescent when viewed in lights, without hairs. Pleural membrane bare. Katepisternum longer than deep, bare. ***Legs***. Foreleg: coxa and trochanter yellow; femur yellow with distal 1/4 brownish black; tibia (Fig. 26) white with distal 1/4 and basal 1/3 (except outer surface white) brownish black, outer median portion largely iridescent in lights; basitarsus entirely black, moderately dilated (W: L=1.0:5.1), with thick dorsal hair crest; rest of tarsal segments black. Midleg: coxa and trochanter brownish black except basal 1/2 of trochanter yellow; femur entirely brownish black; tibia in outside view (Fig. 28) yellowish white on basal 1/3 and brown to brownish black on distal 2/3 (except a little more than basal 1/2 of posterior surface and basal 2/3 of anterior surface yellowish white), in certain lights with large area of whitish sheen on posterior surface; basitarsus whitish except distal 1/5 brownish black; rest of tarsal segments brownish black except basal 1/3 of 2nd segment white. Hind leg (Fig. 30): coxa brownish black; trochanter yellow; femur brownish black except basal 1/5 pale yellow; tibia yellowish white on basal 1/2, brown to brownish black on distal 1/2, with brown subbasal patch, in certain lights with large area of whitish sheen on posterior surface; tibia 0.83× as wide as femur; tarsal segments brownish black except a little more than basal 2/3 of basitarsus and basal 1/2 of 2nd tarsomere whitish; basitarsus W:L=1.0:5.3, calcipala short, W:L ratio 1.1:1.0; pedisulcus distinct. Tarsal claws (Fig. 32) each with small, subbasal tooth. ***Wing***. Length 2.6 mm; costa with spinules and hairs; subcosta with several hairs; basal section of vein R bare; hair tuft at base of stem vein dark brown; basal cell absent. ***Abdomen***. Basal scale black with fringe of dark hairs; 2nd segment dark brown with large, dorsolateral, whitish iridescent spots broadly connected in middle; tergites 3-5 small, dark brown; tergites 6-8 large, black, shining, with dark hairs. ***Genitalia*** (Figs. 33 & 34). Ventral surface of abdominal segment 7 with submedian pair of weak sternal plates of irregular form. Sternite 8 well sclerotized, bare medially, with ca. 24 long stout hairs laterally on each side; anterior gonapophysis inverted trapezoidal, membraneous, covered with 17-19 short hairs and numerous microsetae except narrow portion along posterior margin bare; inner border nearly straight, narrowly sclerotized. Genital fork of inverted-Y form, with well sclerotized slender stem; arms slender, each with strongly sclerotized distal ridge and distinct projection directed anteriorly. Paraproct in ventral view a little wider than long, with ca. 8 minute sensilla on anterointernal surface, covered with ca. 36 short and long hairs ventrally and laterally; in lateral view paraproct not produced ventroposteriorly below cercus. Cercus in lateral view rounded posteriorly, ca. 0.5× as long as wide, covered with ca. 20 short hairs. Spermatheca nearly globular, well sclerotized except tube and small area of tubal base, with no definite reticulate pattern except near base, with minute internal setae.

Male. Body length 2.6 mm. ***Head***. As wide as thorax. Upper eye consisting of large facets in 16 horizontal rows and in 14 vertical columns. Clypeus black, whitish gray pruinose, strongly iridescent when illuminated, sparsely covered with dark brown hairs. Antenna composed of 2+9 segments, dark brown except base of pedicel and base of 1st flagellar segment pale yellow; 1st flagellomere elongated, 1.8× as long as 2nd one. Maxillary palp composed of 5 segments, proportional lengths of 3rd, 4th and 5th segments 1.0:1.3:2.3; 3rd segment (Fig. 24) of normal size, with small sensory vesicle 0.27× length of 3rd segment; opening of sensory vesicle small. ***Thorax***. Scutum brownish black, with silvery iridescent pattern composed of anterior pair of spots on shoulders, large transverse spot on prescutellar area contiguous to anterior spots by band along lateral margins; spot on each shoulder curved posteromedially, somewhat pointing posteriorly appearing like a crescent band; scutum uniformly covered with copper-colored, recumbent pubescence, interspersed with long, upright, dark hairs on prescutellar area. Scutellum, postscutellum, pleural membrane and katepisternum as in ♀. ***Legs***. Foreleg: coxa yellow; trochanter dark yellow; femur yellow on basal 3/4 (though somewhat darkened distally), brownish black on distal 1/4; tibia (Fig. 27) white except basal 1/3 and distal 1/3 on inner and lateral surfaces brownish

black, with large area of whitish sheen on outer surface; tarsi black; basitarsus somewhat dilated (W:L=1.0:5.7). Midleg: coxa, trochanter and femur brownish black except basal 1/3 of trochanter yellow; tibia (Fig. 29) brown to brownish black with base yellow, and anterior surface somewhat paler submedially; basal 1/3 on posterior surface with whitish sheen in certain angle of light; basitarsus whitish except distal 1/5 brownish black (its border not well defined); rest of tarsal segments brownish black except base of 2nd and 3rd segments pale. Hind leg (Fig. 31): coxa brownish black; trochanter dark yellow; femur brownish black except basal 1/5 yellow; tibia brownish black except base pale yellow; tarsal segments brownish black except basal 1/2 of basitarsus and a little less than basal 1/2 of 2nd tarsal segment whitish; hind basitarsus enlarged, wedge-shaped (W:L=1.0:3.9), subequal in greatest width to hind femur, but a little narrower than hind tibia; calcipala small, W: L ratio 1.0:1.0; pedisulcus distinct. ***Wing***. Length 2.4 mm; other features as in ♀ except subcosta bare. ***Abdomen***. Basal scale blackish with long, dark hairs. Terga brown to brownish black with dark hairs; segments 2, 4-7 each with pair of silvery iridescent areas dorsolaterally, those on segment 2 broadly connected in middle. ***Genitalia*** (Figs. 35-39). Coxite in ventral view nearly quadrate, 0.57× as long as style; style length 3.7× width at base, nearly parallel-sided throughout its length in ventral view; style spatulate ventrodorsally, with subterminal spine; no prominent subbasal protuberance present. Ventral plate and basal arms in ventral view forming a broad U-shape, having low ventral projection with toothed posterior margins (7 or 8 teeth on each side); plate with midventral ridge anterior to ventral projection, slightly wider than plate, covered with fine appressed setae anteriorly; basal arms long, curved outwardly and forwardly. Median sclerite of shoehorn shape, thin, gradually widened toward apex. Paramere with numerous medium-sized hooks.

Pupa. Body length (excluding gill filaments) 3.0 mm. ***Head***. Integument dark yellow, very sparsely covered with tubercles, with 1 facial and 2 frontal pairs of short, slender, simple trichomes, facial trichomes much longer than frontal ones. ***Thorax***. Integument yellowish brown, sparsely covered with tubercles, with 2 dorsal and 2 lateral pairs of rather long, simple trichomes on anterior 1/2 plus 1 lateral pair of trichomes on posterior 1/2, those trichomes are subequal to each other, but much longer than facial ones. Gill (Fig. 40) with 6 dark brown, slender filaments in pairs, very short-stalked, gradually tapering toward apex, somewhat shorter than pupa, decreasing in length and thickness from dorsal to ventral; dorsal filament of upper pair longest (2.6-2.7 mm), ventral one of lower pair shortest (1.3-1.4 mm), other filaments intermediate in length (1.5-2.1 mm); cuticle of filaments with well-defined annular furrows and ridges forming reticulate pattern, uniformly covered with minute tubercles. ***Abdomen***. Tergum 1 pale yellowish brown with 1 long seta on each side. Tergum 2 on each side with 1 long and 5 short simple setae in transverse row along posterior margin, and on each anterolateral portion with numerous comb-like groups of minute spines. Terga 3 and 4 each with 4 hooked spines along posterior margin on each side. Terga 7 and 8 each with transverse row of spine-combs on each side. Tergum 9 with pair of small, cone-shaped, terminal hooks, and comb-like groups of minute spines. Sternum 4 with a few, minute, simple setae submedially on each side. Sternum 5 with pair of bifid hooks submedially on each side. Sterna 6 and 7 each with pair of bifid inner and simple outer hooks widely spaced on each side. Grapnel-like hooklets absent. ***Cocoon***. Wall-pocket-shaped, thickly woven, with thick anterior rim; there is no ventrolateral extension.

