REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1995	

4. TITLE AND SUBTITLE
Epidemiology of Enterotoxigenic E. coli (ETEC) Diarrhea in Alexandria, Egypt

5. FUNDING NUMBERS
B690 00101 HIX 3421

6. AUTHOR(S)
Abu-Elyazeed, R.R., Churilla, A.M., Kay, B.A. Kay, Wierzba, T., Peruski, L., Campbell, J., Clemens, J., Rao, M., Bourgeois, A.L. and Mourad, A.S.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Naval Medical Research Unit No. 3
PSC 452, Box 5000
FPO AE 09835-0007

8. PERFORMING ORGANIZATION REPORT NUMBER
TR 1/95

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Naval Medical Research and Development
Command, National Naval Medical Center
Building 1, Tower 12
Bethesda, MD 20889-5044

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
U.S. Naval Medical Research Unit No. 3, Cairo, Egypt, November 1993 - October 1994.

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release;
Distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

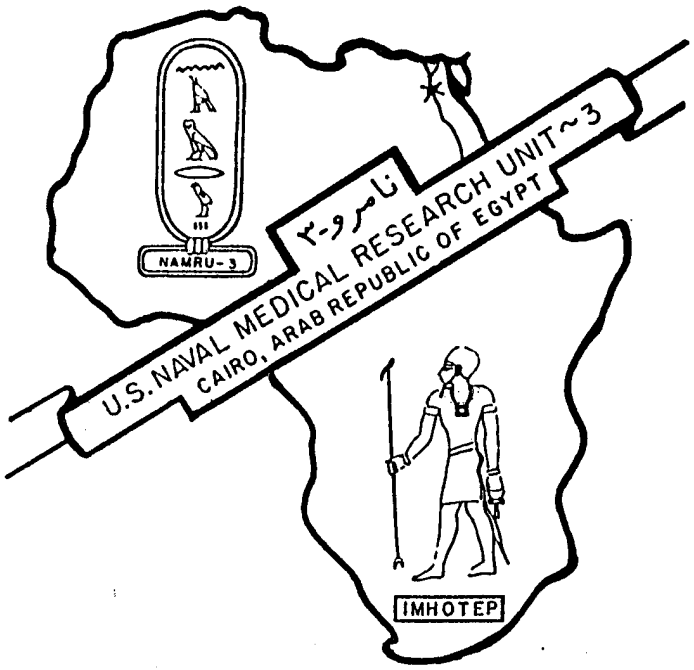
During November 1993 - October 1994, a cohort of newborns and children under the age of two years was assembled in three phases within the Abees field site. A total of 129 subjects was enrolled in the study during that period. Follow-up visits for each child occurred twice weekly and rectal swabs and stool specimens were obtained if the child had diarrheal symptoms. Rectal swabs were transported to the laboratory in Cary-Blair transport medium where they were plated on MacConkey's media, and 5 typical lactose-positive colonies were evaluated for both LT and ST using GM1 ELISA. Colonies that were toxin-positive were evaluated for CFA/I, CFA/II, CFA/III and CFA/IV by slide agglutination using monoclonal or polyclonal sera against colonization factors and their subtypes. During the fifty two weeks of the study, 9496 visits were planned and 7298 (77%) were completed. In about 6% of all completed visits, the child was reported as having loose or liquid stools. Rectal swabs were obtained from 93% of these cases. The rate of diarrhea was 5.9 episodes per year in the age group birth to three years. This rate was inversely related to age, declining from 7.9 episodes per child-year during infancy to 3.4 episodes per child-year during the third year of life. Approximately one fifth of all diarrhea was associated with excretion of ETEC. The rate of ETEC diarrhea between birth and three years was 1.1 episode per child-year and inversely related to age. About 60% of ETEC episodes were LT-/ST+. However, LT+/ST- and LT+/ST+ accounted for 30% and 30% of ETEC episodes, respectively. Twenty three of the 61 (38%) ETEC diarrheal episodes exhibited one or more of CFA I, II or IV. Thirty five of the 61 (57%) ETEC diarrheal episodes were associated with either LT, CFAI, II or IV and thus shared antigens with ET-003 vaccine. The high incidence of ETEC diarrhea in this setting will enable the conduct of ETEC vaccine trials in this area.

14. SUBJECT TERMS
Enterotoxigenic E. coli (ETEC); Epidemiology; Diarrhea; Newborns and children; Alexandria, Egypt.

15. NUMBER OF PAGES
104

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	



TECHNICAL REPORT
1/95

EPIDEMIOLOGY OF ENTEROTOXIGENIC E. COLI (ETEC) DIARRHEA IN ALEXANDRIA, EGYPT

MARCH 1995

U.S. NAVAL MEDICAL RESEARCH UNIT NO.3
(CAIRO, ARAB REPUBLIC OF EGYPT)
PSC 452, BOX 5000

PARTICIPATING INSTITUTIONS AND ORGANIZATIONS

U.S. NAVAL MEDICAL RESEARCH UNIT NO.3

Remon R. Abu-Elyazeed, MD, PhD
LCDR Albert M. Churilla, PhD
Bradford A. Kay, MS, DrPH
Thomas Wierzba, MS, MPH
LT Leonard Peruski, Jr., PhD
CDR James Campbell, PhD

NATIONAL INSTITUTE OF HEALTH

John Clemens, MD
Malla Rao, M. Engg

U.S. NAVAL MEDICAL RESEARCH INSTITUTE

CAPT August L. Bourgeois, PhD

ALEXANDRIA UNIVERSITY

Aly Saleh Mourad, MD, PhD

UNIVERSITY OF GOTEBORG

Ann-Marie Svennerholm, MD, PhD

FINANCIAL SUPPORT

This work is supported by:

1. U.S. Army Medical Research and Development Command
2. U.S. Naval Medical Research and Development Command
3. National Institute of Health
4. World Health Organization

Abstract

During November 1993 - October 1994, a cohort of newborns and children under the age of two years was assembled in three phases, corresponding to these villages (village 800, village 806 and village 807) within the Abees field site. A total of 129 subjects was enrolled in the study during that period. Follow-up visits for each child occurred twice weekly and rectal swabs and stool specimens were obtained if the child had diarrheal symptoms.

Rectal swabs were placed in Cary-Blair transport medium in the field and transported directly to the laboratory. In the laboratory, the swabs were plated on MacConkey's media, and 5 typical lactose-positive colonies were evaluated for both LT and ST using GM1 ELISA. Colonies that were toxin-positive were evaluated for CFA/I, CFA/II, CFA/III and CFA/IV by slide agglutination using monoclonal or polyclonal sera against the colonization factors and their subtypes.

During the fifty two weeks of the study, 9496 visits were planned and 7298 (77%) were completed. In about 6% of all completed visits, the child was reported as having loose or liquid stools. Rectal swabs were obtained from 93% of these cases.

The rate of diarrhea was 5.9 episodes per year in the age group birth to three years. This rate was inversely related to age, declining from 7.9 episodes per child-year during infancy to 3.4 episodes per child-year during the third year of life. Approximately one fifth of all diarrhea were associated with excretion of ETEC.

The rate of ETEC diarrhea between birth and three years was 1.1 episode per child-year and inversely related to age. About 60% of ETEC episodes were LT-/ST+. However, LT+/ST- and LT+/ST+ accounted for 30% and 30% of ETEC episodes, respectively.

Twenty three of the 61 (38%) ETEC diarrheal episodes exhibited one or more of CFA I, II or IV. Thirty five of the 61 (57%) ETEC diarrheal episodes were associated with either LT, CFA I, II or IV and thus shared antigens with the ET-003 vaccine.

The high incidence of ETEC diarrhea in this setting will enable the conduct of ETEC vaccine trials in this area.

TABLE OF CONTENTS

Abstract 3

Introduction 5

Study Objectives 7

Methods 8
Assembly and Baseline Characterization of the Cohort . . 8
Follow-up of the Cohort 9
Procedures for Obtaining Anthropometric Measurements . 10
Evaluation of Fecal Specimens 10
Evaluation of Sera 11

Definitions 11

Quality Control of ETEC results 12

Results to Date 12

Comment 14

Tables 16

References 25

Enclosures 28

Introduction

It has been estimated that among children under 5 years in the developing world nearly 70 million episodes of diarrheal disease due to ETEC occur each year, accounting for approximately 700,000 deaths (1). Although crude, these estimates highlight the high disease burden of ETEC infections in the developing world, and indicate that a vaccine that is effective against ETEC would be of great public health benefit to children in these settings. Accordingly, the development and testing of effective vaccines against ETEC diarrhea has been identified as a specific priority of the Programme for Vaccine Development of the World Health Organization.

The suitability of ETEC infection as a target for vaccination is suggested by the age-related decline both in the incidence of infection and of the ratio of symptomatic to asymptomatic infection among children in endemic areas (2), and by the diminished pathogenicity of ETEC upon reinfection after an earlier infection by an organism bearing the same colonization factor antigen (CFA) (3). This epidemiological pattern suggests that ETEC infection may induce natural immunity, and that a vaccine capable of eliciting this immunity might confer protection.

The relevance of immunity against ETEC toxin and CFAs in explaining this apparent natural protection is suggested by experimental studies, both in humans and animals, in which 1) oral administration of these virulence factors (or non-pathogenic derivatives of the factors) as vaccines led to protection against a challenge dose of virulent ETEC organisms expressing homologous factors (4-6); and 2) ingestion of preformed breastmilk antibodies against virulence factors protected recipients against an ETEC challenge (7). In aggregate, these experimental data suggest that immunogenic ETEC virulence factors may provide the basis for a vaccine that is effective against this infection.

Such extrapolation from experimental studies was confirmed in the 1985 field trial of killed oral cholera vaccines in Bangladesh (8). This trial demonstrated that a vaccine consisting of cholera toxin B subunit, which is antigenically similar to the B subunit of the heat labile enterotoxin of ETEC, conferred 67% protection against all episodes of LT-ETEC diarrhea during the three months after dosing. Notably, this protection was even greater (85%) against clinically severe LT-ETEC diarrhea, and was seen against LT+/ST+ and LT+/ST- episodes. More recently, the same vaccine was demonstrated to protect Finnish tourists from ETEC travellers' diarrhea acquired while on vacation in Morocco (9).

