# SPECIAL REPORT*

# IMMUNOLOGICAL STUDIES ON SOME BACTERIAL AND PARASITIC DISEASES IN THAILAND

Wanpen Chaicumpa

Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 0400, Thailand.

**Abstract.** Over the past two decades a wide range of immunological studies have been carried out on organisms responsible for causing serious bacterial and parasitic diseases in Thailand. Particular emphasis has been placed on cholera, where investigations have been directed towards vaccine development and rapid diagnosis and on diagnosis of typhoid and enterotoxigenic *Escherichia coli.* In the area of parasitic diseases emphasis has been directed especially to immunodiagnosis of helminthic diseases, including gnathostomiasis, paragonimiasis, opisthorchiasis, strongyloidiasis and trichinellosis. These studies are reviewed in this report.

## IMMUNOLOGY OF CHOLERA

### Immunity and vaccine development

At present, no successful measure of cholera control has yet been obtained. Improvement of health education, personal hygiene and sanitation are difficult to carry out in the less developed parts of the world. Immunoprophylaxis, therefore, becomes the focus of attention. However, immunization against cholera has been disappointing because of the lack of a good cholera vaccine. The commonly use vaccine nowadays is a parenteral one which is prepared from heat inactivated, phenol preserved whole cell *Vibrio cholerae* of either biotype or both biotypes. The vaccine gives a short protective period (3-6 months) to adults living in cholera endemic areas (Chaicumpa *et al,* 1980) and offers negligible protection to travellers and children (the age group with the highest cholera attack rates) (Sack, 1984). Besides, the vaccine confers undesirable side effects like fever, malaise, headache, pain and swelling at the injection site to the vaccinees because it is comprised of whole organisms, which contain large amounts of endotoxin. It is evident that a good vaccine against cholera has yet to be developed.

*V. cholerae* is a non-invasive enteropathogen. The pathogenic mechanisms and virulence factors of this organism have been reviewed by many scientists. The ingested pathogenic *V. cholerae* which survive the antiseptic barriers of saliva and gastric acidity penetrate the mucus layer of the small intestine and adhere to the underlying epithelium. Penetration of the mucus layer requires strong motility of the bacteria, which is contributed by the flagella. Adherence to the epithelium is one of the most important steps in cholera pathogenesis. This property enables the organism (1) to resist removal to the large intestine (where some die from antagonistic action of normal flora and others are excreted) by normal intestinal peristalsis, and (2) to multiply near the site of action of the cholera toxin ($Gm_1$ receptors on the enterocyte membrane). The precise mechanism by which *V. cholerae* adheres to the epithelium and the antigens responsible for such adherence (adhesive factors) are the subjects of our intense research (see below) as these may have application to cholera vaccine development. After successful attachment at the epithelium, the organisms multiply and elaborate toxin. The toxin of cholera (CT) consists of a binding portion of 5 identical fragments (B subunits; Mr 11,500 each) and the toxic-active component (A subunit; Mr 28,000). The A subunit is a "nicked" polypeptide comprising two disulphide-linked fragments: $A_1$ and $A_2$. The binding of cholera toxin by the B subunits to the $GM_1$ receptors leads to a translocation of the A subunit through the epithelial cell membrane and release of the $A_1$

* Special report from TROPMED/Thailand

fragment into the cytosol. The $A_1$ fragment enzymatically splits intracellular NAD and, possibly in cooperation with as yet undefined cytosol factors, covalently couples the ADP-ribose moiety of the NAD onto the GTP-binding protein of the adenylate cyclase complex at the inner side of the cell membrane. This results in the accumulation of cyclic AMP intracellularly. The increase in CAMP intracellularly culminates in net secretion of electrolyte-rich fluid into the intestinal lumen. Diarrhea ensues (Holmgren, 1982).

From our studies on intestinal immunity against *V. cholerae,* it was found that the immunity is of humoral type and the protective antibodies must be located in the intestinal tract (Chaicumpa and Rowley, 1972). The protective local antibodies belong to two types: antibacterial antibodies (Freter, 1970) and antibodies to *V. cholerae* secretory products *eg* anti-CT, anti-hemolysin (Finkelstein, 1970, Chaicumpa *et al,* 1980; Samrejrongroj *et al,* 1980). These protective local antibodies may act synergistically or complementarily to each other. In the presence of antibodies to whole *V. cholerae* in the intestine, the vibrios are killed (Chaicumpa and Rowley, 1972). The vibriocidal mechanism is neither complement mediated lysis nor phagocytosis (Chaicumpa and Rowley, 1973). All classes of specific antibodies to whole *V. cholerae* (IgG, IgM, IgA) were found to have the same protective ability against cholera on an equal weight basis (though they have other different biological activities, *eg* agglutinating, precipitating, complement fixing, phagocytosis enhancing) (Steele *et al,* 1974). Our subsequent findings that antibody fragments ($F(ab)'_2$) (Steel *et al,* 1975) and the antibody to *V. cholerae* fragella antigens (Bellamy *et al,* 1975) are also protective led to our believe that specific antibodies to antigens on the cell surface of the vibrios afford protection by preventing or reducing *V. cholerae* attachment to the intestinal epithelium. This effect may be achieved either by agglutinating antibodies [divalent antibodies of any classes including $F(ab)'_2$ fragments, specific to any vibrio surface components *eg* lipopolysaccharide (LPS), outer membrane proteins (OMP), or flagella which agglutinate the vibrios in the intestinal lumen rendering them non-motile, hence unable to penetrate the mucus layer to the epithelium (Bellamy *et al,* 1975)], or by antibodies to *V. cholerae* adhesive factor(s) which mask the adhesive sites and interfere with the functions of these sites, so that the vibrios fail to colonize the intestinal epithelium.

Consequently, the nature of vibrio adhesive factor was of interest. In 1977, we found that rabbit antiserum to *V. cholerae* El Tor cell-bound hemagglutinin was protective against the homologous oral challenge (Chaicumpa and Athasistha, 1977). In 1979, the cell-bound hemagglutinin was isolated and purified from the El Tor vibrios and found that specific antibody to this factor is protective against cholera. The protective ability was the same as the antibodies directed aganist whole organisms or the heat-stable somatic antigens, upon the same weight basis (Chaicumpa and Atthasistha, 1979). Subsequently, the protective mechanism of the antibody to cell-bound hemagglutinin was studied. It was found that the antibody preparations, namely intact IgG, $F(ab)'_2$ and Fab, were able to elicit protection against cholera. The finding that Fab fragments of the anti-cell-bound hemagglutinin were able to afford protection *in vivo* as well as giving rise to a significant reduction of vibrio adherence to isolated epithelial cells *in vitro* implies that masking of the cell-bound hemagglutinin sites directly would be sufficient to confer protection against cholera. Subsequently, the related working hypothesis that this antigen is the vibrio adhesive factor is validated (Foo and Chaicumpa, 1981).

In 1982, we isolated and purified soluble hemagglutinin from classical vibrios. The factor was found to consist of three protein components. One of them was closely associated with lipopolysaccharide. Specific antibodies to this soluble hemagglutinin raised in rabbit by intramuscular injection conferred passive protection against cholera in an experimental model (Chaicumpa *et al,* 1982b). The soluble hemagglutinin-lipopolysaccharide complexes were found to be good intestinal antigens since they elicited high levels of antibodies in the intestine, especially of the IgA class. These specific antibodies persisted for a long period of time at significantly high levels (longer than 6 months). The enteric memory primed by the antigens in the intestinal tract was longer than 3 months. The protective ability of antibodies specific to the soluble hemagglutinin-lipopolysaccharide complexes collected from the intestinal tracts of the experimental animals does not correlate with the levels of vibriocidal antibody. The result seems to indicate that the mechanism of protection

against cholera by the intestinal antibodies is by reducing or preventing the attachment of the vibrios to the epithelium (Chaicumpa and Swasdikosa, 1983).

The immunogenicity of cell-bound hemagglutinin as an oral vaccine was also studied. The experiments revealed that the factor was a good antigen when passively adsorbed onto the surface of sheep red blood cells (which serve as an antigen carrier) and given orally to mice. The antigen not only induced high levels of various antibody classes which persisted in the intestinal tracts for a long time (longer than 6 months) but also the antibodies were protective (Chaicumpa *et al,* 1984).