Mature larva. Body length 5.4-6.0 mm. Body color dark gray. Cephalic apotome pale yellowish white on anterior 1/2, with 3 distinct darkened areas on posterior 1/2 (1 medially just before posterior border, the other 2 submedially between head spots); all head spots pale, except mediolateral spots sometimes somewhat darkened, then appearing negative type; lateral surface of head capsule widely darkened behind eye spot area, with negative lateral spots near posterior border; ventral surface of head capsule (Fig. 41) dark, specially near border of postgenal cleft, with negative spots. Antenna composed of 3 segments and apical sensillum, longer than stem of labral fan; length ratio of segments (from base to tip) 1.00:1.06:0.65. Labral fan with ca. 36 main rays. Mandible (Fig. 42) with mandibular serration composed of 1 large and 1 small tooth, without supernumerary serration; 1st comb-tooth much longer than others. Hypostomium with 9 small anterior teeth, of which median and each corner tooth are longer than others; 5 or 6 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Fig. 41) deep, widest at base, anterior 2/3 gradually narrowed toward anterior tip, ca. 4.0× length of postgenal bridge. Thoracic cuticle bare. Abdominal cuticle bare except dorsolateral areas on last segment moderately covered with short, uncolored setae. Rectal papilla compound, each of 3 lobes with 10 or 11 slender, finger-like, secondary lobules. Anal sclerite X-shaped, with



Figs. 21-42 *Simulium* (*Simulium*) *sumatraense* sp. nov. 21, ♀ fronto-ocular area; 22-24, 3rd maxillary palpal segment with sensory vesicle (22 & 23, ♀; 24, ♂); 25, ♀ cibarium; 26 & 27, fore tibia (26, ♀; 27, ♂); 28 & 29, mid tibia (28, ♀; 29, ♂); 30 & 31, hind leg (30, ♀; 31, ♂); 32, ♀ claw; 33 & 34, ♀ genitalia —— 33, 8th sternite, anterior gonapophyses, genital fork, paraprocts, cerci and spermatheca *in situ* (ventral view); 34, paraproct and cercus (lateral view); 35-39, ♂ genitalia —— 35, coxite, style, ventral plate and parameres *in situ* (left coxite and style omitted, ventral view); 36 & 37, ventral plate (36, lateral view; 37, end view); 38, paramere (left side only, end view); 39, median sclerite; 40, pupal gill filaments (outside view, scale bar 0.2 mm); 41, larval head capsule showing hypostomium and postgenal cleft (ventral view); 42, apex of larval mandible.

broadened anterior arms ca. 0.5× length of posterior ones. Posterior circlet with ca. 88 rows of hooklets with up to 13 hooklets per row. Ventral papillae absent.

TYPE SPECIMENS. WEST SUMATRA: Holotype ♀ (BMNH), slide-mounted, together with pupal exuvia, just south of Lake Dibawah, ca. 1,580 m in alt., 21. VIII. 1994, by H. Takaoka. Paratypes: 1 ♂, slide-mounted, together with pupal exuvia, 1 pupa, 3 mature larvae (BMNH), same data as holotype; 1 ♀, 1 mature larva (BAU), just southeast of Lake Diatas, along the road from Alahanpanjang to Surian, ca. 1,470 m in alt., 12. VIII. 1994, by H. Takaoka.

ECOLOGICAL NOTES. Pupae and larvae were found on twigs caught in the water of two small streams (one, 0.4-0.6 m wide, shaded, running through the natural forest beyond the cultivated field for tea plantation, and the other, 0.3-0.5 m wide, partially shaded, running from the forest into the rice field).

REMARKS. This species is closely related to *S.* (*S.*) *celsum* and *S.* (*S.*) *nebulicola* from Java (Takaoka and Davies, 1996). The genitalia of both sexes are almost the same in these three species. However this species differs from these two known species in the adults by the leg coloration: the femora of both female and male midlegs of this species are entirely dark while those of the other two species are pale at least basally, and also the female hind tibia has a subbasal dark spot in this species but lacks such a spot in the other two species. The pupa of this species is almost the same as that of *S.* (*S.*) *celsum,* but differs from that of *S.* (*S.*) *nebulicola* by the longer gill filaments and the presence of the terminal hooks. This species appears to be distinguished in the larva from the other two species by the unicolored body.

The female of this species is somewhat similar to that of *S.* (*S.*) *fuscopilosum* Edwards from Peninsular Malaysia (Takaoka and Davies, 1995) in several characters including the unpatterned scutum, claws with a subbasal tooth, genital fork with a strong projection on each arm, paraproct not produced ventrally, hind tibia with a subbasal dark spot. However, there are differences in the cibarium, sensory vesicle, leg coloration and shape of the anterior gonapophyses.

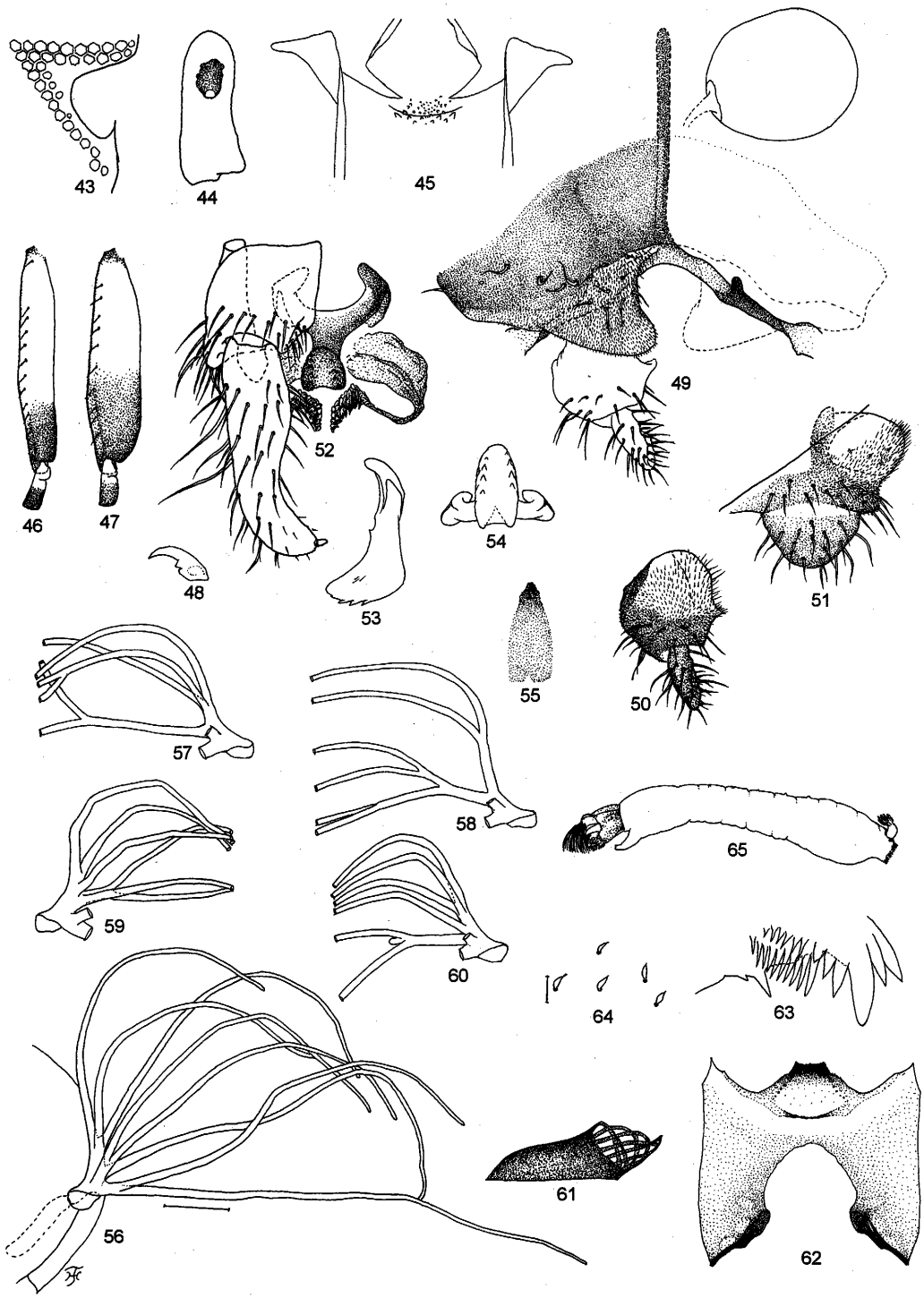
Simulium (*Simulium*) *minangkabaum* sp. nov.