These data have encouraged the development of killed oral vaccines containing a more diverse array of ETEC virulence factor antigens. A prototype oral ETEC vaccine that has shown promise in recent Phase 1 and 2 human studies in Sweden consists of CT- B subunit, together with killed cells and several CFAs (10). Limited human clinical studies indicate that the vaccine is safe and immunogenic; it is reasonable to expect that this vaccine will be suitable for efficacy-testing in an endemic area for ETEC in the near future.

Critical to the testing of future ETEC vaccines is the development of field sites in which ETEC is endemic, and in which the epidemiology of ETEC is characterized in suitable detail for estimation of sample size requirements and other aspects of study design. In this regard, it is important that the occurrence of ETEC by toxin and CFA phenotype be known, since vaccine protection is expected to be homologous with respect to these virulence factors, and since the distribution of these factors is known to vary substantially in different areas (11). Also important for the evaluation of ETEC vaccines is the demarcation of an appropriate definition of "ETEC diarrhea", since excretion of ETEC does not prove its etiological role in an episode of diarrhea, and since episodes in which ETEC is not excreted are not expected to be prevented by vaccination.

Beyond definition of these epidemiological features, a more generic concern is the selection of appropriate laboratory tests for assessing immunological responses to vaccination and for providing immunological correlates of vaccine protection. Although a considerable body of evidence points to the importance of immunity to LT and CFAs in conferring natural immune protection, as described earlier, an earlier case-control evaluation of LT-ETEC infections in rural Bangladesh suggested that higher serum IgG antibody titers against LT, CFA/I, and CFA/II were not associated with a lower susceptibility to infection by ETEC expressing homologous factors (12). Serological studies are needed for the evaluation of immune status and vaccine performance in children, in whom intestinal immunity is not readily measurable and collection of large volumes of blood for measurement of cellular immunity may not be ethical. It is therefore critical to resolve current uncertainties about whether serological measures of immunity against ETEC virulence factors do in fact reflect the degree of host immunity against ETEC infections.

Rural Egypt would appear to be an ideal site for the evaluation of new ETEC vaccines. In rural Egypt, ETEC strains have been shown to be associated with ca. 80, 120, 110, and 50 diarrhea episodes per 100 child-years in the 0-0.5, 0.5-1, 1-2, and 2-3 year old age groups, respectively (13). In

addition, ETEC is one of the most frequent enteropathogens recovered from children suffering from fatal or potentially fatal diarrhea episodes in Egyptian children (14).

The present report describes the first year of progress of a study designed to address these epidemiological and immunological issues. The study is a prospective evaluation of a cohort of infants and children at high risk for ETEC infection and disease in rural Egypt. The research, which was initiated in December, 1993, is being conducted in Abees, Egypt, a rural, agricultural area (population base of approximately 20,000) located at the mouth of the Nile delta within 20 Km of Alexandria, Egypt's second largest city. Collaborating institutions for the research include the U.S. Navy, through its research unit in Cairo, Egypt (U.S. Navy Medical Research Unit No. 3: NAMRU-3), the National Institute of Child Health and Human Development (NICHD) of the U.S. National Institutes of Health, and the Faculty of Medicine, Alexandria University, Alexandria, Egypt (Departments of Microbiology and Community Medicine).

To date, the collaboration has resulted in enrollment of 131 infants and children residing in the Abees area, who are now under continuous, longitudinal surveillance for diarrhea. The children are also visited monthly for collection of surveillance fecal cultures, and at less frequent intervals for collection of blood specimens and measurement of anthropometric parameters. To support this effort, we have set up a modern enteric disease microbiology laboratory at the University of Alexandria; and we have instituted a rigorous, computerized data management system to support the field and laboratory activities. This effort has been reviewed and endorsed by the Egyptian Ministry of Health (MOH). It is noteworthy and encouraging that the MOH has also approved small-scale safety and immunogenicity studies of the recombinant B subunit- killed whole cell vaccine (rBS-WC), including studies to be undertaken in infants and young children.

Study Objectives

This project contains both overt and latent goals.

Overt Goals

The overt goals of the study are to characterize the descriptive epidemiology of pediatric ETEC diarrhea in this site, and to evaluate the analytical hypothesis that natural immune protection against enteric infection by ETEC is governed at least partly by antibodies directed against specific virulence factors, including heat-labile enterotoxin and colonization factors, and that serum antibodies to these

factors reflect this natural immunity.

Specific objectives related to these overt goals are:
1) To determine the age-specific incidence rate of ETEC diarrhea from birth to 35 months, by toxin and colonization factor (CFA) phenotype;
2) To ascertain the strength of attribution of diarrheal symptoms to fecal isolation of ETEC;
3) To evaluate the protective relationship between titers of serum IgG antibodies to ETEC toxins and CFAs and the risk of diarrhea due to ETEC manifesting these virulence factors.

Latent Goals

The latent goal of the project is to develop Abees into a field site for testing of ETEC vaccines.

Specific objectives related to this latent goal include:
1) To develop the field and laboratory infrastucture necessary for detecting and etiologically characterizing ETEC diarrhea by toxin and CFA phenotype, and for evaluating serological responses to ETEC virulence factors;
2) To provide background incidence data for estimating sample size requirements for future vaccine trials;
3) To develop a clinical case definition for ETEC having optimal specificity of etiologic attribution.

Methods

Assembly and Baseline Characterization of the Cohort

Beginning in November 1993, a cohort of newborns and children under the age of two years was assembled from villages in the Abees field area. In November the methods for the trial were pre-tested in village 800 of Abees (villages are identified by number in this study area). This village has remained under follow-up. The next phase of enrollment (Phase I), initiated in January, 1994, added children in village 806 to the study. Village 807 was added to the study in July, 1994 (Phase II), and village 801 was enrolled in December, 1994 (Phase III). Enrollment of the Phase I, II, and III villages was staggered by approximately six-month intervals in order to prevent disruption of the study by too rapid accrual of new subjects.

Just prior to assembly for each phase, study villages were mapped and houses were numbered (see maps in Appendix). A house-by-house census was then conducted over the ensuing two-weeks, followed by one week for computerization and cleaning of the census files. In the census, families were visited and characterized according to selected sociodemographic characteristics as well as to features of household hygiene, particularly those related to water use, food storage and

preparation, and defecation (see CH and CI data forms in the Appendix).

All children were evaluated for eligibility within 8-21 days of birth (for newborns) or within three weeks of the census (for non-newborns age under two years). At this time, a parent or guardian of each potential participant was approached about the study and written informed consent (see Appendix for form) for participation was solicited. The occurrence of new births was determined by two different mechanisms during the period of assembly for each phase: 1) interviews with each village leader once per week beginning one week after the census; and 2) review of the birth registration records at the local site for birth registrations once per week, beginning one week after the census. Only newborns in originally censused families were included in the study.

Eligibility criteria for these potentially eligible children were (see EN data form in Appendix): 1) if a newborn, no major congenital anomalies (e.g., cyanotic congenital heart disease), and no severe illness (e.g., respiratory distress syndrome (RDS) at the time of a physical examination by a physician (age 8-21 days); and 2) if not a newborn, no chronic illness at the time of a physical examination by a physician (within three weeks of the census).

During this first visit, eligible children were characterized according to breast-feeding status (vide infra), and anthropometric measurements (height, weight, and mid-arm circumference) were collected. In addition, structured observations were made about household hygiene and storage of food (see HY data form in Appendix).

Follow-up of the Cohort

Further follow-up visits for each child occurred twice each week. Twice weekly visits for each child continued until the child reached 36 months of age or until the end of the study period (48 months after the inception of the first phase), whichever occurred first. All children will be followed for at least 12 months. Ongoing monitoring for death and out-migration was accomplished as part of these follow-up visits.

At the twice-weekly visits (see WS data form in Appendix), interviewers inquired about diarrheal symptoms over the past four days (or since the last visit, if this came sooner) and obtained a stool specimen and rectal swabs in Cary Blair medium and in phosphate-buffered saline if such symptoms had occurred (specimens were transmitted to the laboratory along the LT form, see Appendix). If symptoms were present, further

information was obtained about the severity of the illness; oral rehydration solution was supplied, and medical referral was offered as appropriate. Information about diet, with a particular focus on breast-feeding status, was also obtained at these visits.

Once each month surveillance rectal swabs (evaluated in the same way as the fecal specimens obtained during diarrheal episodes, vide infra: see MS form in Appendix)) were collected. Every three months weight, height, and mid-arm circumference were measured (see AB form in Appendix). In May and September, 1994 blood specimens (two .1 ml fingersticks) were collected from children aged six months or older (see AB form in Appendix). This age constraint was imposed to limit bleeding to children in whom serum antibody measurements would not be obfuscated by passively derived maternal antibodies. Blood collected in these surveys was diluted 1:10 with sterile PBS in the field; the diluted specimens were immediately transported to a central laboratory and centrifuged, yielding two aliquots of serum that are stored at -20C for future testing.

At the six-monthly visits, structured observations about household hygiene were made, in the same manner as at enrollment, and recorded on the HY data form shown in the Appendix. Formal censuses of each village will be repeated at 12-month intervals after initial censuses. These repeat censuses will employ the same methods and data forms as the initial censuses.

Procedures for Obtaining Anthropometric Measurements

Weights were measured with Seca scales while the infant or child was unclothed or lightly clothed. These weights were calculated as the difference between the weight of the mother alone and the mother holding the child, and were recorded to the nearest 100 grams. Height was measured with a length board (horizontal wooden platform with a sliding foot board) if the child was younger than two years of age and a height stick if the child was two years old or more. Height was recorded to the nearest 0.1 cm. Mid-arm circumference was measured on the left upper arm with a tape measure and was recorded to the nearest mm.

Evaluation of Fecal Specimens

Rectal swabs were placed in duplicate in Cary Blair transport medium and in phosphate buffered saline in the field; stools were refrigerated and transported directly to the laboratory. In the central laboratory, the swab placed in Cary Blair media was plated on MacConkey's media, and 5 typical lactose-positive colonies were evaluated for both LT and ST using GM1

ELISA (16). Colonies that were toxin-positive were evaluated for CFA/I, CFA/II, CFA/III, CFA IV, PCF 0159:H4, and PCF 0166 by slide agglutination using monoclonal or polyclonal sera against the colonization factors and their subtypes (17,18). Additional evaluations employing conventional microbiological techniques were used to assess these specimens for Shigella, Salmonella, Campylobacter, and vibrionaceae. Rectal swabs in PBS and stool specimens were immediately placed in -20C freezers for storage and later evaluation. Evaluations of E. coli colonies for ETEC employed the ER data form (see Appendix); those for conventional pathogens employ the LR form (also in Appendix).