From the pathogenic mechanism of *V. cholerae* outlined above it could be speculated that complete resistance to cholera would require three kinds of specific antibodies available to be in the gut. The first is agglutinating antibody which would agglutinate the newly arrived *V. cholerae* in the intestinal lumen thus render then non-motile and unable to penetrate the intestinal mucus layer. Vibrio antigens which give rise to this *in vivo* agglutination of the vibrios should be antigens on the outer most surface of the vibrios such as lipopolysaccharide (LPS), outer membrane protein (OMP), or H antigen. Among these antigens, the lipopolysaccharide is the most predominant one and easy to prepare. Any invading *V. cholerae* which could escape the agglutinating effect of the first antibody may penetrate the mucus and make an attempt to adhere to the epithelial cells. At this stage, the second antibody, namely antibody to vibrio adhesive factor(s) (ie LPS, cell-bound hemagglutinin, fimbriae or outer membrane proteins) would become important as the host defense mechanism. This would be achieved by masking or interfering with the vibrio adhesive sites so that the organisms fail to attach to the epithelium. Once the vibrios have colonized, they would produce the exo-enterotoxin. The third antibody, *viz* antitoxin would be required at this stage to prevent the B subunits of the toxin from binding to the $GM_1$ receptor on the epithelial cell membrane.

*V. cholerae* antigen(s) administered orally have been established as the most efficient means of stimulating the gut mucosal immune response which accounts for protection in cholera (Newby and Stoke, 1984;). Live attenuated *V. cholerae* strains in which the cholera toxin (CT) genes have been deleted by either chemical treatment or genetic engineering have been prepared and tested in volunteers (Levine *et al,* 1983; Migasena *et al,* 1989). These strains had high immunogenicity when given orally. However most of them were associated with undesirable side effects, *ie* diarrhea in the vaccinees. Besides, most of the so-produced mutants have the potential of reverting back to wild type *V. cholerae.* A *Salmonella typhi* Ty21a - *V. cholerae* hybrid was produced and tested in volunteers of both cholera non-endemic and cholera endemic areas. The hybrid vaccine was found to confer good immunogenicity but poor protective efficacy (Tacket *et al,* 1990). Concurrently, an oral vaccine prepared from killed whole *V. cholerae* of both biotypes and serotypes plus B subunits of CT was developed and tested in volunteers and in the field in cholera endemic areas (Black *et al,* 1983; Clemens *et al,* 1986). At the same time, refined *V. cholerae* antigens which have immunogenic, synergistic and inherent adjuvant properties have been defined, isolated and evaluated as a combined oral vaccine in both animals and humans (Chaicumpa *et al,* 1987; Pierce *et al,* 1982; Chongsanguan *et al,* 1991). In 1987, a combined oral cholera vaccine prepared from LPS, cell-bound hemagglutinin (CHA) and procholeragenoid (P) (the heat-treated high molecular weight CT with toxicity reduced to less than 5% of the holotoxin) was tested in animals (Chaicumpa *et al,* 1987). The vaccine exhibited marked synergism compared to vaccines using one or two of these antigens after two doses of immunization at 14 day intervals. The synergistic effect was found to be due to the increases in antibody producing cells of all antigenic specificities. A vaccine consisting of similar combined-LPS, CHA and P was tested in Thai male volunteers. The rates of the intestinal antibody responses were 50% for anti-LPS, 80% for anti-CHA and 100% for anti-toxin, respectively (Chongsa-nguan *et al,* 1991).

The oral vaccines prepared from killed whole *V. cholerae* or refined antigens are apparently safe, with no untoward reaction but relatively low immunogenicity as compared to the live vaccines. None of them can be delivered in a single dose which is protective enough to have public health significance. In general multiple, spaced-doses of the killed/refined antigen vaccines are required for eliciting adequate degrees of immune response in the hosts. In the light of this evidence, the means to increase the magnitude and duration of the protective immunity evoked by the killed/refined

antigen vaccines is a perceived necessity. Thus, the need of an adjuvant and/or a safe delivery vehicle (to protect the antigens from intestinal proteolysis) for oral vaccines has been emphasized by the World Health Organization since 1979. This would expect to lessen the number and size of doses and enhance efficacy of an oral vaccine.

Liposomes, concentric spheres of phospholipid bilayers, can serve as delivery vehicles for substances which are entrapped in/associated with them. Earlier studies have shown that molecules such as drugs (Gregoriadis *et al,* 1974) or antigens (Allison and Gregoriadis 1974; New *et al,* 1985) can be entrapped in spaces of liposomes, incorporated into lipid bilayers (Banerji and Alving, 1981; Dancey *et al,* 1977), or adsorbed to the liposomal surfaces (Bakouch and Gerlier, 1986).

In 1990, a liposome-associated cholera vaccine consisting of *V. cholerae* LPS, CHA and P was orally administered to rats. It was found that the liposomes displayed an adjuvant property in terms of evoking a higher immune response to *V. cholerae* antigens, as measured by the appearance of specific antibody-producting cells in the intestine, than when the antigens were fed alone. Comparative studies on immunogenicities of a liposome associated vaccine consisting of LPS, CHA and P, and a vaccine which is composed of the antigens in free form in Thai volunteers will be performed shortly with the support of WHO.

**Rapid detection of cholera**

Cholera has two properties which make it a disease of exceptional importance. Firstly, it is one of a small number of infectious diseases which intermittently assume global spread, becoming pandemic. Until now, there have been altogether 7 well-recorded cholera pandemics. Secondly, it is one of the most rapidly killing of all communicable disease. Not only does untreated cholera kill large numbers of people, but it does so with very great rapidity due to its drastic pathogenicity.

Cholera is spread as a fecal-oral disease, and people acquire the infection by ingesting fecally contaminated water or food. In situations where water is the vehicle of the disease, it need not be only drinking water that is responsible, since contaminated water may be consumed in other forms, *eg,* water used for washing dishes or bathing. The role of fomites, fingers, bed linen or other soiled objects in transmission of cholera is important, especially when there is overcrowding and hygiene is very poor as in slums, prisons, refugee and military camps.

Rapid detection of cholera cases is not only critical for saving patients' lives but also for controlling explosive outbreaks which usually occur within a day or two after a single unrecognized case. The disease is commonly and rapidly disseminated by a patient, convalescent carrier or individual with inapparent or mild infection whose fecal matter contaminates water supplies, food, or household contacts who live under conditions of poor personal hygiene.

In fact, rapid diagnosis of cholera can be performed by dropping the patient's diarrheic stool on a glass slide and observing it under dark-field illumination. Live *V. cholerae* have a characteristic "darting movement" which is inhibited by adding specific antiserum. However, the procedure works well only with an experienced observer. In common practice, when a cholera case is suspected in an area remote from an available microbiology laboratory, a few drops of stool or rectal swab are put in a suitable transport medium, *eg* Cary-Blair medium. The *V. cholerae* organisms survive in this kind of transport medium for few days. The specimen is then sent to the laboratory for further processing, *ie* growing in alkaline peptone water, pH 8.4. This medium allows much more rapid growth of *V. cholerae* than of enterobacteriaceae; after 6-8 hours, smears can be stained with fluorescein-labeled specific *V. cholerae* antiserum for quick presumptive identification. The requirements of an expensive fluorescence microscope and fluorescein-antibody conjugate limit the method from widespread application, especially in the field in developing countries where the public health problem of cholera is real. The 6-8 hour culture in alkaline peptone, or the diarrheic stool directly may be streaked on thiosulphate-citrate-bile salt-sucrose (TCBS) agar or taurocholate gelatin agar (TGA) (the usual enteric media are suboptimal and often inhibitory for *V. cholerae*). The colonies of *V. cholerae* are gram-negative, curved rods that are oxidase-positive, and give rise to acid in TSI agar. They are lysine and ornithine decarboxylase positive. For final identification, the organisms in typical colonies are subjected to an agglutination test against *V. cholerae* polyvalent antiserum. Further biotyping may be performed by chicken

red cell agglutination, cholera group IV phage and polymyxin B sensitivity tests and production of hemolysin. The whole process of *V. cholerae* isolation from the diarrheic stool would take, at a minimim, 2 days and by that time the disease might have spread explosively. Thus, rapid detection of cholera is required.

Recently we have commenced development of a rapid, simple, highly sensitive and specific immunological assay using monoclonal antibody based techniques for detecting *V. cholerae* antigens in stools of diarrheic patients and contaminated seafoods. Monoclonal antibodies specific to *V. cholerae* $O_1$ (both Ogawa and Inaba serotypes) were successfully produced. These antibodies are currently being used to develop a suitable immunological assay, *eg* monoclonal-based plate ELISA, dot-blot ELISA or passive latex test (Chaicumpa *et al,* 1992 a, b).