DESCRIPTION. **FEMALE**. Body length ca. 2.5 mm. ***Head***. Narrower than width of thorax. Frons brownish black, shiny, with several dark hairs along each lateral margin and above antennal base; frontal ratio 1.20:1.00: 1.13; frons-head ratio 1.0:4.0. Fronto-ocular area (Fig. 43) narrowly developed, with triangular lateral tip. Clypeus brownish black, shiny, thickly white pruinose, covered moderately with dark hairs except median portion on upper 1/2 largely bare. Antenna composed of 2+9 segments, brown except scape, pedicel and base of 1st flagellar segment yellow in dorsal view. Maxillary palp brownish black, composed of 5 segments with proportional length of 3rd, 4th and 5th segments 1.0:1.2: 2.4; 3rd segment (Fig. 44) of moderate size; sensory vesicle medium in size, elliptical, with rugged surface, 0.24× length of 3rd segment, with medium round opening near distal end. Maxillary lacinia with 11 inner and 13 outer teeth. Mandible with ca. 28 inner and 12 or 13 outer teeth. Cibarium (Fig. 45) with ca. 12 minute tubercles. ***Thorax***. Scutum brownish black, shiny, thinly white pruinose, covered moderately with yellow pubescence, interspersed with long, upstanding, dark hairs on prescutellar area. Scutellum brownish black, with long, dark hairs and yellow pubescence. Postscutellum brownish black, shiny, white pruinose, without hairs. Pleural membrane bare. Katepisternum longer than deep, bare. ***Legs***. Foreleg: coxa and trochanter yellow; femur yellow on basal 1/2, dark yellow to yellowish brown on distal 1/2; tibia white with distal 1/6 brown, and with median anterior portion largely white sheeny in lights; basitarsus entirely brownish black, dilated (W:L=1.0:5.4); rest tarsal segments brownish black. Midleg: coxa brownish black; trochanter and femur brown with basal 1/3 or 1/2 of trochanter yellow; tibia white except distal 1/6 brown, posterior surface largely with a white sheen when illuminated; tarsi brownish with basal 3/4 of basitarsus, basal 2/5 of 2nd segment and base of 3rd segment white. Hind leg: coxa brownish black; trochanter yellow; femur dark brown to brownish black except base yellow; tibia white with a little less than distal 1/2 brown, posterior surface along basal 1/2 or a little more with a white sheen when illuminated; tarsi brown with a little less than basal 3/4 of basitarsus and basal 1/2 of 2nd segment white; basitarsus (Fig. 46) parallel-sided, W:L=1.0:6.1, its width ca. 0.7× that of tibia and ca. 0.6× that of femur; calcipala short, L:W ratio 1.0:1.1, and ca. 0.6× as wide as distal tip of basitarsus; pedisulcus distinct. All tarsal claws (Fig. 48) with small subbasal tooth. All femora and tibiae covered with scale-like setae on outer surface. ***Wing***. Length 2.3 mm; costa with spinules and hairs; subcosta haired except distal 1/5 or 1/6 bare; basal section of vein R bare; hair tuft of stem vein dark brown; basal cell absent. ***Abdomen***. Basal scale brown-

ish black with fringe of pale hairs; all segments brownish black; tergite 2 with pair of large, dorsolateral silvery iridescent spots broadly connected to each other in middle; tergites 3-5 small and dull; tergites 6-8 large, shiny, with dark hairs. ***Genitalia*** (Figs. 49-51). Ventral surface of abdominal segment 7 without developed sternite. Sternite 8 well sclerotized, bare medially but with 8-14 dark, long, stout hairs laterally on each side; anterior gonapophysis tongue-like in shape, membraneous, bent ventrally, covered with 6-8 somewhat long hairs as well as numerous microsetae; inner borders concave, widely separated in middle from each other. Genital fork of inverted-Y form, with well sclerotized stem; arms narrow, each with strongly sclerotized distal bulge and with distinct projection directed anterodorsally. Paraproct enlarged, nearly as long as wide in ventral view, somewhat produced ventrally and ventroanteriorly, covered with 9-14 short, stout hairs in lateral view; surface of anteroventral corner densely covered with pale setae; inside surface largely concave and well sclerotized anteriorly and with several minute setae. Cercus semilunar in lateral view, covered with numerous short hairs. Spermatheca nearly globular, well sclerotized with definite reticulate pattern, and with minute internal setae; tube and small area of tubal base unsclerotized.

Male. Body length 2.5-2.8 mm. ***Head***. Slightly wider than thorax. Upper eye consisting of large facets in 18 horizontal rows and in 16 or 17 vertical columns. Clypeus black, silvery pruinose, strongly iridescent when illuminated, moderately covered with dark hairs except median portion largely bare. Antenna composed of 2+9 segments, dark brown; 1st flagellomere 1.5× as long as 2nd flagellomere. Maxillary palp composed of 5 segments, proportional lengths of 3rd, 4th and 5th segments 1.0: 1.4: 3.1; 3rd segment of normal size, with small sensory vesicle. ***Thorax***. Scutum black, with silvery and blueish iridescent pattern composed of anterior pair of large spots on shoulders, large transverse spot on prescutellar area contiguous to anterior spots by broad band along lateral margins; anterior pair of spots widely separated from each other in middle, curved posteromedially with posteromedial end somewhat pointed; scutum uniformly covered with brown pubescence, interspersed with long, upright, dark hairs on prescutellar area. Scutellum dark brown with long, upright, dark hairs. Postscutellum dark brown, white pruinose, shiny. Katepisternum and pleural membrane as in ♀. ***Legs***. Foreleg: coxa and trochanter pale yellow; femur brown with basal 1/5 or 1/4 yellow or dark yellow; tibia dark brown with outer surface mostly white, with a white sheen when illuminated; tarsi brownish black; basitarsus somewhat dilated, W:L=1.0:5.5. Midleg: coxa brownish black; trochanter and femur dark brown to brownish black except base of trochanter yellow; tibia dark brown with posterior surface of basal 1/3 white, with a white sheen when illuminated; tarsi brown with basal 1/2 or a little less of basitarsus pale yellow. Hind leg: coxa brownish black; trochanter yellow; femur brownish black except base yellow; tibia brownish black except extreme base white; tarsi brownish black except basal 1/2 of basitarsus and of 2nd segment whitish; basitarsus (Fig. 47) enlarged, W:L=1.0:4.0, and only slightly narrower than femur at greatest width, which is slightly narrower than tibia; calcipala small, pedisulcus distinct. ***Wing***. Length 2.2 mm; other features as in ♀ except subcosta bare. ***Abdomen***. Basal scale brownish black with long dark hairs; tergites brown to brownish black with dark hairs; segments 2, 5-7 each with pair of silvery and blueish iridescent spots dorsolaterally; those on segment 2 broadly connected in middle. ***Genitalia*** (Figs. 52-55). Coxite nearly quadrate. Style elongate, ca. 2.0× as long as coxite, narrowed from basal 1/3 to apical tip, and with apical spine; style flattened dorsoventrally, having no basal protuberance and the posterior third curved medially. Ventral plate broadly Y-shaped, with narrow body in ventral view, having median process directed ventrally, and with a few fine setae on each lateral side; posterior surface of median process with vertical row of 4 small spines on each lateral margin; basal arms stout, diverged from each other, with apical tip remarkably curved inward. Parameres wide, each with several small hooks. Median sclerite wide, thin, and with small medial notch apically.

Pupa. Body length (excluding gill filaments) ca. 2.8 mm. ***Head***. Integument yellow, covered moderately or sparsely with small tubercles except frontal surface almost bare, and with 1 facial and 2 frontal pairs of simple trichomes (facial trichomes much longer than frontal ones). ***Thorax***. Integument yellow, without any protruding ridges, covered moderately with small tubercles laterally and posterodorsally but almost bare anterodorsally; thorax anteriorly with 3 dorsal and 2 dorsolateral pairs of long, simple and somewhat stout trichomes (occasionally 1 of those bifid) and also posteriorly with 1 lateral pair of simple trichomes. Gill (Fig. 56) usually with 8 short, slender filaments basically arranged in 2 groups (i.e., inner and outer groups), inner group mostly consisting of 1 middle and 3 dorsal filaments (i.e., 2 paired and 1 individual filaments) or rarely consisting of 2 dorsal and 2 middle paired filaments, and outer group consisting of 2 middle and 2 ventral paired filaments with stalk of variable lengths; in about 1/3 of



Figs. 43-65 *Simulium* (*Simulium*) *minangkabaum* sp. nov. 43, ♀ fronto-ocular area; 44, ♀ 3rd maxillary palpal segment with sensory vesicle; 45, ♀ cibarium; 46 & 47, hind basitarsus and 2nd tarsal segment (46, ♀; 47, ♂); 48, ♀ claw; 49-51, ♀ genitalia —— 49, 8th sternite, anterior gonapophyses, genital fork, paraproct, cercus and spermatheca *in situ* (ventral view); 50 & 51, paraproct and cercus (50, ventral view; 51, lateral view); 52-55, ♂ genitalia —— 52, coxite, style, ventral plate and parameres *in situ* (left coxite and style omitted, ventral view); 53 & 54, ventral plate (53, lateral view; 54, end view); 55, median sclerite; 56, right pupal gill (outside view, scale bar 0.2 mm); 57-60, basal 1/2 of pupal gill showing various arrangements of inner group filaments (outside view, 4 filaments of outer group omitted); 61, cocoon (lateral view); 62, larval head capsule showing hypostomium and postgenal cleft (ventral view); 63, apex of larval mandible; 64, uncolored setae on dorsal surface of larval thorax and abdomen (scale bar 0.01 mm); 65, whole body of mature larva showing dorsal protuberances on abdominal segments 1-5 (lateral view).