Beginning next year, stored frozen stool specimens (or swabs in PBS if stool is not available) from symptomatic subjects will be evaluated for rotavirus with ELISA (19). All microbiological evaluations were conducted either on-site at the University of Alexandria laboratories or in the NAMRU-3 laboratories in Cairo.

Evaluation of Sera

Rather than testing all serum specimens, a focused approach comparing pre-onset sera of ETEC cases with sera from controls will be used. Sera will be evaluated for IgG anti-LT antibodies (anti-ST antibodies do not occur in response to ST-ETEC infections) with GM1-ELISA (12). ELISA will also be used to measure IgG antibodies against purified CFA I, CFA II (CS1+CS3) and CFA IV (12). All such tests will be deferred until the field phase of the project has been completed.

Definitions

A "diarrheal day" was defined as the occurrence of at least three unformed stools (or at least one, if bloody) in a 24-hour period. For breast-fed infants, the mother must also have indicated that there had been an increase in frequency or a decrease in consistency of stools, in relation to the normal pattern of stooling. "Episodes" of diarrhea began on the first diarrheal day after at least three consecutive non-diarrheal days and ended on the first diarrheal day to be followed by at least three consecutive non-diarrheal days. "ETEC diarrhea" denoted an episode in which ETEC was isolated within 2 days of the beginning or the end of the episode. Other isolation of ETEC was termed "asymptomatic".

Definitions of breast-feeding categories (applied to the 24-hour period preceding the interview) were as follows: 1) "Exclusive breast-feeding": intake of only breast milk from mother or wet nurse, or expressed breast milk, and no other liquids or solids except drops or syrups consisting of vitamins, mineral supplements or medicines; 2) "predominant

breast-feeding": intake of predominantly breast milk but ingestion of other fluids including water, water-based drinks (sweetened and flavored water, teas, infusions), fruit juice, oral rehydration solution, drop and syrup forms of vitamins, minerals and medicines, and ritual fluids (in limited quantities; 3) "full breast feeding": "exclusive breast-feeding" and "predominant breast-feeding" together; 4) "breast-feeding": receipt of breast milk (directly from the breast or expressed); 5) "complementary feeding: receipt of both breast milk and solid (or semi-solid) food; and 6) "bottle-feeding": receipt of liquid or semi-solid food from a bottle with a nipple/teat (20).

Quality Control of ETEC results

The original plan was for the University of Alexandria to perform the ETEC testing. However, due to logistical constraints, this testing has been shifted to the Central Research Laboratory at NAMRU-3. While University of Alexandria personnel were performing the tests, the following quality control procedures were followed.

Alexandria would test all isolates for ST, LT and CFA content. NAMRU-3 would confirm all positive findings and an additional 10% of isolates negative by University of Alexandria tests results. A sampling of approximately 10% of positive strains, which included isolates for which NAMRU-3 and Alexandria had differing results, was sent to the laboratory of Professer Svennerholm, at the Department of Microbiology and Immunology, University of Goteborg, Goteborg, Sweden.

Under the original agreement, approximately 660 isolates were tested by the University of Alexandria. All 660 isolates were retested by NAMRU-3. A sampling of 68 isolates was sent to Professor Svennerholm's laboratory for confirmation of results. This included a selection of positive isolates, and all isolates with differing results between NAMRU-3 and the University of Alexandria laboratory tests.

Results to Date

Prior to the initiation of the study, three villages (800, 806, 807) were enumerated (Table 1). In all villages, 17% of children were under the age of five years. Half of all villagers were females. For those eighteen years and older, 63% had no formal education and most worked as housewives (40%), farmers/fisherman (25%) or salaried employees (14%) such as teachers, civil servants and military.

Most families (69%) lived in village houses, with the remainder living in apartments. Most homes have three to four rooms (60%) and electricity (94%) (Table 2). Families were likely

to own radios, televisions and washing machines with ownership representing 71%, 77% and 57% respectively. Nearly 100% of families received their water from the municipal water supply. Sanitary latrines were found in 45% of homes and 62% had a garbage container either in or outside of the home.

Of the 132 children who were approached for this study, all 132 were found to be eligible (Table 3). Of these, 129 or 98% were enrolled at baseline. Of these, slightly more were males (52%) and one-quarter were newborns (Table 4).

For the first fifty-two weeks of the project over 9496 visits were planned (Table 5). Of these, 7298 (77%) visits were completed. Out of all completed visits, 472 (6%) were reported as having liquid or watery stools. From these cases, we obtained a rectal swab from 93% and a stool specimen from 47%.

During the first year of the study, 1187 monthly visits were planned, of which 63% were completed (Table 6). A total of 693 rectal swabs was obtained from 58% of all planned visits. Few visits were made in weeks 9-12 or 45-48 of the study due to religious holidays and a lapse in the research contract, respectively. Six of the thirteen four week periods achieved greater than 90% of all planned visits.

The number of children eligible (>6 months of age) for a blood draw in May, September and January was 58, 90 and 156 respectively. Of these, 93% in May, 93% in September and 86% in January provided .1 ml of blood (Table 7).

Table 8 presents overall and age-specific rates of diarrhea in the study cohort followed between December, 1993 and October, 1994. In the age group, birth to three years, the overall rate of diarrhea was 5.9 episodes per year. This rate was inversely related to age, declining from 7.9 episodes per child-year during infancy to 3.4 episodes per child-year during the third year of life.

Approximately one fifth of all diarrheal episodes were associated with excretion of ETEC (Table 8), and the overall rate of ETEC diarrhea between birth and three years was 1.1 episode per child-year. Slightly more than half of the ETEC episodes were LT-/ST+, with LT+/ST-, and LT+/ST+ accounting for approximately 30% and 20% of episodes, respectively. The rates of ETEC diarrhea exceeded one episode per child-year during the first 24 months of life, and declined to .5 episode per child-year during the third year of life.

Table 9 demonstrates that 23 (38%) of the 61 ETEC diarrheal episodes exhibited one or more CFAs; the vast majority (21) had CFA I, II, or IV. 35 diarrheal episodes were associated

with ETEC isolates that exhibited either LT, CFA I, CFA II, or CFA IV, and thus shared antigens with the ET-003 vaccine. Such "shared vaccine antigens" ETEC episodes had an incidence of .6 episode per child-year in the birth-to-three year age group, and thereby accounted for 57% of all ETEC episodes detected in this age group.

Comment

It is striking that the estimates of age-specific incidence of ETEC diarrhea provided by our surveillance (Table 8) are virtually identical to those from an earlier study [13] by University of Texas investigators working in Bilbeis, a different field site also located in Lower Egypt. The consistency of these estimates not only reinforces the credibility of our findings, but also underscores the fact that ETEC diarrhea is a major public health problem in Lower Egypt. Clearly, the high rates of ETEC diarrhea in this setting will enable powerful evaluations of ETEC vaccines, with many possibilities for subgroup analyses that can examine detailed scientific and public health questions about vaccine performance.

Additional features of ETEC incidence that will facilitate the conduct of ETEC vaccine trials in Lower Egypt include a sustained high incidence of disease, extending into the third year of life, and a high proportion of ETEC diarrheal episodes due to ETEC isolates sharing antigens with the ET-003 vaccine. The former means that meaningful follow-up of participants in a trial can extend to 36 months of age, with the result that a child receiving a placebo at 9 months of age can be expected to experience on the order of two episodes of ETEC diarrhea before terminating follow-up at 36 months. The latter implies that a high fraction of all ETEC episodes observed in this setting can be expected to be prevented by the immunity elicited by ET-003 vaccine.

The high incidence of ETEC diarrhea seen in the Abees cohort is particularly striking in view of two features that probably led to underestimation of this disease outcome in our surveillance. First, only 77% of scheduled visits were completed, primarily due to absenteeism of respondents. Strategies to increase this rate will undoubtedly elevate ETEC diarrheal incidence. Second, much of the testing of fecal E. coli colonies for toxins and CFAs occurred many months after the diarrheal episode. It is known that ETEC toxins and CFAs are located on plasmids, and that stored E. coli isolates tend to lose plasmids over time. Thus, a more rapid strategy for testing of fecal E. coli isolates can be expected to further elevate the observed incidence of ETEC diarrhea, and of ETEC diarrhea associated with isolates sharing vaccine antigens.

Also encouraging was the extremely high rate of compliance with collection of fingerstick blood specimens during the three serological surveys (Table 7). This will greatly facilitate the analysis of seroimmunological correlates of natural protection against ETEC diarrhea, and also augurs well for the blood collection activities that will inevitably be required in the Phase II and Phase III testing of ET-003.

It was somewhat disappointing, though not unexpected, that rates of collection of stool were substantially lower than rates of collection of rectal swabs, both in the weekly (Table 5) and in the monthly (Table 6) surveillance. Whereas rectal swabs can be collected at the discretion of the field team at the time of a home visit, collection of stool requires considerable cooperation by respondents and substantially more field work, since children are frequently unable to produce stools during home visits. Fortunately, rectal swabs are perfectly adequate for the evaluation of bacterial enteropathogens, and it is only for viral and parasitic agents that stools are needed. Should the latter become foci of future research evaluations, more effective (and innovative) field strategies for stool collection will be required.

Finally, it is noteworthy that ETEC diarrhea has a high incidence in Abees, despite ready availability of potable water and the relative affluence of this field site. This observation could mean that economic development <u>per se</u> may not be sufficient to effect a major decline in ETEC incidence, and that an effective ETEC vaccine will be particularly important for interventions to control ETEC in this part of Egypt.