**Epidemiology of cholera**

In 1981, an epidemiological and bacteriological stool survey was conducted by the Department of Tropical Hygiene in collaboration with the Department of Microbiology and Immunology, at the Faculty of Tropical Medicine, Mahidol University. The areas of the study were two villages in Tambon Bang Ya Phraek in Samut Sakorn, Thailand. The isolated *V. cholerae* were El Tor, Ogawa. Higher point prevalence was observed in one village. Children of five years or older and females seemed to be predisposed to the infection. Health habits and practices were also explored. The vehicles of transmission seemed to be water for domestic use and seafoods consumed either raw of half-cooked. Vaccination with parenteral whole cell vaccines did not protect any individuals from harboring the organism (Miquel, 1981).

However, isolations of vibrios from rectal swabs (Tharavanij *et al,* 1970a) and stools (Miguel, 1981) are not entirely satisfactory because of the intermittent excretion of the organisms into the intestinal tract. Often the bacteria are lodged in the gall bladder and could be detected only after purgation (Tharavanij *et al,* 1970b; Levine *et al,* 1983). Besides, laboratory procedures for isolating vibrios from a large number of specimens as in a survey of carriers is very costly in terms of manpower, transportation, media, chemicals and other expendable supplies.

Thus in 1986, a survey on the level of antibodies to various *V. cholerae* antigens (anti-lipopolysaccharide, anti-cell-bound hemagglutinin, and antitoxin) was conducted. The subjects studied were Thai individuals of various age groups (new-borns to < 15 years old) living in areas of either low (<25 cases/100,000 population) or high (>25 cases/100,000 population) cholera morbidity. The antibody assessment was performed using vibriocidal assay and a solid phase enzyme-linked immunosorbent assay in which purified LPS, CHA and CT were used as the antigens. It was found that Thai individuals acquired the vibriocidal antibody early in life. Fifty percent of individuals aged 5 to 15 years old had detectable titers ranging from 1 : 5 to 1 : 125 or higher. Thai adults who lived in areas with high cholera endemicity (H) had significantly higher vibriocidal antibody levels than their counterparts who lived in areas with low cholera endemicity (L). Lipopolysaccharide was not the only antigen responsible for stimulating the vibriocidal antibody production. Adult levels of all classes of anti-CHA in L populations were higher than those in H populations, while anti-LPS (total immunoglobulins, IgG) and IgA were similar but the IgM of L was higher than that of H. The levels of all classes of anti-CT from H seemed to increase with age except at school age (5 years to 15 years) when there were marked decreases of all antibody classes (Chongsa-nguan *et al,* 1986).

## TYPHOID

**Diagnosis of typhoid**

A rapid, specific, and sensitive method is needed for the diagnosis of typhoid fever. Current strategies are based on detection of *Salmonella typhi* antigen(s) (Sivadasan *et al,* 1984) or DNA in clinical specimens (Rubin *et al,* 1985). DNA hybridization assays are, however, not suited for use in a clinical laboratory in a developing country where typhoid occurs sporadically. Serological assays to detect *S. typhi* antigens have been hampered by the lack of polyclonal antibody specificity (Appassakij *et al,* 1987; Araj and Chugh, 1987; Banchuin *et al,* 1987). Recently monoclonal antibodies directed against specific bacterial antigens have been developed (Chaicumpa *et al,* 1988b). In 1988 we reported the use of a monoclonal antibody to detect antigen 9 of *S. typhi* in urine specimens col-

lected from patients with typhoid fever. The test was shown to have 100% specificity and 65% sensitivity when performed on a single urine specimen (Chaicumpa *et al,* 1988b). In 1992, monoclonal antibody specific for antigen 9 of group D*Salmonella* was used in an indirect enzyme-linked immunosorbent assay (ELISA) for detecting the antigen in urine specimens collected from patients with clinical typhoid fever in Jakarta, Indonesia. The ELISA had a sensitivity of 95% in identifying patients in whom *S. typhi* was isolated from hemocultures, 73% in patients in whom *S. typhi* was isolated from stool, and 40% in patients in whom the organism was isolated from bone marrow cultures. Among patients in whom *S. typhi* was isolated from blood cultures, the ELISA had a sensitivity of 65% when a single urine specimen was examined and 95% when serially collected urine specimens were examined. A dot blot immunoassay performed on introcellulose filter in parallel had a sensitivity of 85% vs 83% for the plate ELISA in whom *S. typhi* was isolated from blood, bone marrow and/or stool. Since *S. typhi* antigen is intermittently excreted in the urine of patients with typhoid fever, serially collected urine from patients with typhoid should be tested for antigen 9 (Chaicumpa *et al,* 1992a).

In developing parts of the world where the public health problem due to enteric fever is real, the clinicians are, however, more familiar with the widal test for measuring typhoid antibodies and the assay is routinely requested when clinical features compatible with typhoid are seen. In the light of these issues, we then evaluated the status of the widal test in the diagnosis of typhoid fever in Thailand. From the evaluation, the sensitivity, specificity and the positive and negative predictive values of the widal test, when the reciprocal titer at 160 against typhoid 0 antigen was used as a limit between positive and negative reactions, were 86%, 98%, 92% and 97%, respectively (Chongsanguan *et al,* 1989).

### Immune response to parenteral typhoid vaccine

The heat-killed phenol preserved parenteral typhoid vaccine has been widely used for public health programs in many countries including Thailand for many decades. In Thailand, the vaccine is prepared and distributed by the Government Pharmaceutical Organization, Ministry of Public Health. However, no assessment for efficacy of this type of vaccine was performed. In 1985, we therefore undertook an experimental study which was accomplished by vaccinating human volunteers and observing the magnitude and duration of immune response elicited by the vaccine (Chaicumpa *et al,* 1985).

## ENTEROTOXIGENIC *ESCHERICHIA COLI*

### DNA hybridization

A collaborative study on the detection of enterotoxins produced by enterotoxigenic *E. coli* using DNA probes has been conducted since 1981 between the Department of Bacteriology, Armed Forces Research Institute of Medical Science (AFRIMS), Bangkok, and the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University. Publications derived from the collaboration are shown in the references (Escheverria *et al,* 1982, 1983, 1984a, b, 1985, 1987; Maidin *et al* 1982; Moseley *et al,* 1983).

### Detection of heat-labile enterotoxin of *E. coli* by serological tests

Reagents for serological techniques which are cheap, uncomplicated and may be performed in a remote health center were produced in our laboratory for detecting heat-labile enterotoxin of *E. coli.* These included the reagents for staphylococcal co-agglutination test and the reverse passive hemagglutination tests. Specificity, sensitivity and accuracy to the two tests using our locally produced reagents were compared with the Biken test, DNA hybridization and Y1 adrenal cell assay. The staphylococcal co-agglutination test was found to have 100% specificity, sensitivity and accuracy while the reverse passive hemagglutination test gave variable results (Chaicumpa *et al,* 1988a).

## NATURAL KILLER CELLS IN MALARIA

In 1982, we demonstrated that the natural killer cell activity, as measured by spontaneous cell-mediated cytotoxic assay, in peripheral blood of malaria patients during the convalescence period was markedly lower than the activity observed during the acute phase of illness. This was found

in spite of the accompanying leukocytosis in the convalescence stage of human malaria (Chaicumpa *et al,* 1982a). We, then, put forward hypotheses to explain the above findings. It was speculated that (1) the natural killer cells in the null cell fraction of lymphocytes participated actively in host defence against the malaria parasites during the acute phase of illness. This might lead to the destruction or exhaustion of the natural killer cells in the peripheral blood. As a consequence the body compensated by releasing more but poorly equipped young natural killer cells into the blood. The result was a higher number, but lower activity of null cells during the convalescence period. It was also reasonable to assume that (2) the chemotherapeutic agents used in the treatment of the patients exerted some inhibitory effect on the function of natural killer cells. Thus the cells were inefficient in the cytotoxic assay. The possibility existed also that (3) the increase in the number of null cells during the convalescence phase of malaria was due to the release of more NK cells from bone marrow. These cells participated in antibody dependent cell-mediated cytotoxicity against malaria infected cells during the convalescence period when there was a high level of specific circulating antibodies. As a result the total number of null cells increased while the number of the natural killer cells remained low (perhaps most of them died during the acute illness). Our experiment in 1983 indicated that although the anti-malarial drugs namely chloroquine, quinine, pyrimethamine and sulfadoxine/pyrimethamine combination had some effect on NK cell activity; the effect could not account for all of the marked reduction of the activity observed in malaria patients during the convalescence period. Thus the second possibility outlined above was ruled out (Chaicumpa *et al,* 1983b). In 1988, experiments were designed to study variation of subpopulations of lymphocytes in peripheral blood of patients during acute and convalescence phases of malaria in comparison with the normal controls. Cells positive for specific surface markers (labeled with specific monoclonal antibodies) were enumerated by a fluorescent activated cell sorter. It was found that, during the convalescence phase absolute numbers and percentages of Leu-$7^+$, Leu-$1^+$ and Leu-$7^+$, Leu-$1^-$ cells which had low NK cell activity were significantly higher than during acute illness. The evidence supports the first possibility outlined above (Tongtawe *et al,* 1988). Purified populations of null cells caused the release of $^{51}Cr$ from labeled *Plasmodium falciparum* infected red cells (Chaicumpa *et al,* 1983a).

