

SEATO MEDICAL RESEARCH STUDY ON DIARRHEAL DISEASES

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Period of Report: 1 April 1966 - 31 March 1967

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General:

This study includes projects concerned with the effects of antibiotics on gut function and structure (including histochemistry), the prevalence of lactose intolerance in Thais and Americans living in Thailand; the effect of daily lactose feeding on lactose metabolism in Thais, the effects of acute diarrhea on gut function and structure in Thais, the effect of ceroid pigment deposition on intestinal function in northeast Thailand, and the quantitative changes in the fecal flora of healthy and diarrheic persons. The following projects were started: a long-term study of the effect of a tropical environment on gut morphology and function in Peace Corps volunteers and in Special Forces personnel, and in the latter, the effect of daily folic acid; a study of the immunological defense mechanisms of the Thai intestine in both normal subjects and in those with acute diarrhea; and a quantitative study of the bacterial flora of upper gastrointestinal fluids aspirated from persons with and without diarrhea.

In conjunction with the department of medicine of the Royal Thai Army Hospital, several well-documented cases of malabsorption due to tropical sprue were thoroughly studied and added much to our knowledge of this disease in Thailand. Fortunately, tropical sprue appears to be quite rare in the Bangkok area, and to our knowledge, has never been documented in an American living anywhere in Thailand.

Study Report

Bacteriologic Survey of Stools from Patients with Acute Diarrhea

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The objectives of this study were to determine the types, frequency of occurrence, and pattern of antibiotic sensitivity of salmonellae and shigellae among patients with acute or chronic diarrhea. Stools were studied in an effort to determine the incidence of other Enterobacteriaceae and their relationships to this disease.

This study included specimens from inpatients and outpatients of both sexes from hospitals throughout Thailand. Most of the specimens were collected during the acute phase of the disease from patients hospitalized with diarrhea. During the first 11 months of this reporting period the laboratory procedure was as follows: In the Bangkok area either fecal specimen or three rectal swabs, moistened in alkaline peptone broth, were obtained from each patient. Two of the swabs were placed in enrichment broths (alkaline peptone, selenite-F) and the third streaked directly onto SS and MC agar plates. The alkaline peptone broth was subcultured to alkaline lauryl sulfate tellurite agar for isolation of vibrios. After overnight incubation at 37 C, the selenite-F broth was subcultured on SS and DC plates. Specimens from outside Bangkok were submitted in a holding medium designed for the transport of enteric bacteria. Upon arrival at the laboratory, plates of SS and MC were streaked and tubes of selenite-F and alkaline peptone broth inoculated. The enrichment broths were subcultured as outlined above. All plates were examined after 24 and 48 hours incubation. Lactose-negative colonies were transferred to Kligler's iron agar slants and subsequently to a battery of media to determine patterns of biochemical activity. Those isolates showing biochemical patterns typical of salmonellae, shigellae, or vibrios were definitively identified in accordance with the serological methods described by Edwards and Ewing.

During the last month, the procedures for processing stool specimens were modified to incorporate a technique for the selection of suspicious colonies from initial isolation media by using transmitted oblique light and a stereoscopic dissecting microscope. A more rapid method for determining carbohydrate fermentation patterns is also being evaluated. These techniques are described more fully below.

During this reporting period a total of 2935 specimens were examined from 1786 (1306 Thai, 480 caucasian) patients with acute diarrhea. Recognized diarrheal agents were isolated from 21% of these specimens. Approximately 15.9% of the specimens yielded salmonellae, 5.3% shigellae and 26.6% paracolons (Table I). These data are consistent with the recovery rates which have been remarkably stable during the preceding four years. Enteropathogenic Escherichia coli (E.E. coli) were recovered from 7.8% of 2231 (2062 Thai, 169 caucasian) isolates from children under 6 years of age (Table IV) E.E. coli were found

with twice the frequency among Thais as Caucasian (8.1% Thai, 4.1% Caucasian); however, a larger number of caucasian children must be studied to determine the significance of these data. The finding of enteropathogenic serotypes in infants and small children suffering from acute diarrhea emphasizes the role of these organisms as a pathogen for this age group.