Table 1

Demographic characteristics of sample population by village for Abees, Egypt, January 1993

Characteristics	Villages 800 n (%)	806 n (%)	807 n (%)	All n (%)
Years of age				
0 - 1	37 (5)	59 (8)	44 (6)	140 (6)
2 - 5	68 (9)	80 (11)	109 (15)	257 (11)
6 - 12	139 (18)	160 (21)	171 (23)	470 (21)
13 - 17	119 (15)	93 (12)	71 (10)	283 (12)
18 - 35	248 (31)	234 (31)	213 (29)	695 (30)
>35	177 (22)	130 (17)	132 (18)	439 (19)
Gender				
Male	399 (51)	371 (49)	371 (50)	1141 (50)
Female	389 (49)	385 (51)	371 (50)	1143 (50)
Years of School[a]				
0[b]	159 (38)	244 (69)	297 (87)	700 (63)
1 - 5	49 (12)	38 (11)	22 (7)	109 (10)
5 - 10	80 (19)	48 (14)	12 (4)	140 (13)
>10	130 (31)	25 (7)	10 (3)	165 (15)
Employment[a]				
Farmers/fishermen	35 (8)	127 (35)	116 (34)	278 (25)
Housewives	137 (32)	145 (40)	169 (49)	451 (40)
Laborers	34 (8)	8 (2)	15 (5)	57 (5)
Merchants	3 (1)	1 (0)	0 (0)	4 (0)
Salaried Workers[c]	110 (26)	28 (8)	16 (5)	154 (14)
Students	41 (10)	6 (2)	4 (1)	51 (5)
Others	10 (2)	13 (4)	16 (5)	39 (3)
Not working	55 (13)	36 (10)	6 (2)	97 (9)
Marital Status[a]				
Married	256 (60)	266 (73)	258 (77)	780 (69)
Widow/divorced	32 (8)	21 (6)	28 (8)	81 (7)
Never married	137 (32)	77 (21)	51 (15)	265 (24)

a) for age > 17 years
b) includes persons who can not read and write
c) includes teacher, driver, civil servant, health worker, army, etc.

Table 2

Household characteristics of sample population by village Abees, Egypt

Characteristics	Villages 800 n (%)	806 n (%)	807 n (%)	All n (%)
Type of House				
Village house	58 (55)	62 (71)	75 (83)	195 (69)
Apartment	48 (45)	26 (29)	15 (17)	89 (31)
Rooms per house				
1 - 2	15 (14)	17 (19)	29 (32)	61 (22)
3 - 4	64 (60)	54 (61)	51 (57)	169 (60)
>4	27 (26)	17 (19)	10 (11)	54 (19)
Electricity				
Yes	105 (99)	82 (93)	79 (88)	266 (94)
No	1 (1)	6 (7)	11 (11)	18 (6)
Household possessions[a]				
Radio	86 (81)	62 (71)	53 (60)	201 (71)
Television	93 (88)	67 (76)	58 (64)	218 (77)
Washing Machine	84 (79)	47 (53)	32 (36)	163 (57)
Bicycle	9 (9)	8 (9)	2 (2)	19 (7)
Car/truck	7 (7)	6 (7)	5 (6)	18 (6)
Potable water source				
Municipal	104 (98)	87 (99)	90 (100)	281 (99)
Other	2 (2)	1 (1)	53 (0)	3 (1)
Sanitary Latrine[b]				
Yes	37 (35)	78 (89)	76 (84)	129 (45)
No	69 (65)	10 (11)	14 (16)	155 (55)
Garbage Container[c]				
Yes	84 (79)	56 (64)	37 (41)	177 (62)
No	22 (21)	32 (36)	53 (59)	107 (38)

a) number responding yes to each item
b) sanitary latrine:latrine with disposal of waste into sealed pit or municipal sewage system
c) in or outside of home

Table 3

Number of children approached, number eligible and number enrolled in cohort by village

	Villages			
Characteristics	800	806	807	All
Approached	37	59	36	132
Eligible	37	59	36	132
Enrolled	37	59	33	129

Table 4

Sex and age at enrollment by village

Characteristics	Villages 800 n (%)	806 n (%)	807 n (%)	All n (%)
Gender				
Male	25 (68)	28 (47)	14 (42)	67 (52)
Female	12 (32)	31 (53)	19 (53)	62 (48)
Age at enrollment in months				
Newborns	10 (27)	19 (32)	3 (9)	32 (25)
2 to 12	16 (43)	29 (49)	20 (61)	65 (50)
13 to 24	11 (30)	11 (19)	10 (30)	32 (25)

Table 5

Percent success for completing child visits and obtaining rectal swabs and stool specimens during weekly surveillance

Study Weeks	Visits Planned	Completed Visits n (%)	Reported Cases[a] n (%)	Rectal Swabs Taken n (%)[b]	Stools Specimens n (%)[b]
1-4	216	189 (88)	25 (13)	21 (84)	19 (90)
5-8	592	412 (70)	25 (6)	25 (100)	16 (64)
9-12	600	515 (86)	31 (6)	30 (97)	14 (47)
13-16	616	514 (83)	18 (4)	16 (89)	6 (38)
17-20	624	517 (83)	17 (3)	17 (100)	9 (53)
21-24	648	547 (84)	33 (6)	32 (97)	17 (53)
25-28	648	468 (72)	36 (8)	28 (78)	20 (71)
29-32	672	584 (87)	61 (10)	51 (84)	24 (47)
33-36	904	718 (79)	70 (10)	63 (90)	21 (33)
37-40	920	779 (85)	61 (8)	61 (100)	33 (54)
41-44	1000	880 (88)	57 (6)	56 (98)	24 (43)
45-48	1024	518 (51)	21 (4)	21 (100)	10 (48)
49-52	1032	657 (64)	17 (3)	17 (100)	7 (41)
Total	9496	7298 (77)	472 (6)	438 (93)	220 (47)

a) reported cases of liquid or loose stools
b) swabs or stools taken / reported cases * 100

Table 6

Percent success for completing child visits and obtaining rectal swabs during monthly surveillance

Study Weeks	Number Enrolled	Number of Surveillance Visits Planned	Completed Visits n (%)[a]	Number of Rectal Swabs Taken n (%)[b]
1-4	27	27	24 (89)	22 (81)
5-8	47	74	67 (91)	65 (88)
9-12	1	75	0 (0)	0 (00)
13-16	2	77	65 (84)	64 (83)
17-20	1	78	68 (87)	67 (86)
21-24	3	81	71 (88)	70 (86)
25-28	0	81	29 (36)	29 (36)
29-32	3	84	43 (51)	43 (51)
33-36	29	113	72 (65)	70 (62)
37-40	2	115	103 (90)	103 (90)
41-44	10	125	107 (86)	107 (86)
45-48	3	128	0 (0)	0 (00)
49-52	1	129	53 (41)	53 (41)
Total	129	1187	702 (59)	693 (58)

a) visits made / visits planned * 100
b) swabs taken / visits planned * 100

Table 7

Success of three blood draws for children six months and older in Abees

	Blood Collection Dates		
Collection status	May '94 n (%)	September '94 n (%)	January '95 n (%)
Refused	4 (7)	6 (7)	22 (14)
Given	54 (93)	84 (93)	134 (86)
Total	58 (100)	90 (100)	156 (100)

Table 8

Incidence of Diarrhea in Infants and Children Followed in the Abees Cohort Study, December, 1993 - October, 1994

Age at Follow-up	All Diarrhea	ETEC Diarrhea			
		LT	ST	LT/ST	All ETEC
0-11 mos	188* (7.9)	10 (0.4)	16 (0.7)	6 (0.3)	32 (1.3)
12-23 mos	106 (4.6)	7 (0.3)	13 (0.6)	5 (0.2)	25 (1.1)
24-35 mos	26 (3.4)	0 (0.0)	3 (0.4)	1 (0.1)	4 (0.5)
Total	320 (5.9)	17 (0.3)	32 (0.6)	12 (0.2)	61 (1.1)

* Episodes (incidence per child-year) for cited age range.

Table 9

Incidence of ETEC Diarrhea, by CFA Phenotype, in Infants and Children Followed in the Abees Cohort Study, December, 1993 - October, 1994

Age at Follow-up	CFA Phenotype[+]					Shared Vaccine Antigens[***]
	I	II	IV	Mixed	Other[*]	
0-11	1[**](0.1)	4 (0.2)	6 (0.3)	1 (0.1)	1 (0.1)	21 (0.9)
12-23 mos	2 (0.1)	0 (0.0)	4 (0.2)	1 (0.1)	1 (0.1)	12 (0.5)
24-35 mos	1 (0.1)	1 (0.1)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.3)
Total	4 (0.1)	5 (0.1)	10 (0.2)	2 (0.1)	2 (0.1)	35 (0.6)

[+]CFA I = Only CFA I; CFA II = Only CFA II; CFA IV = Only CFA IV; "Mixed" = a combination of CFAs including I, II, or IV.

[*]"Other" denotes a CFA other than I, II, or IV (e.g., PCF0159, PCF0166).

[**]Episodes (incidence per child-year) for cited age range.

[***]"Shared vaccine antigens" denotes ETEC diarrhea episodes associated with expression of LT or of CFAs I, II or IV.

References

1. Institute of Medicine. New Vaccine Development: Establishing Priorities (Vol II). Washington: National Academy Press. 1986: 178-85.

2. Black RE, Merson MH, Huq I, Alim AR, Yunus M. Incidence and severity of rotavirus and Escherichia coli diarrhoea in rural Bangladesh: implications for vaccine development. Lancet. 1981; 1: 141-3.

3. Cravioto A, Reyes R, Trujillo F, Uribe F, Navarro A, De La Roca J, Hernandez J, Perez G, Vazquez V. Risk of diarrhea during the first year of life associated with initial and subsequent colonization by specific enteropathogens. Am J Epidemiol. 1990; 131: 886-904.

4. Levine MM, Black RE, Clements ML, et. al. Volunteer studies in development of vaccines against cholera and enterotoxigenic Escherichia coli: a review. In: Holme T, Holmgren J, Merson M, Mollby R (eds.) Acute Enteric Infections in Children: New Prospects for Treatment and Prevention. New York; Elsevier. 1981: 443-59.

5. Ahren CM and Svennerholm A.-M. Synergistic protective effect of antibodies against Escherichia coli enterotoxin and colonization factor antigens. Infect Immun 1982; 38: 74-9.

6. Klipstein FA, Engert RF, Clements JD. Protection in rats immunized with Escherichia coli heat stable enterotoxin. Infect Immun 1981; 34: 637-9.

7. Tacket CO, Losonsky G, Link H, Levine M. Protection by milk immunoglobulin concentrate against oral challenge with enterotoxigenic Escherichia coli. N Engl J Med. 1988; 318: 1240-3.

8. Clemens J, Sack D, Harris J, Chakraborty J, Neogy P, Stanton B, Huda N, Khan M, Kay B, Khan M, Ansaruzzaman M, Yunus M, Rao M, Svennerholm A-M, Holmgren J. Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic Escherichia coli: Results of a large-scale field trial. J Infect Dis. 1988; 158: 372-7.