## IMMUNODIAGNOSIS OF HELMINTHIC INFECTIONS

### Gnathostomiasis

Human gnathostomiasis caused by the nematode *Gnathostoma spinigerum* is prevalent in Thailand and many Asian countries. Man acquires the disease by consuming raw meat containing infective larvae of the parasite. The common clinical features include an intermittent cutaneous or subcutaneous migration of the larvae to the central nervous system resulting in intracranial hemorrhage or eosinophilic meningoencephalitis which can be fatal (Daengsvang, 1986). However, the clinical features are not readily distinguishable from other parasitic diseases, namely angiostrongyliasis, trichinosis and cutaneous larva migrans caused by other parasites (Beaver, 1969; Bhaibulaya and Charoenlarp, 1983; Punyagupta, 1978; Kazacos, 1986). Currently, definite diagnosis of human gnathostomiasis can only be made following a recovery of the parasite from the infected host which is rarely successful. Attempts have been made to diagnose this disease by immunological methods which detected antibodies. However, the tests have been hampered by the complex and cross-reactive nature of the antigens used. Studies by ELISA have shown that patients with parasitologically confirmed gnathostomiasis have serum IgG as well as IgE responses against the crude somatic extract of advanced third-stage larvae (L3) of the parasite. Due to the use of crude antigens, the assays were also positive for some patients with other parasitic infections (Suntharasamai *et al,* 1985; Dharmkrong-at *et al,* 1986; Soesatyo *et al,* 1987; Tada *et al,* 1987; Maleewong *et al,* 1988). As with most parasites, little is known about specific immunogen(s) of *Gnathostoma spinigerum.* Our preliminary work on the analysis of the crude L3 water extract revealed that the preparation was highly complex, comprising more than 40 polypeptides. Among them, 20 components were immunogenic in humans as shown by SDS-PAGE and Western blot analysis using sera of parasitologically confirmed gnathostomiasis patients. The Mr of the antigenic proteins ranged from 13 to 150 kDa (Nopparatana *et al,* 1988). Our recent

study on Western blot analysis and immune complexes formation between crude L3 extract and sera of four groups of individuals, *ie* parasitologically confirmed gnathostomiasis, clinically diagnosed gnathostomiasis, other parasitic infections and normal parasite-free controls, showed that the specific L3 component was a 24 kDa protein (Tapchaisri *et al,* 1991).

Later, we purified this specific antigen from the L3. The L3 were ground and extracted with water. Purification procedures involved gel filtration, chromatofocusing and anion exchange column chromatography, while characterization of the specific antigen was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining, Western blot analysis and isoelectric focusing. The specific antigen which has a pI of 8.5 was used as antigen in the indirect enzyme-linked immunosorbent assay (ELISA) to detect specific antibody in four groups of individuals, namely five parasitologically diagnosed gnathostomiasis patients (group 1); 15 clinically diagnosed gnathostomiasis patients (group 2); 136 patients with other parasitic infections (group 3); and 25 normal healthy parasite-free controls. Sensitivity, specificity and predictive values (positive and negative) of the assay were 100%. The anatomical sites of the 24 kDa antigen were also studied electronmicroscopically (Nopparatana *et al,* 1992).

However, the percentage recovery of the antigen purified from the crude extract of the advanced third-stage larvae (L3) was only 0.23. Moreover, the L3 themselves had to be collected individually from livers of naturally infected eels. The infection rates of these eels vary greatly with season and geographical distribution. Thus the supply of the L3 is rather inconsistent. The situation emphasizes the need for production of the antigen by recombinant DNA technology. Alternatively, it could also be possible to replace the antigen with an anti-idiotype antibody. The monoclonal antibodies specific to the Mr 24,000 protein were thus produced for preparing such anti-idiotype antibody (Chaicumpa *et al,* 1991a). The monoclonal antibodies obtained from *in vitro* culture of the hybridoma in serum-free medium were used to immunized Balb/c mice to raise the anti-idiotype antibodies. The IgG fraction obtained from the immunized mice was cleaved by papain under conditions which yielded Fv fractions. The Fv fractions of the anti-idiotype are currently being used for detecting antibody specific to *G. spinigerum* 24 kDa antigen (Chaicumpa *et al,* 1992a).

**Paragonimiasis heterotremus**

Diagnosis of paragonimiasis is based on both clinical features as well as laboratory tests. The most reliable means is the finding of *Paragonimus* eggs from sputum and/or feces of the infected individuals, while serology plays a supplementary role to the parasitological method. However, the immunological tests become the most practical, useful and dependable assays in extrapulmonary paragonimiasis or during the lag period between initial infection with metacercariae and sexual maturation of the egg-laying adult worms.

Although significant progress has been made in improvement and development of procedures for diagnosis of parasitic infections, it is generally accepted that specificity and sensitivity of the immunodiagnostic procedures aimed at detecting specific antibodies depend on purity and specificity of the antigens and the kinds of serological test used. Worms and their products are antigenically more complex than viruses and bacteria and also exist in different developmental stages within the host, expressing a degree of cross-ractivity with antigens of other parasites.

In 1991, crude and partially purified adult worm antigens were used for immunodiagnosis of human paragonimiasis caused by *Paragonimus heterotremus.* Partial characterization of the antigenic fractions was carried out leading to identification of a *Paragnonimus heterotremus* specific antigen. Adult *Paragonimus heterotremus* were recovered from the lungs and pleural cavity of cats orally infected with metacercariae. The worms were ground and extracted with distilled water. The soluble crude antigen (CA) contained about 40% proteins which could be fractionated by gel filtration on Sephadex G-200 into three fractions, $F_1$, $F_2$ and $F_3$. The CA and its Sephadex fractions were used in an indirect enzyme-linked immunosorbent assay (ELISA) for detecting antibodies to *P. heterotremus* in three groups of patients, *ie* patients whose sputum and/or feces revealed *P. heterotremus* eggs (group 1), patients with other parasitic infections (group 2), bacterially proven tuberculosis patients (group 3) and healthy, parasite-free controls (group 4). The sensitivity and

specificity of the assay when the $F_1$ was used as the antigen were 100%. Western blot analysis revealed that specific antigen of *P. heterotremus* was a non-protein component of Mr 35 kDa (Indrawati *et al,* 1991).

**Opisthorchiasis viverrini**

Opisthorchiasis caused by *Opisthorchis viverrini* is one of the major public health problems in Thailand (Sadun, 1955; Harinasuta and Vajrasathira, 1960; Wykoff, *et al,* 1965). Diagnosis of this liver fluke infection is usually done by detecting parasite eggs in stool and the intensity of infection is expressed as the number of egg(s) per gram of feces (Harinasuta and Vajrasathira, 1960; Asavanich, 1977). However, the detection of eggs in the stool is an insensitive method and is reliable only in the hands of experienced personnel. In light infections or when egg output is low as a result of biliary obstruction, stool examination may be falsely negative. The diagnosis is even more difficult in mixed parasitic infections, as eggs of *O. viverrini* are difficult to distinguish from those of other intestinal flukes such as *Prosthodendrium molenkampi, Phaneropsolus bonnei, Haplorchis taichui* and *Echinostoma* spp (Sirisinha, 1986).

Various immunological techniques have been used for the serodiagnosis of *O. viverrini* infection by the detection of antibodies in the sera of infected individuals. When a crude extract of the parasite was used as the antigen, cross-reaction was found with sera of patients with other parasitic infections such as gnathostomiasis, and schistosomiasis (Janechaiwat *et al,* 1980). Recently, an Mr 89,000 specific antigen of *O. viverrini* has been identified (Wongratanacheewin and Sirisinha, 1986). The finding raises the possibility of using this specific antigen in antibody detection assays. However, the development of such assays is hampered by the lack of an adequate supply of the parasite antigen. In addition, antibody detection cannot differentiate between present and past, cured infection. An alternative approach for the diagnosis of active opisthorchiasis would be by serological assays which detect antigens in the clinical specimen(s) of the patient. Specific monoclonal antibodies against *O. viverrini* antigens would offer greater specificity and uniformity of such assays than polyclonal reagents. Our experiments on the production of specific monoclonal antibodies against *O. viverrini* were reported in 1991.