The data in Table II show 18 species to be represented among the 468 salmonellae isolates. S. paratyphi B was the predominating organism and accounted for 58.5% (274/468) of the salmonellae isolates. S. derby accounted for 19.0% with approximately equal numbers of S. weltevreden, S. anatum, and S. montevideo comprising another 11.7%. Isolates of the salmonellae species most frequently recovered, were found to be evenly distributed throughout the year. One exception was noted when an epidemic caused by S. derby resulted in 69 isolates during a one month period. The isolates of S. montevideo decreased for the second consecutive year. In 1964, S. montevideo was the predominant enteropathogen and accounted for 51% of the salmonellae isolates. Recovery percentages dropped to 11.2% in 1965 and to low of 4.0% during this reporting period. The recovery rates for salmonellae (5.8%) and shigellae (5.2%) were approximately equal among the caucasians in this study, whereas salmonellae were 3 times as prevalent as shigellae among the Thai nationals. The significance of these data is unknown at this time, and will require additional investigation.

There were 11 species among the 155 shigellae isolates obtained during this reporting period (Table III). The most frequently encountered species were: Sh. flexneri 3, 40.6%; Sh. flexneri 2, 22.6%; Sh. sonnei form 1, 16.8%; and Sh. dysenteriae 1, 7.7%. Isolations of the various species of shigellae were well distributed throughout the year with no single epidemic accounting for an abnormal frequency for any given species.

Agglutinating and non-agglutinating vibrios were rarely isolated in the Bangkok area. Outbreaks of cholera requiring the assistance of personnel from this laboratory will be reported under the Study Report on Cholera.

Antibiotic sensitivities were determined for 406 enteric pathogens in an effort to maintain a continuing surveillance of the drug susceptibilities of these organisms in Thailand. Six antibiotics (tetracycline, colistin, kanamycin sulfate, chloramphenicol, neomycin sulfate, nalidixic acid) were used in a tube dilution procedure, and the results obtained with 159 salmonellae, 155 shigellae, and 92 E.E. coli isolates are shown in Tables V through X. These data show nalidixic acid and colistin to be uniformly effective in vitro, with the organisms exhibiting varying degrees of resistance to the other drugs. Determinations are still in progress, therefore, a detailed comparison with the studies conducted by Dr. Noyes in 1963-1964 has not been made. There is, however, an apparent increase in resistance, and examples of this shift in drug susceptibility can best be illustrated by the results obtained with the predominating Salmonella and Shigella isolates. These data show 30 of 40 Sh. flexneri 3 isolates to be resistant to <100 mcg of tetracycline, whereas Noyes found only 2 of 17 resistant at this level. Twenty of 21 S. paratyphi B are resistant to <100 mcg of either tetracycline or neomycin sulfate. Two years ago 7 of 10 and 6 of 10 S. paratyphi B isolates were resistant to these levels, respectively.

In July, a survey for enteric pathogens among the children and attendants at the Central Preventorium for Children (Nondhaburi Province) was conducted to provide baseline information prior to conducting a proposed longitudinal study in this group. Two hundred and forty seven stool specimens obtained from 227 persons (152 children under 6 years of age and 75 adults) resulted in the isolation of 27 strains of salmonellae (11%), 4 strains of shigellae (1.7%) and 39 strains of E.E. coli (16%). Additional breakdown of the results showing the recovery of pathogens from asymptomatic versus individuals with diarrhea is presented in Tables XI and XII.