9. Peltola H, Siitonen A, Kyronseppa H, Mattila L, Oksanen P, Kataja M, Cadoz M. Prevention of travellers' diarrhoea by oral B-subunit/whole-cell cholera vaccine. Lancet. 1991; 338: 1285-9.

10. Svennerholm A-M, Holmgren D, Sack D. Development of oral vaccines against enterotoxigenic Escherichia coli diarrhoea. Vaccine. 1989; 7: 196-8.

11. McConnell MM and Rowe B. Colonization factor antigens of enterotoxigenic Escherichia coli in relation to immunity and vaccine development. Development of Vaccines against Cholera and Diarrhoea due to Enterotoxigenic Escherichia coli. World Health Organization Meeting. Baltimore, December, 1988.

12. Clemens J, Svennerholm A-M, Harris JR, et. al. Seroepidemiological evaluation of anti-toxic and anti-colonization factor immunity against infections by LT-producing Escherichia coli in rural Bangladesh. J Infect Dis. 1990; 162: 448-53.

13. Zaki A, DuPont H, el Alamy M, et al. The detection of enteropathogens in acute diarrhea in a family cohort population in rural Egypt. Am J Trop Med Hyg. 1986; 35: 1013-22.

14. Shukry S, Zaki A, DuPont H, Shoukry I, El Tagi M, Hamed Z. Detection of enteropathogens in fatal and potentially fatal diarrhea in Cairo, Egypt. J Clin Micro. 1986; 24: 959-62.

15. National Center for Health Statistics. NCHS growth curves for children birth-18 years, United States. Washington, DC: National Center for Health Statistics, US Department of Health, Education, and Welfare, 1977 (Vital and health statistics, Series 2; DHEW publication no. 78-1650).

16. Echeverria P, Seriwatana J, Sethabutr O, Chatkaeomorakot A. Detection of diarrheogenic Escherichia coli using nucleotide probes. In: Macario A, Conway de Macario E (eds). Gene Probes for Bacteria. New York: Academic Press, 1990: 95-141.

17. Lopez-Vidal Y, Klemm P, Svennerholm A-M. Monoclonal antibodies against different epitopes on colonization factor antigen I of enterotoxin-producing Escherichia coli. J. Clin Microbiol 1988; 26: 1967-72.

18. Gothefors L, Ahren C, Stoll B, et. al. Presence of colonization factor antigens in fresh isolates of fecal Esherichia coli: a prospective study. J Infect Dis 1985; 152: 1128-32.

19. Ward R, Clemens J, Knowlton D, Rao M, van Loon F, Huda N, Ahmed F, Schiff G, Sack D. Evidence that protection against rotavirus diarrhea after natural infection is not dependent on serotype-specific neutralizing antibody. J Infect Dis. 1992; 166: 1251-7.

20. Division of Diarrheal and Acute Respiratory Disease Control. Indicators for assessing breast-feeding practices. Report of an Informal Meeting. Geneva, 11-12 June, 1991.

21. Greenland S and Thomas D. On the need for the rare disease assumption in case-control studies. Am J Epidemiol 1982; 116: 547-53.

22. Kleinbaum D, Kupper L, Morgenstern H. Epidemiological Research: Principles and Quantitative Methods. Belmont,Ca.: Lifetime Learning Publications, 1982.

23. Breslow NE and Day NE. Statistical Methods in Cancer Research. Volume II. The Design and Analysis of Cohort Studies. IARC Scientific Publications No. 82. International Agency for Research on Cancer, Lyon, 1987.

Enclosures

I. LIST OF DATA FORMS

Enclosure 1. CH - Census Households Form

This form contains information about the house's condition, the type of house, number of rooms, presence or absence of electricity, water supply, and other housing and environmental conditions.

Enclosure 2. CI - Census Individual Form

This form includes information about the subjects resident in the house, i.e. name, sex, relation to the head of household, age, marital status, level of education, occupation, and more detailed information about children less than 24 months of age.

Enclosure 3. EN - Enrollment Form

To be used by the physician in the field to enroll children in the study.

NB - Newborn Form

This form is identical to the EN Form, except that the header (identification information) is filled out in the field. In the EN Form, the header comes from pre-printed labels.

Enclosure 4. WS - Weekly Surveillance Form

To be used by the field workers during the twice-weekly visits. It contains information about the child's diet and any symptoms, with an emphasis on diarrheal symptoms.

Enclosure 5. MS - Monthly Surveillance Form

To be used during the monthly visit. It contains information about the child's diet, vaccination status, and any diarrheal symptoms, and recent antibiotic treatment.

Enclosure 6. AB - Blood Collection and Storage Form

This form contains information on whether a blood sample was obtained and on the location of the frozen serum from this sample.

Enclosure 7. Anthropometry Data Form

This form is a list of the children who need anthropometric measurements taken during that week and their personal identification information. Anthropometric data are filled in on this form.

Enclosure 8. HY - Hygiene Form

This form contains detailed information about housing and environmental conditions.

Enclosure 9. LT - Laboratory Transmittal Form

The left half of this form is used in the field to indicate which specimens have been collected and to identify the child from whom they were collected. The right half of this form is used in the laboratory to indicate which specimens have been received by the laboratory and their storage location.

Enclosure 10. LR - Microbiology Laboratory Results Form

The bottom half of this form is used by the microbiology laboratory during the work-up of the specimen. The results of the identification are recorded on the upper half of the form.

Enclosure 11. ER - ETEC Results Form

This form is used to record the results of LT/ST toxin testing and CFA testing of *E. coli* isolates from each patient as well as the storage location of each isolate.

II. CONSENT FORM

Enclosure 12. Consent Form

III. MANUAL OF STANDARD OPERATING PROCEDURES

Enclosure 13. Manual of Standard Operating Procedures for the Field

Enclosure 14. Manual of Standard Operating Procedures for the Laboratory

IV. MAPS OF STUDY VILLAGES

Enclosure 15. Abees 800

Enclosure 16. Abees 806

Enclosure 17. Abees 807

No Label

Form: CH._ (A,B,C,D) Serial #:_ _ _ _ _ _ Date: _ _/_ _/_ _
d d m m y y

Village:_ _ _
(800-850)

HHld # _ _ _ Name HHld _____ Religion ___
A=Muslim
B=Christian
C=Other

1. What is the type of this house?
   A. Village house
   B. Flat
   C. Small independent house
   D. Other

2. Is the house or flat owned or rented?
   A. Owned exclusively
   B. Part ownership
   C. Rented

3. What is main composition of the walls of the dwelling?
   A. Cement
   B. Red bricks
   C. White bricks
   D. Wooden planks
   E. Mud bricks
   F. Tin
   G. Sticks
   H. Other, specify

   _____

4. What is the main composition of the roof of the dwelling?
   A. Cement
   B. Wood
   C. Wood and mud
   D. Tin
   E. Other, specify

   _____

5. What is the main composition of the floor of the dwelling?
   A. Ceramic tiles
   B. Wood
   C. Cement tiles
   D. Earth and sand
   E. Other, specify

   _____

6. What is the number of rooms in the house, excluding toilets and unattached kitchens? _ _

ENCLOSURE(1)

7. What is the number of rooms used for sleeping in the house? _ _

8. Does the house have electricity? Y. Yes
N. No

9. Is there a container used for garbage inside the house? Y. Yes
N. No

10. Is there a container used for garbage outside the house? Y. Yes
N. No

11. What is the main source of drinking water for the household?
A. Municipal
B. Water truck
C. Tubewell
D. Open well
E. Canal
F. Other, specify

_____

12. Please indicate other sources of water used for drinking:

a. Municipal	Y. Yes	N. No	Z. Unc
b. Water truck	Y. Yes	N. No	Z. Unc
c. Tubewell	Y. Yes	N. No	Z. Unc
d. Open well	Y. Yes	N. No	Z. Unc
e. Canal	Y. Yes	N. No	Z. Unc

13. What is the main source of cooking water for the household?
A. Municipal
B. Water truck
C. Tubewell
D. Open well
E. Canal
F. Other, specify

_____

14. Please indicate other sources of water used for cooking:

a. Municipal	Y. Yes	N. No	Z. Unc
b. Water truck	Y. Yes	N. No	Z. Unc
c. Tubewell	Y. Yes	N. No	Z. Unc
d. Open well	Y. Yes	N. No	Z. Unc
e. Canal	Y. Yes	N. No	Z. Unc

15. What is the main source of washing water for the household?

A. Municipal
B. Water truck
C. Tubewell
D. Open well
E. Canal
F. Other, specify

_____

16. Please indicate other sources of water used for washing:

a. Municipal	Y. Yes	N. No	Z. Unc
b. Water truck	Y. Yes	N. No	Z. Unc
c. Tubewell	Y. Yes	N. No	Z. Unc
d. Open well	Y. Yes	N. No	Z. Unc
e. Canal	Y. Yes	N. No	Z. Unc

17. What is the main source of bathing water for the household?

A. Municipal
B. Water truck
C. Tubewell
D. Open well
E. Canal
F. Other, specify

_____

18. Please indicate other sources of water used for bathing:

a. Municipal	Y. Yes	N. No	Z. Unc
b. Water truck	Y. Yes	N. No	Z. Unc
c. Tubewell	Y. Yes	N. No	Z. Unc
d. Open well	Y. Yes	N. No	Z. Unc
e. Canal	Y. Yes	N. No	Z. Unc

19. How many kitchens are used by the household? _

20. Is the use of these kitchens exclusively by your household, or shared with another household?

A. All exclusive
B. Some exclusive, some shared
C. All shared
X. Not applicable

21. How many latrines are used by members of your household? _

The following three questions pertain to the latrine most commmonly used by household members.

22. Please give the location of the latrine.
   A. In house or flat
   B. Outside the house or flat, but inside the compound.
   C. Outside of the compound.
   X. Not applicable.

23. What type latrine is it?
   A. Modern toilet.
   B. Local with flush.
   C. Local without flush.
   D. Pit.
   E. Other, specify _____
   X. Not applicable.

24. What type of drainage does the latrine have?
   A. Municipal sewage.
   B. Sealed pit.
   C. Unsealed pit.
   D. Drains to environment.
   E. Other, specify _____
   X. Not applicable.

The following three questions pertain to the latrine next most commmonly used by household members.