A Balb/c mouse was immunized with a crude soluble antigen of *Opisthorchis viverrini* adult worms (OVAA) over a period of seven months. Spleen cells from the immune mouse were fused with Sp2/0 myeloma cells using polyethylene glycol 3,350 as fusogen. The ratio of spleen cells : myeloma cells was 10 : 1. The selective medium used was 15% fetal bovine serum supplemented RPMI-1640 medium containing hypoxanthine and azaserine. Among the 264 wells containing the fused cells, cells of 96 wells (36%) produced antibodies to the immunizing agent. Antibodies produced by 56 out of the 96 polyhybrids reacted with crude extracts of *Paragnonimus heterotremus.* Supernates from 20, 12, 6, 5, 3, 2, 1, 1, 1 and 1 polyhybrids reacted with extracts of *Echinostoma revoluntum, Taenia saginata, E. malayanum, E. ilocanum, S. stercoralis, Trichinella spiralis, P. westermani, Schistosoma monsoni, Ascaris* ssp. and *Entamoeba histolytica,* respectively. Seventeen polyhybrids produced antibodies specific only to the OVAA. Three of the 17 polyclones were subjected to monocloning and 31 specific monoclones were obtained. Antibodies produced by the 31 monoclones could be classified according to their tissue specificities into three groups. The first group reacted strongly to the worm integument and weakly with the muscles while those belonging to the second group reacted only to muscles of the worms. The monoclonal antibodies of the third group gave positive reaction to both muscles and tegument (Chaicumpa *et al,* 1991b). Some of these monoclonal antibodies were used in a sandwich enzyme-linked immunosorbent assay (ELISA) for detecting *Opisthorchis viverrini* antigen in fecal extracts of 4 groups of individuals. These were 24 patients with *O. viverrini* infection only (group 1), 31 patients with *O. viverrini* and other parasitic infections (group 2), 141 patients with other parasitic infections (group 3) and 21 normal, parasite-free individuals (group 4). The first antibody used in the ELISA was polyclonal immunoglobulin G prepared from the serum of a rabbit previously immunized with crude extract of *O. viverrini.* The second antibody was a monoclonal antibody specific to the antigen located in the worm tegument and muscular tissue. Sensitivity of the assay was 31% while specificity was 100%. Considerations for improving the sensitivity were given in our paper (Chaicumpa *et al,* 1992b).

**Strongyloidiasis**

The finding of *Strongyloides stercoralis* larvae in stool, duodenal fluid, sputum or, occasionally, other tissue fluids of the infected persons is the most reliable diagnosis of human strongyloidiasis. However, in a mild infection, this may be extremely difficult and time consuming as the larvae are frequently absent or are present only in small numbers in the clinical specimens (Napier, 1949; Jones and Abadie, 1954; Beal *et al,* 1970; Pelletier, 1984; Gill and Bell, 1979). In the latter situation, immunological assays are not only supplementary and practical but are also the most dependable means.

Several immunological tests based on detecting serum antibodies against *S. stercoralis* in the in fected individuals have now been described, and their application as screening tests is becoming increasingly accepted. These include intradermal test (ID) (Brannon and Faust, 1949; Sato *et al,* 1986; Genta, 1986) indirect immunofluorescence test (IFA) (Dafalla, 1972; Grove and Blair, 1981), radioallergo-sorbent test (RAST) (McRury *et al,* 1986; Badaro *et al,* 1987), and enzyme-linked immunosorbent assay (ELISA) (Neva *et al,* 1981; Carroll *et al,* 1981; Sato *et al,* 1985; Genta, 1986; McRury *et al,* 1986; Gam *et al,* 1987; Badaro *et al,* 1987). The ELISA has been useful also in evaluating treatment as the titers become low or negligible after chemotherapeutic cure while persisting antibody levels were observed in the existing infection.

The antigens used in the above mentioned tests, however, were either crude soluble antigens or frozen sections of the filariform larvae of either human or animal strongyloid worms. The sensitivity of the assays varied from 83 to 92%. False positive reactions occurred with serum of patients who harbored other nematodes. The existing situation indicates the use of specific antigenic preparation in the assays.

In 1991, crude antigen (CA) was prepared from *Strongyloides stercoralis* filariform larvae obtained from *in vitro* culture of human feces containing rhabditiform larvae. The lyóphilized filariform larvae were ground and ultrasonicated in distilled water then the soluble antigenic preparation was delipidized. The protein content of the crude soluble antigen was 20% of the original dried larvae. The CA was passed through a gel filtration chromatography column and yielded three different protein fractions, $F_1$, $F_2$ and $F_3$. CA and its fractions were used in an ELISA for detecting antibodies to *S. stercoralis* in serum samples of 5 groups of individuals. These were patients with parasitologically confirmed strongyloidiasis (group 1), patients with mixed *S. stercoralis* and other parasitic infections (group 2), non strongyloidiasis patients with other worm infestation(s) (group 3), normal parasite-free Thais (group 4) and normal parasite-free Swedes (group 5). It was found that $F_2$ was the best antigen in the ELISA. The sensitivity, specificity and positive and negative predictive values of the test using $F_2$ as the antigen were 95.0%, 96.4%, 95.0% and 96.4%, respectively (Mangali *et al,* 1991).

**Trichinellosis**

Trichinellosis is one of the food-borne parasitic zoonoses. It is caused by a nematode *Trichinella spiralis.* Humans get infection by consuming raw or improperly cooked meat contaminated with encysted infective larvae of the parasite. Within the host intestine the larvae excyst and develop from the first stage larvae to the second, third and fourth stage larvae and adult males and females, respectively (intestinal phase). After fertilization, the male worms die, while the female produce first stage larvae. These larvae enter the blood circulation of the host (circulatory phase) and finally encyst in various musculatures (muscular phase). Clinical features of the infected individual include myalgia, periorbital or facial edema, eosinophilia ( > 50 cells/ μl), elevation of creatine kinase ( > 60 units/l) and/or elevation of lactate dehydrogenase ( > 240 units/l) (Feldmeier *et al,* 1987). The patient may have diarrhea during the intestinal phase.

Definite diagnosis of human trichinellosis is made by the finding of the encysted larva in a muscule biopsy using a trichinoscope. However, this direct observation has low sensitivity which can be negative in the case of light infection and intestinal or cirulatory phases. Besides, complications may occur in the patient after performing the biopsy. The trichinoscopy itself is time consuming and eye fatique can occur when several specifmens have to be inspected.

Several immunological tests based on detecting serum antibodies have been developed for diagnosis of trichinellosis (Gould, 1970; Kagan and Norman 1970; Au *et al,* 1983; Feldmeire *et al,* 1987; Kham-

booruang and Thammasonthi, 1987). Among them, the indirect version of the enzyme-linked immunosorbent assay (ELISA) gained the most popularity due to its sensitivity and reproducibility (Taylor *et al,* 1980; Knapen *et al,* 1980; Gamble *et al,* 1983). Two kinds of antigens were commonly used in the tests. These were the excretory--secretory (ES) and the crude somatic antigens prepared from either adult worms, newborn larvae or excysted infective larvae ($L_1$). The ES antigen prepared from adult worms was poorly immunogenic (Ko and Yeung, 1989). In swine trichinellosis, the ES antiggen used in the indirect ELISA gave higher specificity than the crude somatic antigen (Zarlenga and Gamble, 1990; Su and Prestwood, 1991). Antigenic analysis using SDS-PAGE and immunoblotting in swine trichinellosis revealed that the ES antigenic components which were common among *T. spiralis* strains were the 45, 49 and 53 kDa proteins (Murrell *et al,* 1986). For human trichinellosis, IgG-ELISA was found to give higher sensitivity than the IgM-ELISA when the crude somatic antigen of $L_1$ was used (Knapen *et al,* 1982).

We have prepared ES antigen from *in vitro* cultures of the $L_1$ in serum-free medium and used the antigen in the IgG-ELISA for detecting antibodies to *T. spiralis* in sera serially collected from patients with parasitologically confirmed trichinellosis. The specificity of the assay was evaluated by comparing the results with those for healthy, parasite free controls and patient with other parasitic infections.