Table 1 Variation in the number of pupal gill filaments of *Simulium* (*Simulium*) *minangkabaum* sp. nov. Numbers of pupae collected at two localities with different numbers of gill filaments are shown

Localities	No. filaments (left, right or vice versa) (8, 8)	(8, 9)	(9, 9)	(9, 10)	(10, 10)
Talang	9	0	1	1	2
Lake Diatas	28	0	1	2	10

the pupae examined (Table 1), gill with 9 or 10 filaments, caused by variations in filament number of inner group (Figs. 57-60), e.g., individual filament of dorsal triplet is further divided into 2 filaments, with stalk of variable lengths, or additional filament arising from the base of dorsal triplet, and/or middle filament is branched into 2 filaments with stalk of variable lengths; middle filament is occasionally branched into 4 filaments in pairs while individual filament of dorsal triplet disappears; gill filaments yellow to brown, as dark as or darker than thoracic integument, increasing in length from dorsal to ventral (ca. 0.8-1.6 mm long), tapering toward tip, with moderate annular ridges and furrows throughout their length, forming reticulate pattern, covered with minute tubercles. ***Abdomen***. Tergum 1 pale yellowish brown, with 1 simple, slender hair on each side. Tergum 2 on each side with 1 long, simple seta and 5 short, simple, spinous setae, 3 or 4 of which are somewhat stout. Terga 3 and 4 each with 4 hooked spines along posterior margin on each side. Tergum 8 with transverse row of distinct spine-combs on each side. Tergum 9 with pair of small but distinct, cone-shaped, terminal hooks. Sternum 4 with a few, minute, simple setae submedially on each side. Sternum 5 with pair of bifid hooks submedially on each side. Sterna 6 and 7 each with pair of bifid inner and simple outer hooks widely spaced on each side. Grapnel-like hooklets absent. ***Cocoon*** (Fig. 61). Shoe-shaped, with rather low front wall, thickly woven, but anteriorly with several open interspaces or windows in webs.

Mature larva. Body length 5.5-6.1 mm. Body color pale reddish brown or yellowish green to greenish gray. Cephalic apotome usually pale yellow to yellow on anterior 1/2, but darkened to varying extent on posterior 1/2, with median narrow portion darkest just before the posterior margin; anterior median longitudinal spot usually appearing positive, while other head spots appearing positive or negative depending on the extent of darkness of the ground color, or merged in ground color with pale submedial space on each side of posterior longitudinal spot; ventral surface of head capsule (Fig. 62) uniformly darkened to varying extent except near posterior margin of hypostomium widely pale yellow. Antenna composed of 3 segments and apical sensillum, longer than stem of labral fan; length ratio of segments (from base to tip) 1.0: 1.3: 0.6. Labral fan with 54-58 main rays. Mandible (Fig. 63) with 2nd and 3rd comb-teeth of equal size, shorter than 1st one; mandibular serration composed of 1 medium and 1 small tooth but without supernumerary serration. Hypostomal teeth 9 in number, small, with median and each corner tooth longer than others; lateral margins markedly serrate apically; 5 or 6 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Fig. 62) very deep, rounded, ca. 4.0× as long as postgenal bridge. Thoracic and abdominal cuticle moderately covered with very minute, flat, uncolored setae (Fig. 64) dorsally and dorsolaterally. Abdominal segments 1-5 each with dorsolateral pair of conical protuberances (Fig. 65). Rectal papilla compound, each of 3 lobes with 8-11 finger-like secondary lobules. Anal sclerite X-shaped, with broadened anterior arms ca. 0.7× as long as posterior ones. Last abdominal segment bulged laterally but lacking ventral papillae. Posterior circlet with 90-96 rows of hooklets with up to 16 hooklets per row.

TYPE SPECIMENS. WEST SUMATRA: Holotype ♀ (BMNH), slide-mounted with pupal exuvia, just southeast of Lake Diatas, along the road from Alahanpanjang to Surian, ca. 1,460 m in alt., 12. VIII. 1994, by H. Takaoka. Paratypes: 1 ♀ (BAU), 1 ♂ (BMNH), both slide-mounted, 4 ♀, 4 ♂, 4 pupal exuviae, 2 mature larvae (BAU), 3 ♀, 3 ♂, 6 pupal exuviae, 4 mature larvae (BMNH), all in alcohol, same dasa as holotype. Other paratypes: 4 ♀, 4 ♂, 2 pupal exuviae, 1 mature larva (BZM), just south of Talang, along the road from Solok to Padang, ca. 970 m in alt., 9. VIII. 1994, by H. Takaoka.

ECOLOGICAL NOTES. Pupae and larvae of this new species were found attached to trailing grasses, and leaves and stems of aquatic plant in water in two medium, moderately flowing streams (3-5 m wide, 0.2-0.4 m deep), exposed to the sun, running through hilly cultivated fields. Water temperature was 20°C.

REMARKS. This new species most closely resembles, in the female, *S.* (*S.*) *argentipes* Edwards, which was originally described from female specimens collected from Peninsular Malaysia (Edwards, 1928), in many characters including the genitalia with subtle differences

(Takaoka and Davies, 1995). There are only slight differences in the leg coloration between the two species: in the latter species, the fore femur was almost brown, the fore tibia was largely white but brown on the distal tip and along the inner margin, and the hind tibia white with the distal 1/3 brown. There may be some differences in the other characters, such as the cibarium, mandible, maxillary palp and sensory vesicle, of which no detailed descriptions are, however, available for the latter species.

The other related known species, i.e., *S.* (*S.*) *beludense* from Sabah (Takaoka, 1996), *S.* (*S.*) *canlaonense* from the Philippines (Takaoka, 1983), *S.* (*S.*) *tanae* from south China (Xue, 1992), and *S.* (*S.*) *bidentatum* from Japan and China (Takaoka, 1976) are different from this new species by the coloration of the female fore femora, which are almost brownish in the former two known species, and almost yellowish in the latter two.

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HOOKWORM INFECTION RECORDED AT AN UNIVERSITY TEACHING HOSPITAL IN KATHMANDU, NEPAL OVER ONE DECADE PERIOD

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Abstract: We report an annual prevalence of hookworm infection among Nepalese as studied at Parasitology Laboratory of Department of Pathology, Tribhuvan University Teaching Hospital, Kathmandu, Nepal over one decade period (1985-1994). A total of 4,164 to 9,440 (average 7,857) fecal samples were examined each year for the presence of various intestinal parasites. The annual positivity rate for hookworm infection ranged from 3.8 to 10.7 percent. Throughout the study period, a higher incidence of hookworm infection was observed among adults compared with children. Hookworm infection was more common among females compared with their male counterparts except for the year 1989 and 1990. Of the two species of human hookworms, 67.0% were *Ancylostoma duodenale* while remaining 33.0% were *Necator americanus*.

Key words: hookworm, *Ancylostoma duodenale, Necator americanus,* Nepal

INTRODUCTION

Hookworms are among the most common human parasite and are transmitted by fecal contamination of the soil. Approximately one billion people, mainly in developing countries, are affected by hookworm infection (Gilles, 1985; Capello *et al.*, 1995). The hallmark of hookworm infection is gastro-intestinal blood loss and iron deficiency anemia. Blood loss is attributed to the direct ingestion by worms and bleeding from trauma produced by worm attachment and feeding. The loss of blood into the gut is proportional to the worm load (Gilles, 1985) but becomes disproportionately great when worms are crowded (Beaver *et al.*, 1964). Hookworm infection in children cause both physical and intellectual growth retardation. Heavy infection may cause death due to severe hemorrhage and shock. Zimmermann (1946) reported 60.0% fatality rate due to ancylostomiasis in children of less than 16 months in Guam. An association between malaria and hookworm infection in the causation of severe anemia has also been reported (Williams and Naraqi, 1979; Fleming, 1989). Thus, the hookworm infection has a significant impact both on health and economic productivity of an individual.

Hookworm infection rate elsewhere in the world reportedly vary from less than 5.0% to as high as 90.0% (Maldonado, 1993) and 93.0% (Adams *et al.*, 1994), Mani *et al.* (1993) from southern part of neighboring country India reported an incidence rate of 45.0% among primary school children. An unusual outbreak of hookworm disease after a *Kabaddi* game (a local game which results in much body contact with the ground) has also been reported from North India (Koshy *et al.*, 1978). Infantile hookworm disease has also been reported. Yu *et al.* (1995) have reviewed hundreds of cases of infantile hookworm disease in China. Chaudhary and Jayswal (1984) have also reported infantile hookworm disease from India.