25. Please give the location of the latrine.
   A. In house or flat
   B. Outside the house or flat, but inside the compound.
   C. Outside of the compound.
   X. Not applicable.

26. What type latrine is it?
   A. Modern toilet.
   B. Local with flush.
   C. Local without flush.
   D. Pit.
   E. Other, specify _____
   X. Not applicable.

27. What type of drainage does the latrine have?
   A. Municipal sewage.
   B. Sealed pit.
   C. Unsealed pit.
   D. Drains to environment.
   E. Other, specify _____
   X. Not applicable.

28. Please indicate whether each of the following items are owned by the household:

a. Radio	Y. Yes	N. No	Z. Unc
b. TV (Black and white)	Y. Yes	N. No	Z. Unc
c. TV (Color)	Y. Yes	N. No	Z. Unc
d. VCR (video casette recorder)	Y. Yes	N. No	Z. Unc
e. Fan	Y. Yes	N. No	Z. Unc
f. Refrigerator	Y. Yes	N. No	Z. Unc
g. Washing machine	Y. Yes	N. No	Z. Unc
h. Sewing machine	Y. Yes	N. No	Z. Unc
i. Bicycle	Y. Yes	N. No	Z. Unc
j. Motorcycle	Y. Yes	N. No	Z. Unc
k. Water heater	Y. Yes	N. No	Z. Unc
l. Oven	Y. Yes	N. No	Z. Unc
m. Car or taxi	Y. Yes	N. No	Z. Unc
n. Truck or trailer	Y. Yes	N. No	Z. Unc
o. Tractor	Y. Yes	N. No	Z. Unc
p. Farmland	Y. Yes	N. No	Z. Unc
q. Land for building	Y. Yes	N. No	Z. Unc
r. Shop or factory	Y. Yes	N. No	Z. Unc
s. Another home	Y. Yes	N. No	Z. Unc

29. Please indicate <u>the number</u> of each of the following animals that are owned by the household:

a. Chickens	_ _
b. Ducks	_ _
c. Pigeons	_ _
d. Rabbits	_ _
e. Sheep & Goats	_ _
g. Cows	_ _
h. Buffalo	_ _
i. Camels	_ _
j. Donkeys	_ _
k. Horses	_ _

30. Initials of first, middle, and last names of data collector _ _ _

31. Initials of first, middle, and last names of reviewer _ _ _

32. Initials of first, middle, and last names of first data entry technician _ _ _

33. Initials of first, middle, and last names of second data entry technician _ _ _

No label

Header: CI

Date of visit: _ _/ _ _/ _ _
d d m m y y

Form serial number: _ _ _ _ _ _

Village number: _ _ _ House number: _ _ _

Name of Head of Household: _____

Individual information (for each household member)

1. Name: _____

2. Sex: M. Male F. Female

3. Relation to head of household:
A= HH Head
B= Spouse
C= Father
D= Mother
E= Son
F= Daughter
G= Sister
H= Brother
I= Grandson
J= Granddaughter
K= Sister-in-law
L= Brother-in-law
M= Nephew
N= Niece
O= Other, specify:

_____

4. Nuclear family number (begin with head of household's): _ _ +

5. Date of birth for subjects <36 mos: _ _/ _ _/ _ _
(99 = unk) d d m m y y

6. Source of date of birth for subjects < 36 mos:
A. From Record
B. Not from record

7. Age in yrs for subjects ≥3 yrs: - - -

8. Marital status:
A. Currently married
B. Engaged
C. Widowed
D. Divorced
E. Never married

ENCLOSURE(a2)

9. For married women, husband's individual code: - -
(XX= Husband does not reside in household)

10. Able to read? Y. Yes N. No Z. Unc

11. Able to write? Y. Yes N. No Z. Unc

12. Level of education:
A. No schooling
B. Some elementary (<5 completed yrs)
C. Finished elementary (5-7 completed yrs)
D. Finished preparatory (8-10 completed yrs)
E. Finished secondary (11 completed yrs), but no university
F. At least some university
Z. Unc

13. Occupation:
A. Manual laborer
B. Housewife
C. Student
D. Merchant/butcher
E. Teacher
F. Farmer
G. Fisherman
H. Driver
I. Army/police
J. Civil servant
K. Baker
L. Pension
M. Religious work
N. Health worker(not physician)
O. Garbage collector
P. Professional
R. Other, specify:

_____

S. None

Answer the following 6 questions for children <24 mos of age:

14. Who is the child's caretaker?
A. Natural mother
B. Other

15. Individual number of the child's caretaker: _ _

16. Individual number of the child's father (XX= Not in household): _ _

17. Was the child born as one of multiple births?

A: No single
B: Yes, twin
C: Yes, triplet (or more)

18. If the child was born as one of multiple births, give the individual numbers of the other births who are are still alive (XX= Not applicable):

_ _
_ _

_ _

Answer the following 5 questions for females >15 yrs who have given birth to at least one child:

19. Give the number of births who are still alive today: _ _

20. Give the number that were born alive: _ _

21. Give the number who were born alive but who died during the first year of life: _ _

22. Give the number who died between one and five years of age: _ _

23. Give the number who died after five years of age: _ _

(Note: 1+3+4+5=2)

Answer the next 2 questions for women >15 and <45 years of age

24. Is the person currently pregnant _ (Y/N/Z)

25. If the persom is pregnant, what is the first day of her LMP

_ _/ _ _/ _ _
dd mm yy

+ Nuclear family refers to the hierarchy of:
1) parents and their children under 15 yrs of age
2) all other married couples (each assigned a number)
3) all other individuals (each assigned a number)

Form: EN._ (A,B,C,D)

Serial #: _ _ _ _ _ _

Preprinted Label:

Study: EN Week: E

Village #: _ _ _

House #: _ _ _

Child name:_____

Child sex: M. Male
F. Female

Child's age in mos: _ _

Child registration # (RID): _ _ _ _ _ _

Caretaker name: _____

Caretaker RID: _ _ _ _ _ _

To be entered by data collector:

1. Date of visit: $\underline{d}\ \underline{d}/\ \underline{m}\ \underline{m}/\ \underline{y}\ \underline{y}$

2. Date of birth: from birth certificate or father's I.D. $\underline{d}\ \underline{d}/\ \underline{m}\ \underline{m}/\ \underline{y}\ \underline{y}$ (ZZ= unc)

3. Was the child born before the expected date of delivery?
   Y. Yes
   N. No
   Z. Uncertain

4. If born premature, how many weeks early?
   ($\underline{Z}\underline{Z}$= unc;
   XX= not applicable)

5. Where was the child born?
   A. Parents' home
   B. Another home
   C. Hospital
   D. Clinic
   E. Other

ENCLOSURE(3)

6. Were there problems during labor or delivery?
   - Y. Yes
   - N. No
   - Z. Uncertain

   If yes or uncertain, specify: _____

7. Does the child suffer from any chronic (lasting >1 month) illnesses?
   - Y. Yes
   - N. No
   - Z. Uncertain

   If yes or uncertain, specify: _____

8. Was the child born with any congenital anomalies?
   - Y. Yes
   - N. No
   - Z. Uncertain

   If yes or uncertain, specify: _____

9. Was the child born as one of multiple births?
   - A. No single
   - B. Yes, twin
   - C. Yes, triplet (or more)

10. During the first week of life, was the child fed the mother's colostrum?
    - Y. Yes
    - N. No
    - Z. Uncertain

11. If fed colostrum (#10=Y), what portion of the mother's colostrum was given?
    - A. All
    - B. Some
    - Z. Colostrum given, but amount uncertain
    - X. Not applicable

12. Was the child fed sugar water during the first week of life?
    - Y. Yes
    - N. No
    - Z. Uncertain

13. Was the child ever breast-fed?
    - Y. Yes
    - N. No
    - Z. Uncertain

14. How old was the child when breast-feeding was initiated?
    __ __ days
    (00= day of birth;
    ZZ= uncertain;
    XX= not applicable)

15. Is the child still breast-fed?
    - Y. Yes
    - N. No
    - Z. Uncertain

16. If Q#15=N, what was the age of the child when breast feeding stopped?

__ __ months
(00= first month of life;
ZZ= uncertain;
XX= not applicable)

17. Does the child take water or water-based fluids?

Y. Yes
N. No
Z. Uncertain

18. If yes (Q#17=Y), at what age did this begin?

__ __ months
(00= first month of life;
ZZ= uncertain;
XX= not applicable)

19. Does the child take animal milk (natural or reconstituted)?

Y. Yes
N. No
Z. Uncertain

20. If yes (Q#19=Y), at what age did this begin?

__ __ months
(00= first month of life;
ZZ= uncertain;
XX= not applicable)

21. Does the child take formula?

Y. Yes
N. No
Z. Uncertain

22. If yes (Q#21=Y), at what age did this begin?

__ __ months
(00= first month of life;
ZZ= uncertain;
XX= not applicable)

23. Does the child take other fluids not mentioned above?

Y. Yes
N. No
Z. Uncertain

24. If yes (Q#23=Y), at what age did this begin?

__ __ months
(00= first month of life;
ZZ= uncertain;
XX= not applicable)

25. Does the child take foods from any of the following categories: cereals, breads/cakes/bisquits, meats, fruits, rice, vegetables?

Y. Yes
N. No
Z. Uncertain

26. If yes (Q21=Y), at what age did this begin?

__ __ months
(00= first month of life;
ZZ= uncertain;
XX= not applicable)

27. Has the child received measles vaccine?

A. Yes, documented by certificate
B. Yes, only by recollection
C. No
Z. Uncertain

28. If measles vaccine was received (Q#27= A or B), when was it received ?

_ _/ _ _/ _ _
d d m m y y
(ZZ= uncertain; XX/XX/XX= not applicable)

29. Height (length): _ _ _. _ cm
(999.9= uncertain)

30. Weight: _ _._ kg
(99.9= uncertain)

31. Mid-arm circumference: _ _. _ cm
(99.9= uncertain)

32. Interviewer initials (first, middle, last names) _ _ _

For office use:

33. Eligible?

Y. Yes
N. No
Z. Uncertain

34. Informed consent obtained?

Y. Yes
N. No
Z. Uncertain

35. Reviewer initials (first, middle, last names) _ _ _

36. First data entry technician's initials (first,middle, last names) _ _ _

37. Second data entry technician's initials (first,middle, last names) _ _ _

Form WS._ (A,B,C,D)

Serial # _ _ _ _ _ _

Pre-printed Label:

Study: WS

Week # _ _ _ Visit # _ (1/2)

Village #: _ _ _

House #: _ _ _

Child name:_____

Child sex: M. Male
F. Female

Child's date of birth: _ _/ _ _/ _ _
d d m m y y

Child's age in mos: _ _

Child registration # (RID): _ _ _ _ _ _

Caretaker name: _____
(First/Middle/ Last)

Caretaker RID: _ _ _ _ _ _

Portion to be filled out by data collector:

1) Date of visit: _ _/ _ _/ _ _
d d m m y y

2) Informant for the interview:
A. Mother
B. Other
C. No informant (no interview conducted)

3) Is the child still an infant (<13 mos): Y. Yes
N. No

ENCLOSURE(4)

The following questions pertain to the date of the interview together with the previous 3 days:

4) If the child is still an infant, is the child still taking breastmilk?