The specificity of the assay was 100%. The sensitivity of the test was also 100% when performed on sera of group 1 collected at days 57 and 120 after the infection. Sera collected at earlier time (day 23) and those collected 700 days after the infection had negligible reactivity. Thus IgG-ELISA using ES antigen of the $L_1$ was not only for diagnosis but also useful in evaluation of cure of the disease. Western blot analysis revealed that specific antigens of the *T. spiralis* were 94, 67, 63 and 39 kDa components (Mahannop *et al,* 1992).

## REFERENCES


Allison AC, Gregoriadis G. Liposomes as immunological adjuvants. *Nature* 1974; 252 : 252.

Appassakij H, Banchuin N, Sarasombath S, *et al.* Enzyme-linked immunosorbent assay for detection of *Salmonella typhi* protein antigen. *J Clin Microbiol* 1987; 25 : 273-7.

Araj GF, Chugh TD. Detection of *Salmonella* spp. in clinical specimens by captured enzyme-linked immunosorbent assay. *J Clin Microbiol* 1987; 25 : 2150-3.

Asavanich CK. Opisthorchiasis. *Siriraj Hosp Gaz* 1977; 29 : 1804-7.

Au ACS, Ko RC, Simon JW, Ridell RJ, Wong FWT, Tempelr MJ. Study of acute trichinosis in Churka. Specificity of enzyme-linked immunosorbent assay for IgM and IgE antibodies to *Trichinella* larval antigens in diagnosis. *Trans R Soc Trop Med Hyg* 1983; 412-5.

Badaro R, Carvalho EM, Santos RB, Gam AA, Genta RM. Parasite-specific humoral responses in different clinical form of strongyloidiasis. *Trans R Soc Trop Med Hyg* 1987; 81 : 149-50.

Banchuin N, Appassakij H, Sarasombath S, *et al.* Detection of *Salmonella typhi* protein antigen in serum and urine : a value for diagnosis of typhoid fever in an endemic area. *Asian Pacific J Allerg Immunol* 1987; 5 : 155-9.

Bakouch O, Gerlier D. Enhancement of immunogenicity of tumor virus antigen by liposomes : the effect of lipid composition. *Immunology* 1986; 58 : 507-13.

Banerji B, Alving CR. Anti-liposome antibodies induced by lipid A. I. Influence of ceramide, glycosphingolipids and phosphocholine on complement damage. *J Immunol* 1981; 126 : 1080-4.

Beal CB, Viens P, Grant RGL, Hughes JM. A new technique for sampling duodenal contents. Demonstration of upper small-bowel pathogens. *Am J Trop Med Hyg* 1970; 19 : 349-52.

Beaver PC. The nature of visceral larva migrans. *J Parasitol.* 1969; 55 : 3-12.

Bellamy JEC, Knop J, Steels EJ, Chaicumpa W, Rowley D. Antibody cross-linking as factor in immunity to cholera in infant mice. *J Infect Dis* 1975; 132 : 181-8.

Bhaibulaya M, Charoenlarp P. Creeping eruption caused by *Gnathostoma spinigerum. Southeast Asian J Trop Med Public Health* 1983; 14 : 266-8.

Black R, Levine MM, Young C, *et al.* Immunogenicity of Ty21a attentuated *Salmonella typhi* given with sodium bicarbonate or in enteric-coated capsules. *Rev Biol Scand* 1983; 53 : 9-14.

Brannon MJC, Faust EC. Preparation and testing of a specific antigen for diagnosis of human strongyloidiasis. *Am J Trop Med Hyg* 1949; 29 : 229-39.

Carrol SM, Karhigasu KT, Grove DI. Serodiagnosis of

human strongyloidiasis by an enzyme-linked immunosorbent assay. *Trans R Soc Trop Med Hyg* 1981; 75 : 706-8.

Chaicumpa W. Vaccines for bacterial diarrhoeas. *Asian Pacific J Allerg Immunol* 1985; 3 : 93-8.

Chaicumpa W, *et al.* WHO Progress report on "Levels of anti *V. cholerae* lipopolysaccharide, anti-vibrio adhesins and antitoxin in Thai individuals of various age groups". WHO, 1985.

Chaicumpa W, Atthasistha N. Study of intestinal immunity against *V. cholerae* : Role of *V. cholerae* haemagglutinin in intestinal immunity. *Southeast Asian J Trop Public Health* 1977; 8 : 13-8.

Chaicumpa W, Atthasishtha N. The study in intestinal immunity against *V. cholerae* : purification of *V. cholerae* El Tor haemagglutinin and the protective role of its antibody in experimental cholera. *Southeast Asian J Trop Med Public Health* 1979; 10 : 73-80.

Chaicumpa W, Atthasistha N, Looareesuwan S, Tharavanij S. Natural killer cells in peripheral blood of healthy individuals and patients with malaria. *Southeast Asian J Trop Med Public Health* 1982a; 13 : 61-8.

Chaicumpa W, Boonthum A, Kalumbaheti t, Tapchaisri P. Cell-bound haemaglutinin (CHA) of *V. cholerae* $O_1$ as protective antigen. *Southeast Asian J Trop Med Public Health* 1984; 15 : 407-13.

Chaicumpa W, Chaisri V, Tapchaisri P, Chongsa-nguan M, Pongponrath E. Oral vaccine against cholera prepared from *Vibrio cholerae* antigens. *Southeast Asian J Trop Med Public Health* 1987; 18 : 142-8.

Chaicumpa W, Chatkaewomorakote A, Patarapotikul J, Looareesuwan S. Effects of natural killer (NK) cells on *Plasmodium falciparum* infected red cells. *Asian Pacific J Allerg Immunol* 1983a; 1 : 135-9.

Chaicumpa W, Pacharaprakiti C, Plueksawan W, Attasishtha N. Assessment of antibody responses and protective immunity in cholera vaccinated subjects. *Southeast Asian J Trop Med Public Health* 1980; 11 : 58-66.

Chaicumpa W, Peungjesda U, Martinez B, Atthasishtha N. Soluble haemagglutinin of classical vibrios : isolation and protection against cholera by its antibodies. *Southeast Asian J Trop Med Public Health* 1982b; 13 : 637-45.

Chaicumpa W, Roca R, Atthasistha N, Chongsupajaisiddhi T. Effects of antimalarial drugs on human natural killer cell activity. *Southeast Asian J Trop Med Public Health* 1983b; 14 : 413-9.

Chaicumpa W, Rowley D. Experimental cholera in infant mice : protective effect of antibody. *J Infect Dis* 1972; 125 : 480-5.

Chaicumpa W, Rowley D. Mechanisms of antibacterial immnity against *Vibrio cholerae* in the intestinal tracts of baby mice. *J Infect Dis* 1973; 128 : 56-61.

Chaicumpa W, Ruangkunaporn Y, Nopparatana C, Chongsa-nguan M, Tapchaisri P, Setasuban P. Monoclonal antibody to a diagnostic Mr. 24,000 antigen of *G. spinigerum. Int J Parasitol* 1991a; 21 : 735-8.

Chaicumpa W, Ruangkunaporn Y, Kalambaheti T, *et al.* Specific monoclonal antibodies to *Opisthorchis viverrini. Int J Parasitol* 1991b; 21 : 969-74.

Chaicumpa W, Ruangkunaporn Y, Burr D, Chongsa-nguan M, Echeverria P. Diagnosis of typhoid fever by detection of *Salmonella typhi* antigen in urine. *J Clin Microbiol* 1992a; 30 : 2513.

Chaicumpa W, Sae Er, Chongsa-nguan M, Echeverria P. Simple serological tests for detecting classical heat-labile enterotoxin (LT-1) of *E. coli. Southeast Asian J Trop Med Public Health* 1988a; 19 : 175-85.

Chaicumpa W, Swasdikosa S. Immunogenicity of soluble haemagglutinin lipopolysaccharide complex of classical *V. cholerae. Southeast Asian J Trop Med Public Health* 1983; 14 : 548-58.

Chaicumpa W, Thin-inta W, Khusmith S, *et al.* Detection with monoclonal antibody of *Salmonella typhi* antigen 9 in specimens from patients. *J Clin Microbiol* 1988b; 26 : 1824-30.

Chaicumpa W, Wechsathanarak Y, Tantivanich S, Plueksawan W. Antibody responses to heat-killed phenol preserved parenteral typhoid vaccine. *Southeast Asian J Trop Med Public Health* 1985; 16 : 371-6.

Chaicumpa W, Ybanez L, Kitikoon V, *et al.* Detection of *O. viverrini* antigens in stools using specific monoclonal antibody. *Int J Parasitol* 1992b; 22 : 527-31.