During the November 1966 visit of Dr. Samuel Formal, Chief Department of Applied Immunology MRAIR, arrangements were made with the Special Forces unit in Thailand to study the diarrheas among their

personnel. Specimens from acute diarrhea were to be collected in holding medium and sent to SMRL for culture. An information sheet was to accompany each specimen. Unfortunately, it has been impossible to obtain fecal specimens from this group, and hope for this aspect of the study has been abandoned. Captain Blaydow, the S.F. surgeon, has submitted 109 questionnaires which provide some information on the amount of diarrhea occurring in this group of approximately 300 men. These men are assigned in small groups to many areas in Thailand, and not all segments have been participating on a regular basis. However, by making a few estimates the following approximations were obtained. There has been approximately 4905 man days of diarrhea out of a total of 28,800 man days incountry. The average time of onset of diarrhea was 7.6 weeks after arrival, with a 4.5 day duration of illness. The average number of stools per day was 6, with 24% complaining of fever and 44% having cramps. The type of unit being studied, and the fact that only those men reporting to the dispensary with diarrhea are included in the statistics, tends to make these data obvious underestimations of the amount of diarrhea occurring in this group. These data do provide some information on the amount of diarrhea in these troops, and is perhaps the closest approximation that can be obtained.

In May of 1966, a survey of bacterial enteropathogens and parasites was conducted in Nakornpanom (northeast) Thailand. The recovery of bacterial pathogens among adults and children with and without diarrhea is presented in Table XIII. The intestinal parasites are listed in Table XIV.

A study was undertaken in August of 1966 to evaluate a rapid technique for the detection and identification of enteropathogenic organisms. The technique was reported by Sanders, A.C. *et al* (Appl. Microbiol. 5: 1957). It was designed for use in diarrheal epidemics, but had not been evaluated using freshly isolated organisms or under field conditions. The rapid technique differs from the classical methodology only after initial isolation, when a 2 hour incubation in "booster broth" is used to screen lactose from non-lactose fermenting organisms. The booster broth serves as a medium for the determination of indol production, urease activity and motility as well as inoculum for pour plates and serological typing. The carbohydrate fermentation patterns of non-lactose fermenting organisms are determined by preparing a pour plate, and placing 4 paper discs saturated with dextrose, lactose, sucrose and mannitol on the surface of the agar. The production of acid and H₂S is detectable, around the appropriate carbohydrate discs, after 6 hours incubation, and further identification is then possible by serologic typing of the organism. Using this technique it is theoretically possible to isolate and identify an enteropathogen within a 2 day period.

Initial evaluation of the rapid technique consisted of a direct comparison with the classic methodology routinely used at SMRL. In order to process the same colony through both methods it was necessary to pick each colony from the isolation medium to a Kligler's iron agar slant to provide sufficient inoculum for use in both identification schemes. Eighty five suspected colonies were processed by both methods and 16 strains of salmonellae and 2 strains of shigellae detected. Results were identical with both methods.

One hundred forty one stool specimens (34 adult, 107 children) from Praputhabath Provincial Hospital provided additional opportunity to evaluate the Sander's technique. Recognized pathogens were isolated from 28.4% of these patients, and the distribution of species among the 13 salmonellae, 13 shigellae and 14 E.E. coli is shown in Table XV. All 13 of the shigellae species were isolated during a small outbreak of shigellosis, which explains the unexpected recovery of equal numbers of shigellae and salmonellae from this central Thailand community. This method appears to be a simple and reliable technique for the rapid identification of enteropathogens. The opportunity has not arisen to conduct field tests during a large epidemic, however, it should prove to be an effective technique for handling large numbers of specimens. Sander's method combined with the use of transmitted oblique illumination (reported below) is currently being evaluated for use as a routine method in the enteric bacteriology section.

Dr. Richard A. Finkelstein with the assistance of Miss Kannikar Punyashthiti investigated the feasibility of using transmitted oblique illumination to reveal colonial differences which might be used for the recognition of enteropathogens. A report of their investigation is included.

Study Report: A "New" Approach to Diagnostic Enteric Bacteriology

Principal Investigator: Richard A. Finkelstein, Ph.D.