Y. Yes
N. No
Z. Uncertain
X. Not applicable (child is not an infant)

5) If the child is still an infant, does breastmilk constitute the majority of all liquids that the child takes?

Y. Yes
N. No
Z. Uncertain
X. Not applicable

6) If the child is still an infant, does the child take non-water-based fluids?

Y. Yes
N. No
Z. Uncertain
X. Not applicable.

7) If the child is still an infant, does the child take solids?

Y. Yes
N. No
Z. Uncertain
X. Not applicable.

8) Has the child been coughing?

Y. Yes
N. No
Z. Uncertain

Vomiting:

9) Has the child been vomiting?

Y. Yes
N. No
Z. Uncertain

10) If the child has been vomiting, what was the first day during the period including today and the the past 3 days that you noticed this vomiting.

0. Today
1. Yesterday
2. 2 days ago.
3. 3 days ago.
Z. Uncertain
X. Not applicable

11) If the child has been vomiting, what was the last day during the period including today and the the past 3 days that you noticed this vomiting?

0. Today
1. Yesterday
2. 2 days ago.
3. 3 days ago.
Z. Uncertain
X. Not applicable

12) During the period including today and the past 3 days, what was the maximum number of times that the child vomited **on any single day**?

(ZZ= Uncertain, XX= Not applicable)

Convulsions:

13) Did you notice that the child had one or more general convulsions during the period including today and the past 3 days?

Y. Yes
N. No
Z. Uncertain

14) If you noticed a convulsion, was the child unarousable for at least 10 minutes after the convulsion?

Y. Yes
N. No
S. Sometimes (with some convulsions but not others)
Z. Uncertain
X. Not applicable

15) If you noticed a convulsion, did the child urinate or defecate during the convulsion?

Y. Yes
N. No
S. Sometimes (with some convulsions but not others)
Z. Uncertain
X. Not applicable

Fever:

16) Did you notice that the child had a fever?

Y. Yes
N. No
Z. Uncertain

17) If the child has had fever, what was the first day during the period including today and the the past 3 days that you noticed this fever?

0. Today
1. Yesterday
2. 2 days ago.
3. 3 days ago.
Z. Uncertain
X. Not applicable

18) If the child has had fever, what was the last day during the period including today and the the past 3 days that you noticed this fever?

0. Today
1. Yesterday
2. 2 days ago.
3. 3 days ago.
Z. Uncertain
X. Not applicable

19) If the child has had a fever during this period, did you notice that the child also had teeth-chattering chills?

Y. Yes
N. No
Z. Uncertain
X. Not applicable

Loose or liquid stools:

20) During the period including today and the past 3 days, did you notice that the child ever had liquid or watery stools?
Y. Yes
N. No
Z. Uncertain

21) During the period including today and the past 3 days, did you notice that the child ever had loose or unformed stools?
Y. Yes
N. No
Z. Uncertain

If the child has had liquid or loose stools, complete the following section; otherwise skip to question 48:

22) Give the number of loose or liquid stools between yesterday at this time and now: (ZZ= uncertain)

23) Give the number of loose or liquid stools all day yesterday: (ZZ= uncertain)

24) Give the number of loose or liquid stools 2 days ago: (ZZ= uncertain)

25) Give the number of loose or liquid stools 3 days ago: (ZZ= uncertain)

26) During this period of loose or liquid stooling, did you ever notice blood in the stool?
Y. Yes
N. No
Z. Uncertain

27) During this period of loose or liquid stooling, did you ever notice that the child appeared thirstier than usual?
Y. Yes
N. No
Z. Uncertain

28) During this period of loose or liquid stooling, did you ever notice that the child appeared less active than usual?
Y. Yes
N. No
Z. Uncertain

29) During this period of loose or liquid stooling, did you ever notice that the child was drowsy and very difficult to awaken?
Y. Yes
N. No
Z. Uncertain

30) During this period of loose or liquid stooling, did you ever notice that the child's eyes seemed sunken?
Y. Yes
N. No
Z. Uncertain

31) During this period of loose or liquid stooling, did you ever notice that the child's rectum prolapsed out of the child's anus?
Y. Yes
N. No
Z. Uncertain

32) During this period of loose or liquid stooling, did you ever notice that no tears came when the child cried?
Y. Yes
N. No
Z. Uncertain

33) During this period of loose or liquid stooling, did you ever notice that the child was so tired or drowsy that he no longer wished to drink?
Y. Yes
N. No
Z. Uncertain

34) Has the child received ORS for these loose or liquid stools?
Y. Yes
N. No
Z. Uncertain

35) Has the child visited a doctor for treatment of these loose liquid stools?
Y. Yes
N. No
Z. Uncertain

36) During this period, do you think that the child was passing loose or liquid stools **more frequently** than usual for the child?
Y. Yes
N. No
Z. Uncertain

37) During this period, do you think that the child's stools were looser or more liquid than usual for the child?
Y. Yes
N. No
Z. Uncertain

38) In your opinion, does this loose or liquid stooling of the child represent "diarrhea"?
Y. Yes
N. No
Z. Uncertain

The following should be directly observed by the data collector:

39) Does the child have sunken eyes?
Y. Yes
N. No
Z. Uncertain

40) Does the skin above the abdomen "tent" after pinching?
Y. Yes
N. No
Z. Uncertain

41) Is the anterior fontanelle depressed?
Y. Yes
N. No
Z. Uncertain
X. Not applicable (fontanelle is closed)

42) Is the mouth parched dry?
Y. Yes
N. No
Z. Uncertain

43) Strength of the radial pulse?
N. Normal
W. Weak
A. Absent
Z. Uncertain

44) Any other abnormal signs:
Y. Yes
N. No
Z. Uncertain

If yes, specify: _____

Specimens collected:

45) Rectal swab in Cary-Blair
Y. Yes **(fill in LT form)**
N. No

46) Rectal swab in PBS
Y. Yes **(fill in LT form)**
N. No

47) Stool
Y. Yes **(fill in LT form)**
N. No

48) Lab accession number
(_ _. = Study prefix)

---

49) Was the visit completed?
Y. Yes
N. No

50) If the visit was not completed, give the reason:

a) No informant	Y. Yes	N. No
b) Refused	Y. Yes	N. No
c) Child was asleep	Y. Yes	N. No
d) Child was away temporarily, but will return	Y. Yes	N. No
e) Child has moved away permanently	Y. Yes	N. No
f) Child died	Y. Yes	N. No

51) Date of outmigration or death (49 e or f= yes) _ _/ _ _/ _ _
d d m m y y

ZZ= uncertain
XX= Not Applicable

52) Revisit required for data
Y. Yes
N. No

53) Revisit required for rectal swab in Cary Blair
Y. Yes
N. No

54) Revisit required for rectal swab in PBS
Y. Yes
N. No

55) Revisit required for stool
Y. Yes
N. No

56) Is there a new birth in the house?
Y. Yes
N. No

57) Interviewer initials (first, middle, last names) _ _ _

58) Reviewer initials (first, middle, last names) _ _ _

59) First data entry technician's initials (first,middle, last names) _ _ _

60) Second data entry technician's initials (first,middle, last names) _ _ _

Form: MS._ (A,B,C,D)

Serial #: _ _ _ _ _ _

Preprinted Label:

Study: MS

Week #: _ _ _

Village #: _ _ _

HHld #: _ _ _ Individual Code _ _

Child's name: _____

Child's sex: M. Male F. Female

Child's date of birth: _ _/ _ _/ _ _
d d m m y y

Child's age: _ _ months

Child's registration number (RID): _ _ _ _ _ _

Caretaker's name: _____

Caretaker's RID: _ _ _ _ _ _

To be filled out by data collector:

1. Date of visit: _ _/ _ _/ _ _
d d m m y y

2. Informant: A. Mother
B. Other
C. No informant (no interview)

Present diet:

3. Does the child still breast-feed? Y. Yes
N. No
Z. Uncertain

4. If yes (to Q#3), does breastmilk constitute most of the child's total fluids? Y. Yes
N. No
Z. Uncertain
X. Not Applicable

ENCLOSURE(5)

5. Does the child currently drink water or water-based fluids, such as tea?
   Y. Yes
   N. No
   Z. Uncertain

6. Does the child currently drink animal milk?
   Y. Yes
   N. No
   Z. Uncertain

7. Does the child currently drink formula or Powdered Milk?
   Y. Yes
   N. No
   Z. Uncertain

8. Does the child currently drink other fluids, apart from those already mentioned?
   Y. Yes
   N. No
   Z. Uncertain

9. When child drinks fluids, milk, or formula does he/she ever use a spoon?
   Y. Yes
   N. No
   Z. Uncertain
   X. Not Applicable

10. When child drinks fluids, milk, or formula does he/she ever use a cup?
   Y. Yes
   N. No
   Z. Uncertain
   X. Not Applicable

11. When child drinks fluids, milk, or formula does he/she ever use a bottle with a nipple?
   Y. Yes
   N. No
   Z. Uncertain
   X. Not Applicable

12. Does the child take foods from any of the following categories: cereals, breads/cakes/bisquits, meats, fruits, rice, vegetables?
   Y. Yes
   N. No
   Z. Uncertain

Vaccination status:

13. During the last 3 months, has the child received measles vaccine?
   A. Yes, documented by certificate
   B. Yes, only by recollection
   C. No
   Z. Uncertain

14. If measles vaccine was received (Q#13= A or B), when was it received ?
   _ _/ _ _/ _ _
   d d m m y y
   (ZZ= uncertain; XX/XX/XX= not applicable)