Chongsa-nguan M, Chaicumpa W, Kalambaheti T, Thin-inta W, Echeverria P, Overtoom R. Current status of widal test in diagnosis of typhoid fever in an endemic area. *Southeast Asian J Trop Med Public Health* 1989; 20 : 493-5.

Chongsa-nguan M, Chaicumpa W, Kalambaheti T, *et al.* Vibriocidal antibody and antibodies to *V. cholerae* lipopolysaccharide, cell-bound haemagglutinin and toxin in Thai population. *Southeast Asian J Trop Med Public Health* 1986; 17 : 558-66.

Chongsa-nguan M, Chaicumpa W, Ruangkunaporn Y, Looareesuwan S. Immunogenicity of two formulations of oral cholera vaccines in Thai volunteers. *Vaccine* 1991; 9 : 53-9.

Clemens JD, Jertbarm M, Sack D, *et al.* Effect of neutralisation of gastric acid on immune responses to an oral subunit, killed whole-cell cholera vaccine. *J Infect Dis* 1986; 154 : 175-8.

Daengsvang S. *Gnathostoma spinigerum* and human gnathostomiasis : a brief review. In : Sucharit S, Areekul S, Sucharit P, Vanuanonta S, Kitikoon V, Chantachum Y, eds. The 25th Anniversary of the Faculty of Tropical Medicine. Bangkok. Krung Siam Press, 1986; 124-47.

Dafalla AA. The indirect fluorescent antibody test for the serodiagnosis of strongyloidiasis. *J Trop Med Hyg* 1972; 75 : 109-11.

Dancey GF, Yasuda T, Kinsky SC. Enhancement of liposomal model membrane immunogenicity by incorporation of lipid A. *J Immunol* 1977; 119 : 1868-73.

Dharmkrong-at A, Migasena S, Suntharasamai P, Bunnag D, Priwan R, Sirisinha S. Enzyme-linked immunosorbent assay for detection of antibody to *Gnathostoma* antigen in patients with intermittent cutaneous migratory swelling. *J Clin Microbiol* 1986; 23 : 847-51.

Echeverria P, Seriwatana J, Chityothin O, Chaicumpa W, Tirapat C. Detection of enterotoxigenic *E. coli* in water by filter hybridization using three enterotoxin gene probes. *J Clin Microbiol* 1982; 16 : 1086-90.

Echeverria P, Seriwatana J, Leksomboon U, Tirapat C, Chaicumpa W. and Rowe B. Identification by DNA hybridization of enterotoxigenic *E. coli* in homes of children with diarrhoea. *Lancet* 1984a; 1 : 63-6.

Echeverria P, Seriwatana J, Patamaroj U, Moseley S, Mc Farland A, Chityothin O, Chaicumpa W. Prevalence of heat stable enterotoxigenic *E. coli* in pigs, water, and people at farms in Thailand as determined by DNA hybridization. *J Clin Microbiol* 1984b; 19 : 489.

Echeverria P. Seriwatana J, Taylor DN, Tirapat C, Chaicumpa W, Rowe B. Identification by DNA hybridization of enterotoxigenic *E. coli* in a longitudinal study of villages in Thailand. *J Infect Dis* 1985; 151 : 124.

Echeverria P, Taylor DN, Seriwatana J, *et al.* Potential sources of enterotoxigenic *E. coli* in homes of children with diarrhoea in Thailand. *Bull WHO* 1987; 65 : 207.

Echeverria P, Tirapat C, Charoenkul D, Yanggratoke S, Chaicumpa W. Epidemiology of bacterial enteric pathogens in rural Thailand: Application of a DNA hybridization assay to detect enterotoxigenic *E. coli.* In : Takeda Y, Miwatani, T eds. Bacterial diarrhoeal diseases. An International Symposium. kDk Tokyo, Japan 1983.

Feldmeite H, Fischer G, Blaumeiser G. Kinetics of humoral response during the acute and the convalescent phase of human trichinosis. *Zentralbl Bakteriol, Mikrobiol Hyg,* ser A 1987; 264 : 221-34.

Finkelstein RA. Antitoxic immunity in experimental cholera : observations with purified antigens and the ligated ileal loop model. *Infect Immun* 1970; 1 : 464.

Foo ESA, Chaicumpa W. Protection against *V. cholerae* infection afforded by fragment of anti-haemagglutinin. *Southeast Asian J Trop Med Public Health* 1981; 12 : 506-12.

Freter R. Mechanism of action of intestinal antibody in experimental cholera II. Antibody-mediated antibacterial reaction at the mucosal surface. *Infect Immun* 1970; 2 : 556.

Gamble HR, Anderson WR, Graham CW, Murrell KD. Diagnosis of swine trichinosis by enzyme-linked immunosorbent assay (ELISA) using an excretory-seecretory antigen. *Vet Parasitol* 1983; 13 : 349-61.

Gam AA, Neva FA, Krotosk WA. Comparativesensitivity and specificity of ELISA and IHA for serodiagnosis of strongyloidiasis with larval antigens. *Am J Trop Med Hyg* 1987; 37 : 15-61.

Genta RM, Douce RW, Walzer PD. Diagnostic implications of parasite-specific immune responses in immunocompromised patients with strongyloidiasis. *J Clin Microbiol* 1986; 23 : 1099-103.

Genta RM, Predictive value of an enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of strongyloidiasis. *Am J Clin Pathol* 1986; 89 : 391-4.

Gill GV, Bell D. *Strongyloides stercoralis* infection in former Far East prisoners of war. *Br Med J* 1979; 2 : 572-4.

Gould SE, Clinical pathology : Diagnostic laboratory procedures. In : Gould, SE, Trichinosis in man and animals. Springfield; Charles C Thomas, 1970; 190-221.

Gregoriadis G, Allison AC. Entrapment of protein in liposomes prevents allergic reactions in pre-immunised mice. *FEBS Lett* 1974; 45 : 71-4.

Grove DI, Blair AJ. Diagnosis of human strongyloidiasis by immunofluorescence, using *Strongyloides ratti* and *S. stercoralis* larvae. Am J Trop Med Hyg 1981; 30 : 344-9.

Harinasuta C, Vajrasthira S. Opisthorchiasis in Thailand. *Ann Trop Med Parasit* 1960; 54 : 100-5.

Holmgren J, Pathogenesis and prevention of cholera *Scan J Infect Dis* 1982; 36 (Suppl) : 58.

Indrawati I, Chaicumpa W, Setasuban P, Ruangkunaporn Y. Studies on immunodiagnosis of human paragonimiasis and specific antigen of *Paragonimus heterotremus*. *Int J Parasitol* 1991; 21 : 395-401.

Janechaiwat J, Tharavanij S, Vajrasathira S, Chaicumpa W. The immunological diagnosis of human opisthorchiasis and the humoral immune response to opisthorchis infection in the hamster. *J Med Assoc Thai* 1980; 63 : 447.

Jones CA, Abadie SH. Studies in human strongyloidiasis II. A comparison of the efficiency of diagnosis by examination of feces and duodenal fluid. *Am J Clin Pathol* 1954; 24 : 1154-8.

Kagan IG, Norman LG. The serology of trichiniasis. In: Gould SE, ed. Trichinosis in Man and Animals. Springfield: Charles C Thomas 1970; 222-68.

Kazacos KR. Trichinosis. *J Am Vet Ass* 1986; 188 : 1272-5.

Khamboonruang C, Thammasonthi W. Enzyme-linked immunosorbent assay (ELISA) in patients infected with *Trichinella spiralis*. In : Kim CW, Ruitenberg EG, Teppema JS, eds. Trichinosis. Proceedings of the Fifth International Conference on Trichinellosis Survey. London: Reedbooks 1987; 263-7.

Knapen VF, Frachimont JH, Ruitenburg EJ. The reliability of the enzyme-linked immunoassay (ELISA) for the detection of swine trichinosis. In : Kim, CW, Ruitenberg EG, Teppema JS, eds. Trichinosis. Proceedings of the Fifth International Conference on Trichnellosis Survey. London: Reedbooks 1980; 399-404.

Knapen VF, Frachimont JH, Verdonk AR, Stumpf KU. Detection of specific immunoglobulin (IgG, IgM, IgA, IgE) and total IgE level in human trichinosis by means of enzyme-linked immunosorbent assay (ELISA). *Am J Trop Med Hyg* 1982; 31 : 973-6.

Levine MM, Kaper JB, Black RE, Clements ML. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. *Microbiol Rev* 1983; 47 : 510-50.