Anemia is a common clinical condition among

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Nepalese to which hookworm infection contribute to a great extent. A good correlation between hookworm infection and hemoglobin depletion has been observed in Nepalese children (Curtale *et al.*, 1993). In Nepal, the hookworm infection rate is variable between less than 5.0% to as high as 83.8% in different specified population (Gianotti, 1990; Rai and Gurung, 1986; Estevez *et al.*, 1983). Thus, it is necessary to consider an effective control measures for the control of hookworm and other soil-transmitted helminths while formulating any public health intervention programme aimed at reducing anemia prevalence in Nepal. Keeping in view of public health importance of hookworm infection, here, we report an annual incidence rate of hookworm infection in Nepal as recorded at Tribhuvan University Teaching Hospital in Kathmandu, Nepal.

Materials and Methods

A total of 4,164 to 9,440 (average 7,857) fecal samples received from patients visiting Tribhuvan University Teaching Hospital for their some kind of ailments were examined for the presence of intestinal parasites each year over a period of one decade (1985 to 1994). Patients were provided a clean, dry and screw capped plastic container for sample collection. An average of 30 samples/day were received. Routine saline preparation (direct smear) was prepared from each sample received and examined microscopically. Concentration technique (formal-ether sedimentation) was applied only to few doubtful cases (anemia and melena). In the later part of the study, 200 hookworm ova positive samples were subjected to culture by Harada and Mori (1955) technique. The hookworm larva thus recovered were identified either as *Ancylostoma duodenale* or *Necator americanus* on the basis of their morphological characters. The findings were stratified against age (children: 15 and less than 15 years and adults: more than 15 years) and sex.

Result and Discussion

The annual rate of hookworm infection varied between 3.8-10.7% (Fig. 1) with a mean of 7.0 percent. A declining trend of incidence was observed till the year 1991. This appears to be due to the behavioral change of Nepalese during recent years. Now a days, most of the Nepalese wear some kind of shoes or slipper even in the remote areas which reduce the chances of skin penetration by infective larva. The farming land area in Kathmandu valley has also been reduced significantly

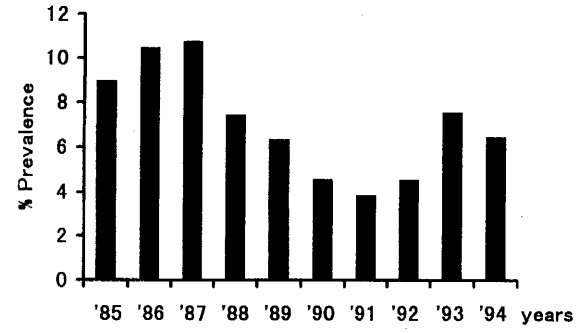

Figure 1 Annual overall rate of hookworm infection in Nepal.

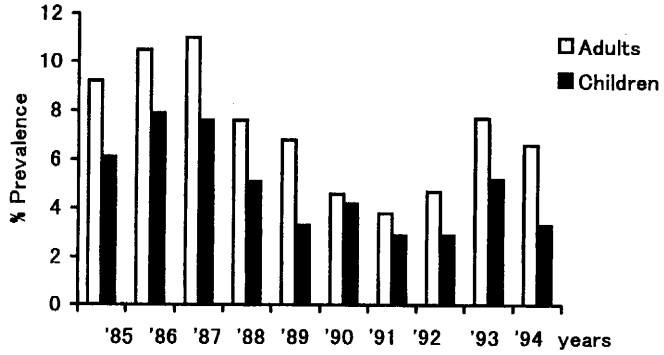

Figure 2 Annual rate of hookworm infection in adults and children.

during recent years mainly due to the influx of people from different parts of the country and construction of houses. This, in turn, reduced the chances of exposure to the infective hookworm larva among the farming community (*Jyapu*) of Kathmandu valley. On the other hand, the increasing trend observed after 1991 may reflect the consequence of unplanned urbanization in Nepal particularly in Kathmandu valley. Most area in Kathmandu valley represent the urban slum in developing countries where sewerage system is virtually non-existent or insufficient even if it is existed. Drinking water is highly contaminated with fecal matter (Adhikari *et al.*, 1986). Even now, some people in Kathmandu valley do not have latrine at their house and go for open defecation that leads to soil contamination. Thus, a street flood up to knee level and with bad smell in some parts of city area, is a common phenomena during rainy season in Kathmandu valley recently. This might have contributed hookworm infection in spite of wearing shoes/slipper resulting into a high incidence during the later part of this study. Further, two rivers- *Bagmati* and *Bishnumati* in the valley are heavily contaminated with sewage during recent years, but some people still utilize the river water for washing and bathing. Udonsi

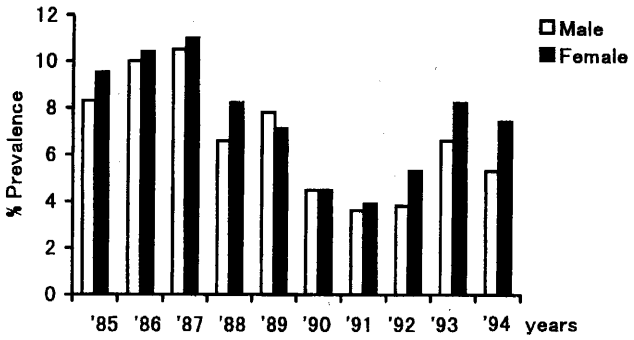

Figure 3 Annual rate of hookworm infection in males and females.

and Amabibi (1992) from Nigeria have also reported water-borne transmission of hookworm among fishermen. Further, an association between urbanization and parasitic infection has also been reported by other investigators (Cromton and Savioli, 1993).

Throughout the study period, adults (more than 15 years of age) showed higher incidence rate compared with children (15 or less than 15 years of age) (Fig. 2). This finding was in agreement with those reported by other investigators (Gilles, 1985; Xu *et al.*, 1995). Further, Bakta *et al.* (1993) from Indonesia have reported an increase in the prevalence and intensity of hookworm infection with age. In this study, females were found more commonly infected compared with their male counterparts except in the year 1989 and 1990 (Fig. 3). However, the differences were not significant statistically. These findings showed that both male and female have almost an equal chance of exposure to hookworm infection in Nepal.

Of the two species of human hookworms, *A. duodenale* is considered to be more pathogenic as it sucks 8 to 9 times more blood (260 μl/worm/day) compared with *N. americanus* (30 μl/worm/day). Keeping in view of this fact, in the latter part of the study period, we cultured 200 hookworm ova positive fecal samples to see the type of human hookworm prevalent in Nepal. Of the total 200 hookworm ova positive samples cultured, 67.0% were *A. duodenale* while the remaining 33.0% were *N. americanus*. This finding revealed that hookworm associated anemia in Nepal is caused mainly by *A. duodenale* and correlated well with a significantly lower hemoglobin level among hookworm infected subjects (Curtale *et al.*, 1993). A higher prevalence of *A. duodenale* appears to be due to its ability to undergo arrested development and to infect also through oral and transmammary/transplacental (Yu *et al.*, 1995) routes other than skin penetration. However, report on infantile hookworm infection was not available from Nepal till date and remains to be the subject of future study. Higher incidence of *A. duodenale* in Nepal was in agreement with the finding of Koshy *et al.* (1978) from North India where they recovered *A. duodenale* from all patients of hookworm outbreak except four who had received antihelminthic treatment and with that reported from China (Yu *et al.*, 1995). About one-fourth of hookworm infection in this study were found to be associated with other parasites most common being *Ascaris lumbricoides* and *Trichuris trichiura* (data not shown).

The incidence rate could be higher had the concentration technique been applied to each of the sample included in this study or had three consecutive samples from each patient been examined. However, we consider that our present finding sufficiently demonstrate the status of human hookworm infection in Nepal. Though, there are reports showing less than 5.0% (Gianotti, 1990) to as high as 83.8% (Entevez *et al.*, 1983) incidence of hookworm infection in Nepal, such a high difference appears to be due to the difference in study population and sample size (less than 40 samples). Since the good nutrition (Roche and Layrisse, 1966) has no protective role against hookworm infection, it is necessary to take appropriate preventive measures including planned urbanization with good sanitary disposal and sewerage facility to prevent the hookworm associated morbidity, mortality and economic losses in Nepal in future.

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Jpn. J. Trop. Med. Hyg., Vol. 25, No. 2, 1997, pp. 85-87

CHARACTERIZATION OF *VIBRIO CHOLERAE* O1 ISOLATED IN LAO PEOPLE'S DEMOCRATIC REPUBLIC

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Abstract: Thirty-four strains of *Vibrio cholerae* O1 serotype Ogawa isolated in Lao People's Democratic Republic in 1995 were examined for their biological behaviour and cholera toxin productivity. Biotyping indicated that the 34 strains belonged to the El Tor biotype. All strains were hemolysin positive and resistant to polymyxin B and cholera phage IV. However, 4 strains were negative for the hemolysin activity as determined by the test tube method, although they were positive for the antigenic activity. Prophage typing identified 31 strains of the Celebes type, including 3 cured Celebes type, and 3 Ubol type. All the strains produced detectable levels of cholera toxin; 15 isolates produced more than 100 ng/m*l*, 10 strains between 10 and 100 ng/m*l* and 9 strains less than 10 ng/m*l*, determined by using AKI medium in a stationary test tube. The maximum production was 200 ng/m*l*.