Associate Investigator: Kannikar Punyashthiti, M.Sc.

Conventional methods of bacteriological diagnosis of enteric disease suffer from the severe shortcoming that results are not usually available during the acute illness of the patient. Accordingly, it would be highly desirable to develop more rapid methods for recognition of enteric pathogens. We, and others, have observed that transmitted oblique illumination reveals differences in color, refractivity and internal structure of bacterial colonies on transparent media which are not apparent with other forms of illumination. These colonial differences are heritable, and the colonial morphology, under defined conditions, is characteristic for different bacterial genera or species. With the use of an ordinary stereoscopic dissecting microscope, the ability of the observer to detect colonial differences is enhanced even further.

With some experience, the technician can be trained to recognize these differences and to associate particular colonial appearances with particular genera and species. Simply by observation, then, he is enabled to make educated guesses regarding the identity of the bacteria present which can, and must, then be confirmed or refuted by selection of appropriate rapid tests. For example, suspecting a *Salmonella* from the colonial appearance, the technician could immediately perform slide agglutination tests for both serological grouping and typing.

With experience, the accuracy of these "guesses" should approach or exceed 90%. This results in great savings of time, materials, and manipulative steps without sacrifice of accuracy and with even some increase in sensitivity over conventional procedures. The technique allows the laboratory to establish a diagnosis in the case of enteric disease before the patient is either recovered or dead.

The system is easy to set up in any laboratory. One needs only a microscope lamp, a mirror, and a low power dissecting microscope. The mirror is placed flat on the table, in front of the microscope, and the light is directed into the mirror and reflected by it upwards, through the Petri dish on the microscope stage, at an angle of approximately 40°. It should be adjusted, by the observer, for maximum contrast.

We have prepared a series of color photographs of colonies of the common enteric pathogens and commensal organisms on MacConkey's agar which serves as a "rogues gallery" for reference while using the technique and as an aid in training.

A comparison between the proposed technique and conventional methodology has been made on a series of 68 consecutive enteric specimens. The conventional method employed a battery of selective and differential isolation media, including MacConkey's agar, and enrichment procedures. Only the MacConkey's plate was used in the "new" technique. The conventional methods yielded a total of two salmonellae strains and five shigellae. Nine salmonellae and the same five shigellae were identified by the proposed technique. One *Salmonella* isolate, which was obtained by the conventional technique only after selective enrichment, was missed using the "new" approach.

Summary:

Bacterial pathogens were recovered from approximately 21% of 2935 stool specimens from individuals with acute diarrhea. Salmonellae were isolated from 15.9%; shigellae, 5.3%; and paracolons, 26.6%. The most frequently isolated enteropathogen was *S. paratyphi B* followed by *S. derby*, *Sh. flexneri*

3 and Sh. flexneri 2. Enteropathogenic E. coli were recovered from 7.8% of the stool specimens from children under 6 years of age. Antibiotic susceptibilities for more than 400 isolates of enteric pathogens, to 6 antibiotics, were determined using a tube dilution technique. All isolates were uniformly susceptible, in vitro, to colistin and naladixic acid with varying resistance to tetracycline, chloramphenicol, neomycin sulfate and kanamycin sulfate. A survey being conducted in a Special Forces unit in Thailand indicates diarrhea not to be a major cause of morbidity in this group. The average time for onset of diarrhea was 7.6 weeks after arrival in country, with a 4.5 day duration of illness. A technique for colonial recognition of enteric organisms using oblique transmitted light and a stereoscopic dissecting microscope was evaluated as a practical procedure for use in the routine enteric bacteriology laboratory. A rapid technique for determining the carbohydrate fermentation patterns of enteric pathogens was also evaluated, and found to be reliable. A combination of these two techniques is currently being evaluated. Preliminary indications are that identification of enteropathogens can be made more readily than when classical procedures are employed.