Mahannop P, Chaicumpa W, Setasuban P, Morakote N, Tapchaisri P. Immunodiagnosis of human trichinellosis using ES antigen. *J Helminthol* 1992; 66.

Maidin A, Tharavanij B, Chaicumpa W. Seasonal variation of enterotoxigenic *E. coli* diarrhea and the cotransfer of plasmids coding for enterotoxin and drug resistance. *Southeast Asian J Trop Med Public Health* 1982; 13 : 385.

Maleewong W, Morakote N, Thamasonthi W, Charuchinda K, Tesana S, Khamboonruang C. Serodiagnosis of human gnathostomiasis. *Southeast Asian J Trop Med Public Health* 1988; 19 : 201-5.

Mangali A, Chaicumpa W, Nontasut P, Chantavanich P, Tapchaisri P, Viravan C. Enzyme linked immunosorbent assay for diagnosis of human strongyloidiasis. *Southeast Asian J Trop Med Public Health* 1991; 22 : 88-92.

McRury J, Messsias ID, Walzer PD, Huitger T, Genta RM. Specific IgE responses in human strongyloidiasis. *Clin Exp Immunol* 1986; 65 : 631-8.

Migasena S, Desakorn V, Suntharasamai P, *et al,* Immunogenicity of two formulations of oral cholera vaccine comprised of killed whole vibrios and the $\beta$-subunit of cholera toxin. *Infect Immun* 1989; 57 : 117-20.

Miquel CA. Epidemiology of cholera in Samut Sakorn, Thailand. Faculty of Tropical Medicine, Mahidol University, Bangkok. 1981. MSc Thesis,

Moseley SL, Echeverria P, Seriwatana J, *et al.* Identification of enterotoxigenic *E. coli* by colony hybridization using three enterotoxin gene probes. *J Infect Dis* 1983; 145 : 863-9.

Murrell KD, Anderson WR, Schad GA, *et al.* Field evaluation of enzyme-linked immonosorbent assay for swine trichinosis. Efficacy of the excretory-secretory antigen. *Am J Vet Res* 1986; 47 : 1046-9.

Napier LE. *Strongyloides stercoralis* infection. *J Trop Med Hyg* 1949; 52 : 25-30.

Neva FA, Gam AA, Burke J. Comparison of larval antigens in an enzyme-linked immunosorvent assay for strongyloidiasis in humans. *J Infect Dis* 1981; 144 : 427-32.

New RR, Theakston RD, Zumbuckl O, Iddon D, Friend J. Liposomal immunisation against snake venoms. *Toxicon* 1985; 23 : 215-9.

Newsby TJ, Stoke CR. The intestinal immune system and oral vaccination. *Vet Immunol Immunopathol* 1984; 6 : 67-105.

Nopparatana C, Chaicumpa W, Tapchaisri P, Setasuban P, Ruangkunaporn Y. Towards as suitable antigen for diagnosis of *Gnathostoma spinigerum* infection. *Int J Parasitol* 1992; (in press).

Nopparatana C, Tapchaisri P, Setasuban P, Chaicumpa W, Dekumyoy P. Antibody responses in human gnathostomiasis. *Southeast Asian J Trop Med Public Health* 1988; 19 : 219-24.

Pelletier LL. Chronic strongyloidiasis in World War II. Far East ex-prisoners of war. *Am J Trop Med Hyg* 1984; 33 : 51-65.

Pierce NF, Cray WCJr. Determinants of the localization, magnitude and duration of a specific mucosal IgA plasma cell response in enterically immunised rats.

*J Immunol* 1982; 128 : 1311-5.

Punyagupta S. Clinical manifestation of eosinophilic meningitis *(Gnathostoma spinigerum). Southeast Asian J Trop Med Public Health* 1978; 9 : 278.

Rubin FA, Kopecko DJ, Noon KF, Baron LS. Development of a DNA probe to detect *Salmonella typhi. J Clin Microbiol* 1985; 22 : 600-5.

Sack DA. Candidate cholera vaccines : field trials. 3rd meeting of the scientific working group on bacterial enteric infections : Cholera : a review of research advances, WHO Geneva, 12-14 Sept, 1984.

Sato Y, Takara M, Otsurau M. Detection of antibodies in strongyloidiasis by enzyme-linked immunosorbent assay (ELISA). *Tran R Soc Trop Med Hyg* 1985; 79 : 51-5.

Sadun EM. Studies on *Opisthorchis viverrini* in Thailand. *Am J Hyg* 1955; 62 : 81-115.

Samrejrongroj P, Chaicumpa W, Riganti M. Effect of antibody to *Vibrio cholerae* El Tor hemolysin on pathological changes of the intestines in cholera. *Southeast Asian J Trop Med Public Health* 1980; 11 : 262-6.

Sato Y, Otsuru M, Takara M, Shiroma Y. Intradermal reactions in strongyloidiasis. *Int J Parasitol* 1986; 16 : 87-91.

Sirisinha S, Rathunasiriwilai W, Puengtornwatanakul S, Sobhon P. Sartongded P, Koonchornboon T. Complement-mediated killing of *Opisthorchis viverrini* via activation of the alternative pathway. *Int J Parasitol* 1986; 16 : 349-56.

Sivadasan K, Kurin B, John TJ. Rapid diagnosis of typhoid fever by antigen detection. *Lancet* 1984; 1 : 134-5.

Soesatyo MHNE, Ratanasiriwilai W, Suntharasamai P, Sirisinha S. IgE responses in human gnathostomiasis. *Trans R Soc Trop Med Hyg* 1987; 81 : 799-801.

Steele EJ, Chaicumpa W, Rowley D. Isolation and biological properties of three classes of rabbit antibody to *Vibrio cholerae. J Infect Dis* 1974; 13 : 93.

Steele EJ, Chaicumpa W, Rowley D. Further evidence for cross-linking as a protective factor in experimental cholera : properties of antibody fragments. *J Infect Dis* 1975; 132 : 175-80.

Su X, Prestwood AK. A dot-ELISA micro western blot test for the detection of swine trichinosis. *J Parasitol,* 1991; 77 : 76-82.

Suntharasamai P, Desakorn V, Migasena S, Bunnag D, Harinasuta T. ELISA for immunodiagnosis of human ghathostomiasis. *Southeast Asian J Trop Med Public Health* 1985; 16 : 274-9.

Tacket CO, Forrest B, Morond R, *et al.* Safety, immunogenicity and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* ty21a. *Infect Immun* 1990; 58 : 1620-7.

Tada I, Araki T, Matsuda H, Araki K, Akahane H, Mimori T. A study on immunodiagnosis of gnathostomiasis by ELISA and double diffusion with special reference to the antigenicity of *Gnathostoma doloresi. Southeast Asian J Trop Med Public Health* 1987; 18 : 444-8.

Tapchaisri P, Nopparatana C, Chaicumpa W, Setasuban P. Specific antigen of *Gnathostoma spinigerum* for immunodiagnosis of human gnathostomiasis. *Int J Parasitol* 1991; 21 : 315-9.

Taylor SM, Kenny J, Mallon T, Davidson WB. The micro-ELISA for antibodies to *Trichinella spiralis.* Elimination of false positive reactions by antigen fractionation and technical improvements. *Zentralbl Vet* 1980; 27 : 764-72.

Tharavanij S, Charoenpong K, Thaveetiyanond S, Supavej S, Santiswatdinont P. Study on cholera carriers in Thailand. *Southeast Asian J Trop Med Public Health* 1970a; 1 : 247.

Tharavanij S, Kunasol P, Charoenpong K, Boonthai P, Supavej S, Srisomporn D. Serological study on cholera in Thailand. *Southeast Asian J Trop Med Public Health* 1970b; 1 : 254.

Tongtawe P, Chaicumpa W, Tapchaisri P, Looareesuwan S, Webster HK. Lymphocyte subpopulations during acute and convalescence phases of malaria. *Asian Pacific J Allerg Immunol* 1988; 6 : 11-8.

Wongratanacheewin S, Sirisinha S. Analysis of *Opisthorchis viverrini* antigens by radioimmunoprecipitation. *Southeast Asian J Trop Med Public Health* 1986; 18 : 511-20.

Wykoff DE, Harinasuta C, Juttijudata P, *et al. Opisthorchis viverrini* in Thailand: the life cycle and comparison with *O. filineus. J Parasitol* 1965; 51 : 207-14.

Zarlenga DS, Gamble SR. Molecular cloning and expression of immunodominant 53-kDa excretory-secretory antigen from *Trichinella spiralis* muscle larvae. *Mol Biochem Parasitol* 1990; 42 : 165-74.