Keywords: *Vibrio cholerae,* Lao PDR, biotyping, prophage typing

INTRODUCTION

The seventh cholera pandemic which began in 1961 shows no signs of declining. El Tor vibrios, the agent responsible for the present pandemic, appears to be more resistant to adverse environmental factors than the classical biotype responsible for earlier cholera pandemics. However, various changes in the epidemic cholera vibrios have been seen during the past 36 years. All epidemic strains of El Tor vibrios were lysogenic until 1966, when "cured" strains suddenly appeared in Cambodia and Thailand (Kuyyakanond *et al.,* 1990). Moreover, strains of *V. cholerae* O1 biotype El Tor that were susceptible to Mukerjee's cholera bacteriophage IV were reported (Iwanaga *et al.,* 1989), and changes in hemolytic property of El Tor vibrios were also observed (Nakasone *et al.,* 1987). In 1992, a new serogroup *V. cholerae* O139 emerged as a causative agent of cholera in India and Bangladesh, which rapidly spread to neighboring countries (Ramamurthy *et al.,* 1993). The changes in epidemic cholera vibrios must be considered for ecology of the organisms and for epidemiology of the disease. Therefore, characterization of cholera vibrios at different places and at different times must be done.

In 1995, *V. cholerae* biotype El Tor was reported from all regions of the world. A total of 208,755 cases were officially reported to WHO, an almost 50% decrease over 1994. The case-fatality rate was reduced globally to 2.4%. According to the "Weekly Epidemiological Record" from WHO, Lao People's Democratic Republic (Lao PDR) reported 9,640 cases in 1994 and 1,365 cases in 1995 with a case fatality rate of 6.3% and 12.7%, respectively. The important efforts made by the government and WHO in the region, as well as the positive response from the donor community, may have contributed to the decrease in the incidence of cholera cases. However, the case-fatality rate in the Lao PDR still remains high. This study examined the characteristics of *V. cholerae* serogroup O1 strains isolated in Lao PDR in 1995.

MATERIAL AND METHODS

Bacterial strains: Thirty-four strains of *V. cholerae* O1 isolated in 1995 from cholera patients and submitted to the National Institute of Hygiene and Epidemiology of Lao PDR were used. *V. cholerae* H218 (biotype classic, serotype Ogawa) was used for the study of bacterio-

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phages. The isolates were stocked in nutrient agar prepared with a double amount of water until use.

Characterization of isolates: The characteristics of each isolate were determined on the basis of serotype, biotype, prophage type and cholera toxin production.

Although biotype is decided essentially by hemolytic properties of the organisms, Voges-Proskauer reaction and susceptibility to cholera phage group IV, El Tor phage 5 and polymyxin B were also examined. Serotype was determined by the slide agglutination method using commercial anti-Ogawa and anti-Inaba sera (Denkaseiken Co., Tokyo, Japan).

Hemolysin activity was titrated by a slight modification (using heart infusion broth with 1% glycerol) of Feely and Pittman's method (Feely and Pittman, 1963). The strains with no hemolytic activity were examined for the antigenic activity of El Tor hemolysin by the reversed passive latex agglutination (RPLA) method (Chinen *et al.*, 1996). The Voges-Proskauer reaction was examined by using Voges-Proskauer soft agar (Eiken Co., Japan).

Susceptibility to polymyxin B was examined with a plate dilution technique to determine the minimum inhibitory concentration (MIC) of the drug. Agar plate containing the drug at concentrations ranging from 100 to 6.25 μg/ml were prepared, and organisms which grew with a drug concentration greater than 12.5 μg/ml were regarded as resistant to polymyxin B.

Susceptibility to cholera phage IV and El Tor phage 5 was examined as follows. First, 10 ml of peptone agar (1% peptone, 0.5% NaCl, 1.5% agar) was poured into a petri dish and solidified. An overnight broth culture of the organisms (0.2 ml) and 4 ml of soft agar (heart infusion broth with 0.5% agar) kept at 43°C were mixed and overlaid on the peptone agar plate. Phage solution (15 μl) at the routine test dilution was spotted on the soft agar plate containing the organisms.

Prophage typing was done basically as described by Takeya and Shimodori (1963) using kappa phage and its host strain H218. Susceptibility of the organisms to kappa phage was examined with the method mentioned above. Production of kappa phage by the organisms was examined as follows. The organisms cultured in broth were killed with chloroform. The killed culture fluid (0.1 ml), a broth culture of strain H218 (0.2 ml), and soft agar (4 ml) were mixed and overlaid on a peptone agar plate. After overnight incubation at 37°C, turbid plaque formation was regarded as kappa phage production. The organisms were classified as Celebes type if they produced kappa phage and were not susceptible to the phage; cured Celebes type did not produce kappa phage but was susceptible; and Ubol type did not produce kappa phage and was not susceptible.

Cholera toxin production: The organisms were cultured in AKI medium (Bacto-Peptone 1.5%; yeast extract 0.4%; NaCl 0.5%; sodium bicarbonate 0.3%) at 37°C for 20 hr in a stationary test tube, after which the cholera toxin in the culture supernatant was titrated by using the reversed passive latex agglutination method (Iwanaga and Yamamoto, 1985).

Table 1 Biotype characterization

	positive[b)]	negative[b)]
Hemolysin[a)]	100	0
Voges-Proskauer	74	26
Susceptibility to:		
cholera phage IV	0	100
El Tor phage 5	100	0
polymyxin B	0	100

a) Determined by biological or antigenic activity.
b) Percentage of the strains.

Table 2 Prophage typing

Prophage type (%)[a)]	susceptibility to kappa phage	production of kappa phage
Celebes (82%)	−	+
cured Celebes (9%)	+	−
Ubol (9%)	−	−

a) Percentage of the strains.

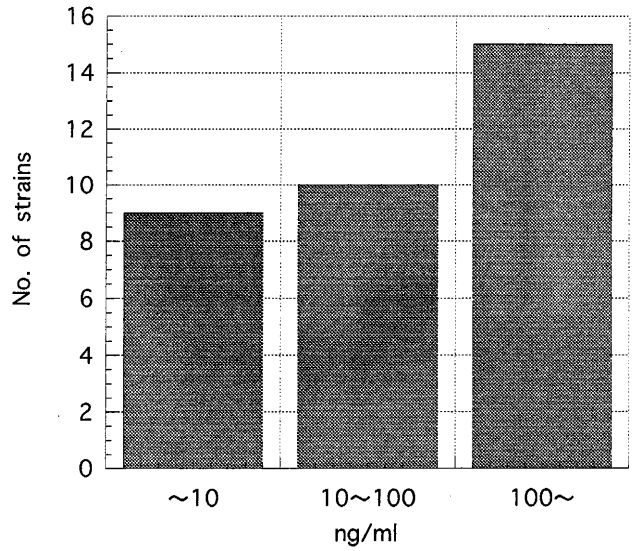

Figure 1 Cholera toxin production in AKI medium in stationary test tube as determined by the reversed passive latex agglutination (RPLA) method.

RESULTS

All the isolates belonged to serotype Ogawa. The biotype-specific reactions are shown in Table 1. Hemolytic activity was negative for 4 strains examined by the method of Feeley and Pittman. However, these strains were positive for the antigenic activity of El Tor hemolysin when tested by the RPLA method. The Voges-Proskauer reaction was positive in 25 strains (74%). The cholera phage IV susceptibility test was negative for all the strains, while 100% of the strains were susceptible to El Tor phage 5. The MIC of polymyxin B against these cholera vibrios was more than 25 $\mu g/ml$, and they were regarded as resistant.

Prophage typing (Table 2) classified the isolates into 28 Celebes type (82%), 3 cured Celebes type (9%), and 3 Ubol type (9%).

All the strains produced detectable levels of cholera toxin (Fig. 1); 15 isolates (44%) produced more than 100 ng/m*l*, 10 strains (29%) between 10 and 100 ng/m*l*, and 9 strains (27%) less than 10 ng/m*l*. The maximum production was 200 ng/m*l*.

DISCUSSION

All *V. cholerae* serogroup O1 strains examined in this study were positive for the production of El Tor hemolysin, as determined by biological activity or by antigenic activity. Historically, the El Tor biotype was identified by its ability to hemolyze sheep erythrocytes, while classical strains were non-hemolytic (Feeley, 1966). However, by 1972, nearly all El Tor isolates worldwide had become non-hemolytic, and thereafter, changes in hemolytic property of El Tor vibrio were observed (Iwanaga *et al.*, 1982). Chinen *et al.* (1996) have suggested the presence of some hemolysin inactivation factor(s) in El Tor vibrios. Although the proportion of hemolytic strains was increased by using glycerol-enriched broth, our findings showed that some strains produced El Tor hemolysin, though biological activity was not detected in the culture supernatants. Therefore, El Tor hemolysin must be antigenically or genetically detected to avoid false negative results.

Susceptibility to cholera phage IV, El Tor phage 5 and polymyxin B were typical findings of El Tor vibrios. Therefore, phage and polymyxin B susceptibility seem to be the most reliable in deciding the biotype of *V. cholerae* O1. Kuyyakanond *et al.* (1990) reported El Tor vibrios susceptible to cholera phage IV, but such a strain was not found in this study.

In the current seventh cholera pandemic, the epidemic strains are regarded as Celebes type El Tor vibrios. In the present study, 31 of 34 isolates (91%) belonged to the Celebes type, including 3 cured type. The remaining 3 isolates were Ubol type.

Iwanaga and Yamamoto (1985) previously noted that cholera toxin production by El Tor vibrios in AKI medium usually ranged from 20 to 100 ng/m*l*. However, an increase in productivity was observed in more recent isolates (Kuyyakanond *et al.*, 1990). We also found that 44% of the isolates produced more than 100 ng/m*l* of cholera toxin. Whether this increased productivity is a general tendency of El Tor vibrios worldwide or just indicates a dispersion of the parameter will be determined only when epidemic strains are examined at different places and at different times.

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ETIOLOGY OF DIARRHEAL DISEASES AND THE HEALTHY CARRIER OF ENTEROPATHOGENS IN VIENTIANE, PEOPLE'S DEMOCRATIC REPUBLIC OF LAO

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Received March 19, 1997/Accepted April 30, 1997

Abstract: Etiologic bacteria of 135 cases with diarrheal diseases and the healthy carrier rate in 211 individuals were studied in People's Democratic Republic of Lao. Diarrheagenic *Escherichia coli, Shigella* and *Campylobacter* were the predominant pathogens isolated. This country does not face the sea, but *Vibrio parahaemolyticus* was isolated from 2 cases. Healthy carrier rate of enteropathogenic *E. coli* as diagnosed by serogroup Class I was 3 times higher than the detection rate in diarrheal patients (10.9% and 3.7%). Healthy carrier rate of *Shigella* was 4.3%, whereas the detection rate of *Shigella* in diarrheal patients was 15.4%. The isolation frequencies of enterotoxigenic *E. coli* and *Campylobacter jejuni* in the patients and healthy individuals were almost the same. Verotoxin producing *E. coli* was found in one healthy individual (Toxin type VT1, O-antigen O111). Rotavirus was not detected from the 29 infant diarrhea examined, but detected from 2 adult cases out of 32 examined.

Keywords: diarrhea, enteropathogen, epidemiology, Laos

INTRODUCTION

Although the numbers of death due to diarrheal disease has markedly decreased during the past 15 years because of intensive world wide promotion of primary health care (PHC) activities including the enhancement of oral rehydration therapy, diarrheal disease is still a leading cause of death among young children in developing countries. Diarrheal diseases are preventable, curable, and controllable with a simple technique. There is no need to die of diarrhea but many children do so. Epidemiological study is the first step to control the diseases in the society. The isolation frequency and the distribution of diarrheagenic bacteria in many countries have been reported, and a different isolation pattern between regions was shown (Adkins *et al.*,1987; Aihara *et al.*, 1991; Iwanaga *et al.*, 1993; Kain *et al.*, 1991; Kim *et al.*, 1989; Yam *et al.*, 1978), but no report from People's Democratic Republic of Lao (Lao PDR) has been published. In this communication, the etiologic agents of diarrhea recently studied in Lao PDR were described.

PATIENTS, MATERIALS AND METHODS

Patients: A total of 135 patients with diarrhea at 3 public hospitals and a private clinic in Vientiane were examined. The patients were examined during the period of 70 days from October 17 to December 27, 1996. Age distribution ranged from 3 months to 73 years including 44 patients older than 15 years, 86 patients younger than 5, 2 patients of 12 years. There were 3 patients whose ages were not recorded.

Specimens: The contents of 135 specimens were rectal swab from 70 patients and stool in the plastic containers from 65 patients.

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Microbiological examinations: The target bacteria for isolation were 4 kinds of diarrheagenic *Escherichia coli* except enteroaggregative *E. coli* (EAggEC), *Shigella, Salmonella, Vibrio, Aeromonas, Plesiomonas* and *Campylobacter* for all specimens. Rotavirus was examined for the stools collected in the plastic containers by using latex agglutination kit, Rotalex (Denkaseiken Co. Tokyo, Japan). Serial 10-fold dilutions of stool samples were made in normal saline solution, and 25 μl of the dilution at 10^{-3}, 10^{-2}, and 10^{-1} were inoculated onto agar plates of modified Drigalsky medium, SS agar medium, and TCBS agar medium, respectively. The inoculated sample was spread over one-third space of the agar plate by a glass bar spreader and then streaked onto the remaining two-thirds space of the plate with a wire inoculation loop. The colonies grown on agar plate were identified by routine laboratory tests. For the diagnosis of diarrheagenic *E. coli*, 4 to 5 possible *E. coli* colonies were subcultured to proliferate on a nutrient agar plate. The proliferated organisms were examined for *E. coli* O group agglutination by using diarrheagenic *E. coli* diagnostic antisera (Denkaseiken Co.). The strains with agglutination were re-examined after heating at 121°C for 15 min. Heat labile enterotoxin (LT) and verotoxin production were examined by using reversed passive latex agglutination kit (VET-RPLA, Denkaseiken Co.). Verotoxin production was examined for the *E. coli* strains which agglutinated with the antisera of enteropathogenic *E. coli* (EPEC) and enteroinvasive *E. coli* (EIEC) serogroup indicated in the commercially purchased preparation. Heat stable enterotoxin (ST) was examined by using immunoenzyme assay kit (COLIST, Denkaseiken Co.). The species of the strains with positive agglutination or positive enterotoxin production were biochemically identified. *Campylobacter*s were isolated on Campylobacter blood-free selective medium (Oxoid) incubated at 42°C for 48 hr and was identified by confirming the morphology with Gram-staining and degradation activity for hippuric acid. Rectal swab samples were directly inoculated onto the agar plate by routine techniques. Rotavirus was examined for the stool samples collected in the containers.

Drug sensitivity test: Twenty-two *Shigella* isolates from 21 patients and 9 strains from healthy carriers were examined for susceptibility against ampicillin (ABPC), tetracycline (TC), cefdinir (CFDN), ofloxacin (OFLX) and erythromycin (EM). Minimum inhibitory concentration (MIC) of the drugs were examined by a plate dilution technique. Heart infusion agar plates containing the drug at the serial 2-fold concentration from 0.025 μg/ml to 100 μg/ml were prepared. The organisms were cultured in heart infusion broth overnight, and the culture fluids were diluted 1:10 with normal saline solution for the inoculum (Ca. 10^7/ml). They were inoculated by using a microplanter (Sakuma Co. MIT#00257), and determination of MIC was made after incubation at 37°C for 24 hr.

Healthy carriers: Carrier of enteropathogens was examined for a total of 211 individuals including 185 healthy children under 6 years of age in "Children's School" and 26 adult staff taking care of the children. All but 15 specimens (stools) were collected in the plastic containers. The methods for isolation and identification of the pathogens were the same with those used for diarrheal stools.

RESULTS

Enteropathogens isolated from the patients: As shown in Table 1, *Shigella* was isolated from 21 patients out of 135 (15.4%). Subgroup distribution revealed *S. flexneri* 14, *S. sonnei* 6 and *S. boydii* 2. One case, a 3 year-old girl, excreted 2 subgroups of *Shigella* (*S. flexneri* 2a, *S. sonnei* 1) and LT producing enterotoxigenic *E. coli* (ETEC). *Salmonella* was isolated from 3 cases, and all 3 strain belonged to O antigen group 4. EPEC serogroup was classified into three groups, Class I, Class

Table 1 Enteropathogens isolated from hospital-visiting diarrheal patients in Vientiane

Isolated pathogens	Case frequency (%)
Shigella spp.	21 (15.4)
Salmonella spp.	3 (2.2)
EPEC (serogroup Class I)	5 (3.7)
EPEC (serogroup Class II)	4 (3.0)
EPEC (serogroup others)	7 (5.2)
EIEC (serogroup)	3 (2.2)
ETEC (LT 4, ST 7)	11 (8.1)
EHEC	0
Vibrio parahemolyticus	2 (1.5)
Vibrio hollizae	1 (0.74)
Aeromonas spp.	0
Campylobacter jejuni	7 (5.2)
Campylobacter coli	2 (1.5)
pathogen positive	57 (42%)
pathogen negative	78 (58%)
Total	135

More than 2 pathogens were isolated from 1 case.
Of 65 examined, 2 cases were positive for rotavirus.

Table 2 Healthy carrier of enteropathogens

Isolated pathogens	Number examined-age		
	Total: 211	<6y: 185	>20y: 26
Shigella spp.	9	8	1
Salmonella spp.	0	0	0
EPEC (serogroup Class I)	23	19	4
EPEC (serogroup Class II)	5	5	0
EPEC (serogroup others)	15	15	0
EIEC (serogroup)	6	5	1
ETEC (LT 2, ST 16, LT/ST 2)	20	20	0
EHEC (VT1 producing)	1	1	0
Vibrio spp.	1	1	0
Aeromonas caviae	1	1	0
Campylobacter jejuni	11	11	0
Campylobacter coli	6	6	0
Rotavirus	0	0	0
pathogen positive cases(%)	83 (39%)	77 (42%)	6 (23%)
pathogen negative cases(%)	128 (61%)	108 (58%)	20 (77%)

More than 2 pathogens were isolated from 16 cases. Serogroup of EHEC was O111.

II, and others. Class I EPEC serogroup includes O26, O55, O86, O111, O119, O125, O126, O127, O128ac, and O142. Class II EPEC includes O18, O44, O112, and O114. Others EPEC includes O1, O146, O151, O157, O158. These antisera were prepared in the batch of diagnostic antisera (Denkaseiken Co.). The case frequency with Class I, Class II and others were 5 (3.7%), 4 (3.0%) and 7 (5.2%), respectively. Three cases excreted EIEC serogroup. ETEC were isolated from 11 cases in total, including 4 LT producing strains and 7 ST producing strains. *V. parahemolyticus* was isolated from 2 adult cases with watery diarrhea containing many leucocytes. *V. hollizae* was isolated from a 2 year old boy with bloody diarrhea. *Aeromonas* and *Plesiomonas* were not isolated at all. *C. jejuni* and *C. coli* were isolated from 7 and 2 cases, respectively. In total, bacterial pathogen positive cases were 57 (42%). Rotavirus was examined for 65 stool samples collected from 29 infants, 32 adults and 4 adolescent. The infatile specimens were all negative but 2 adult cases were positive.

Healthy carrier rate: The isolation frequency of Class I EPEC was higher than the patients with diarrhea, and that of *C. jejuni* was the same with diarrheal patients. As shown in Table 2, 9 healthy carrier of *Shigella* out of 211 examined (4.3%) were found. The subgroup of *Shigella* consists of 4 *S. boydii*, 3 *S. sonnei*,

Table 3 Drug sensitivities of *Shigella* isolates

Number of strains

MIC(μg/ml)	ABPC	TC	EM	CFDN	OFLX
0.025 >	0	0	0	0	1
0.025	0	0	0	0	1
0.05	0	0	0	0	1
0.1	0	0	0	3	28
0.2	0	0	0	17	1
0.39	0	0	0	11	0
0.78	0	0	0	0	0
1.56	1	3	0	0	0
3.13	6	1	1	0	0
6.25	6	0	4	0	0
12.5	3	0	7	0	0
25	0	5	8	0	0
50	0	3	11	1	0
100	0	17	0	0	0
100 <	16	3	1	0	0

ABPC: ampicillin, TC: tetracycline, EM: erythromycin, CFDN: cefdinir, OFLX: ofloxacin

and 2 *S. flexneri*. Class I, Class II, and others EPEC were isolated from 23 (10.9%), 5 (2.4%), and 15 (7.1%), respectively. Six cases were positive for EIEC serogroup. A total of 20 ETEC (9.5%) producing LT, ST, and LT/ST were isolated from 2, 16, and 2 cases, respectively. Enterohemorrhagic *E. coli* (EHEC) producing VT-1 was isolated from only one case, a 2 year-old girl. The strain belongs to the serotype O111.

Drug sensitivities of Shigella: The isolates were generally resistant to ABPC and TC, and sensitive to CFDN and OFLX. The sensitivity to ABPC showed 2 types; one was highly resistant (MIC>100 μg/ml) and another was moderately resistant (MIC=1.56-12.5 μg/ml). Almost 90% of the isolates were highly resistant to TC. On the contrary, MIC of OFLX was 0.1 μg/ml against almost all isolates (Table 3).

DISCUSSION

Surveillance of the disease based on detail epidemiological data is essential for making the strategy to control diseases in the society. Diarrheal disease, one of the leading cause of death in developing countries, can be controlled by implementing a proper strategy. Analysis of the epidemiological data obtained in the present study would play an important role to control diarrheal diseases in the related societies.

Attention to high frequency of shigellosis and high healthy carrier rate of *Shigella* should be paid. Number of cases with shigellosis was 21 out of 135 diarrheal patients (15.6%), and the healthy carrier rate was 4.3% (9/211). Fortunately, the 9 healthy carriers of *Shigella* in the present study did not manifest the illness, and the pathogens spontaneously disappeared in the re-examination at 1 month after the first detection of *Shigella*. In other Asian countries, the reported case frequency was usually more or less 5% of diarrheal patients and the healthy carrier rate was usually less than 2% (Adkins *et al.*, 1987; Aihara *et al.*, 1991; Iwanaga *et al.*, 1993; Kain *et al.*, 1991; Kim *et al.*, 1989; Yam *et al.*, 1978). Shigellosis is a typical "fecal-oral infection". These high frequencies suggest that sanitary condition is not preferable in the society. The enhancement of primary health care activities is required.

Generally, diarrheagenic *E. coli* is most frequently isolated from diarrheal stool in the tropical countries, but identification of the pathogen, especially EPEC, is not so easy. The identification of ETEC and EHEC is routinely made by detection of the toxins. EAggEC is usually not handled in the clinical laboratories, because of lacking the proper diagnostic method. Little is known about the pathogenesis, epidemiology, and serotypes of EAggEC. Exactly speaking, EPEC can be identified by detecting bundle forming pili (BFP) and 94 kDa outer membrane protein (intimin) or their genes (*bfp, eae*) (Donnenberg and Kaper, 1992; Law, 1994; Levine, 1987). Sereny test or examination of a pathogenic gene, such as the gene for invasive plasmid antigen (Ipa) is required for identification of EIEC (Levine, 1987). However, in the routine laboratory diagnosis, so called corresponding O-antigens are examined by slide agglutination test using traditionally prepared antisera. Although the presence of certain O antigens (serogroup Class I) is considered to correspond well to the presence of *bfp* and/or *eae* (Levine, 1987), the diagnosis with serogroups agglutination test may not be reliable. Anti-*E. coli* O antigen sera are commercially available for the screening of diarrheagenic *E. coli*. In Japan, the commercially available antisera include some batches of sera for the screening of EPEC, ETEC, and EIEC. However, it is unclear what percentage of *E. coli* with these serogroups are true pathogens.

C. jejuni is one of the major diarrheagenic organism in worldwide. The isolation frequency in diarrheal stool was 5.2% (7/135) which may be a universal average ratio including the industrialized countries (Adkins *et al.*, 1987; Aihara *et al.*, 1991; Iwanaga *et al.*, 1993; Kain *et al.*,1991; Kim *et al.*, 1989; Yam *et al.*, 1978). However, attention should be paid in the present study to the same isolation frequency, in the healthy population (11/211, 5.2%) and diarrheal patients (7/135, 5.2%).

High healthy carrier rate of enteropathogen was seen not only for *Campylobacter* but also for *Shigella* and diarrheagenic *E. coli*. The carrier rate of EPEC with Class I serogroup was 11.4% (24/211) which is higher than the isolation rate of diarrheal patients (3.7%, 5/135). Some studies reported the isolation rate of ETEC in diarrheal patients and healthy individuals is almost the same, but EPEC was definitely associated with diarrhea (Albert *et al.*, 1995; Kain *et al.*, 1991). In the present study, however, the carrier rate of EPEC was 3 times that of diarrheal patients. The reason for this is unknown, but many healthy individuals may be ready to develop diarrhea at any time.

Drug sensitivity was examined for *Shigella* isolates. The results showed that ABPC and TC were not helpful, but oral cephem and new quinolone were very effective. Almost all isolates were sensitive to nalidixic acid as examined by the disc method (data not shown). Nalidixic acid must be the first drug of choice for shigellosis, because oral cephem and new quinolones are not readily available in developing countries. Anti-

Shigella activity of erythromycin seems poor, but in 1960s, there were many reports in Japan that erythromycin was as effective as tetracycline and chloramphenicol which were excellent antibiotics for the treatment of shigellosis at that time (Saito *et al.*, 1964). In general, rotavirus is frequently detected in infant diarrhea without seasonal variation in the tropical countries (Adkins *et al.*, 1987: Aihara *et al.*, 1991; Iwanaga *et al.*, 1993; Kain *et al.*, 1991; Kim *et al.*, 1989; Yam *et al.*, 1978), whereas the epidemic in Japan is concentrated in the first 3 months of the year (Fujita, 1990). In the present study, rotavirus was detected from only 2 cases out of the 65 examined (3%). It is unknown whether this low frequency is the characteristic of Lao PDR or not. The continuous study throughout the year is required.

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CONTENTS

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