15. Was any antibiotic (oral or injection) treatment given to the child in the past 3 days?
Y. Yes
N. No
Z. Uncertain

If antibiotics were given describe:
what was received _____
what was the reason for receiving it _____

16. Did the Child have loose or liquid stools in the last week?
Y. Yes
N. No
Z. Uncertain

17. If the answer to Q16. is yes then answer the following:

a. What were the maximum no. of stools on a single day? (ZZ=Uncertain)

b. Were the stools ever bloody?
Y. Yes
N. No
Z. Uncertain

c. Were the stools increased in frequency or decreased in consistency with usual?
Y. Yes
N. No
Z. Uncertain

d. Were the stools still loose or liquid on **any** day from 3 days ago till today?
Y. Yes
N. No
Z. Uncertain

If the answer to Q17 d. is Yes:
1) *Collect Rectal Swab (CB and PBS), and stool*
2) *Attach **Yellow** specimen labels to the specimens and Q19 of this form*
3) *Attach **Yellow** label to WS form for today (this form should be filled out in the usual way)*
4) *Attach **Yellow** label to LT form*

If the answer to Q17 d. is No or Uncertain:
1) *Collect Rectal Swab (CB and PBS), and stool*
2) *Attach **Blue** specimen labels to the specimens and Q19 of this form*
3) *Fill out the WS form for today (this form should be filled out in the usual way); no label is required for this form*
4) *Attach **Blue** label to LT form*

Specimens collected during this visit:

18. a. Was a rectal swab in Cary-Blair obtained? — Y. Yes / N. No

    b. Was a rectal swab in PBS obtained? — Y. Yes / N. No

    c. Was stool obtained? — Y. Yes / N. No

19. Lab accession number: Sticker here

Result of visit:

20. Was the visit completed? — Y. Yes / N. No

21. If the visit was not completed, give the reason:

a) No informant	Y. Yes	N. No	X. NA (Visit Complete)
b) Refused	Y. Yes	N. No	X. NA (Visit Complete)
c) Child was asleep	Y. Yes	N. No	X. NA (Visit Complete)
d) Child was away temporarily, but will return	Y. Yes	N. No	X. NA (Visit Complete)
e) Child has moved away permanently	Y. Yes	N. No	X. NA (Visit Complete)
f) Child died	Y. Yes	N. No	X. NA (Visit Complete)

22. Date of outmigration or death (if 21 e. or f. = Yes) — _ _/ _ _/ _ _ (d d / m m / y y)

ZZ= uncertain
XX= Not Applicable

23. Revisit required for data — Y. Yes / N. No ____

24. Revisit required for
    a. rectal swab in CB — Y. Yes N. No ____
    b. rectal swab in PBS — Y. Yes N. No ____
    c. stool — Y. Yes N. No ____

Interviewer initials (first, middle, last names) _ _ _

Reviewer initials (first, middle, last names) _ _ _

First data entry technician's initials (first,middle, last names) _ _ _

Second data entry technician's initials (first,middle, last names) _ _ _

BLOOD COLLECTION AND STORAGE FORM (AB)

TO BE FILLED OUT AT THE CLINIC

Form Name AB. _____ (A,B,C,D)

1. Form Serial Number: _ _ _ _ _ _

****
* *
* *
* *
* *
* LABEL AREA *
* *
* *
* *
* *
* *
* *
* *
****

2. Date of visit: ___/___/___
dd mm yy

3. Was a blood sample obtained ? _____

A. Yes

B. No, refused (age ≥ 6 months)

C. No, under 6 months of age

4. Lab Number (Sticker)

****
* PLACE STICKER HERE *
* *
****

5. Physician/ Nurse initials ___ ___ ___
(first, middle, last names)

TO BE COMPLETED BY THE LABORATORY:

6. Location of frozen serum

Mark X here if no serum ____

Box Number SR ___ ___ ___ ___

Aliquot No.	#1	#2
Row Letter (A-J)	____	____
Column Number (1-9, 0)	____	____

7. Lab Reviewer initials
(first, middle, last names)

___ ___ ___

8. First data entry technician's initials (first, middle, last names)

___ ___ ___

9. Second data entry technician's initials (first, middle, last names)

___ ___ ___

ENCLOSURE(6)

ANTHROPOMETRIC MEASUREMENTS LIST (AP)

NAME	AGE	CID	RID	MOTHER	HT	WT	MAC

ENCLOSURE(7)

Form HY._ (A,B,C,D) Serial _ _ _ _ _ _

Preprinted label:

Study: HY

Week # _ _ _

Village _ _ _ Household # _ _ _

Name of HHld Head _____

RID of HHld Head _ _ _ _ _ _

No. of children under F/U: _

Oldest child's name: _____

Oldest child's sex: M. Male
F. Female

Oldest child's age: _ _ months

Oldest child's RID: _ _ _ _ _ _

To be filled out by data collector:

Dt Visit _ _/_ _/_ _ (dd mm yy) Revisit1 _ _/_ _/_ _ (dd mm yy) Revisit2 _ _/_ _/_ _ (dd mm yy)

The following questions are to be answered by observation:

Q1) For the sleeping rooms (Y=Yes N=No Z=Uncertain):

____ a) Are there barriers to keep animals out of each room?

____ b) Do you see animals in any room?

____ c) Do you see any feces in any room?

____ d) Do you see any uncovered garbage in any room?

Q2) For the eating rooms (Y=Yes N=No Z=Uncertain):

____ a) Are there barriers to keep animals out of each room?

____ b) Do you see animals in any room?

____ c) Do you see any feces in any room?

____ d) Do you see any uncovered garbage in any room?

ENCLOSURE(8)

Q3) For the cooking rooms (Y=Yes N=No Z=Uncertain):

____ a) Are there barriers to keep animals out of each room?

____ b) Do you see animals in any room?

____ c) Do you see any feces in any room?

____ d) Do you see any uncovered garbage in any room?

_____ Q4 a) In the kitchen do you see previously prepared (cooked/uncooked) food for a child < 36 months outside of a refrigerator/cold box?
Y=Yes N=No Z=Uncertain

_____ b) If Q4 a)=Yes, ask: Was all of this food prepared today?
Y=Yes N=No Z=Uncertain X=Not Applicable

_____ c) If Q4 b)=No, observe whether the food prepared before today is covered?
A=Yes all such food is covered
B=Yes, some is covered
C=None is covered
X=Not Applicable

_____ Q5 a) In the kitchen do you see previously prepared (cooked/uncooked) food for older children or adults outside of a refrigerator/cold box?
Y=Yes N=No Z=Uncertain

_____ b) If Q5 a)=Yes, ask: Was all of this food prepared today?
Y=Yes N=No Z=Uncertain X=Not Applicable

_____ c) If Q5 b)=No, observe whether the food prepared before today is covered?
A=Yes all such food is covered
B=Yes, some is covered
C=None is covered
X=Not Applicable

____ Q6) Number of latrines used by the household
(if the answer is 0 skip to Q8)

Q7) For the latrines most commonly (primary) and second most commonly (secondary) used, answer the following:

Enter X for not applicable (NA)

Prim Sec.

___ ___ a) Location of latrine
A= Inside House/flat B=Inside Compound but outside house/flat C=Outside compound
X=NA

___ ___ b) Type of latrine
A=Modern B=Local with flush C=Local no flush
D=Pit E=Other Specify _____
X=NA

___ ___ c) Ask: Where does the latrine drain?
A=Municipal B=Sealed Pit C=Unsealed Pit
D=Drains to Environment E=Other Specify _____
X=NA

___ ___ d) Inside the latrines do you see any feces on the floor?
Y=Yes N=No Z=Uncertain
X=NA

___ ___ e) Are there feces within 3 steps of the outside wall of the latrine? Y=yes N=No Z=Uncertain
X=NA

____ ___ f) Inside the latrines do you see any puddling on the floor?
Y=Yes N=No Z=Uncertain
X=NA

____ ___ g) Are there puddles within 3 steps of the outside wall of the latrine? Y=yes N=No Z=Uncertain
X=NA

____ Q8) Number of containers used for storing drinking water

Q9) For the 3 largest containers cited in Q8

Enter X for Not Applicable (for #1 #2 or #3)

#1	#2	#3	
___	___	___	a) Ask: Is it used exclusively for drinking? Y=Yes N=No Z=Uncertain X=NA
___	___	___	b) Is the container covered? C=Completely P=Partly N=Not Covered X=NA
___	___	___	c) Does the container have a narrow neck? A=Yes, a small childs hand fits through B=Yes but a small childs hand cannot fit through C=Yes, but uncertain whether a hand fits through D=No X=NA
___	____	___	d) Is there a long handled dipper? Y=Yes N=No Z=Uncertain X=NA

___ ___ ___ e) If Yes, Ask: Is dipper always used when taking drinking water?
Y=Yes N=No Z=Uncertain X=NA

___ ____ ___ f) Is there a tap for the container?
Y=Yes N=No Z=Uncertain X=NA

___ ____ ___ g) If Yes, Ask: Is the tap always used when taking drinking water?
Y=Yes N=No Z=Uncertain X=NA

-- -- -- Q10) Interviewer

__ __ __ Q11) Reviewer initials (first, middle, last names)

__ __ __ Q12) First data entry technician's initials (first,middle, last names)

__ __ __ Q13) Second data entry technician's initials (first,middle, last names)

SPECIMEN TRANSMITTAL FORM (LT)

1. Study Phase: LT _____

2. Form Serial Number: _ _ _ _ _ _

3. Date of collection: ___ / ___ / ___
dd mm yy

4. Lab Number (Sticker)

****
* PLACE STICKER HERE *
* *
****

5. Serial # of Visit Form _ _ _ _ _ _

6. Name _____

7. Village Number ___ ___ ___

8. Household Number ___ ___ ___

9. Individual Code ___ ___

10. Child's Registr. # ___ ___ ___ ___ ___ ___

11. Is this a revisit? Y = Yes N = No _____

12. Source of Specimens _____

W = Weekly visit
M = Monthly visit
C = Combined W + M

13. Specimens Collected Y = Yes N = No

RS in Cary-Blair _____

RS in PBS _____

Stool (unpreserved) _____

14. If Stool = Y, when was it produced

T = Today
Y = Yesterday _____
D = Day before Yesterday
Z = Uncertain
X = No stool collected

15. Specimens to be collected on revisit
Y = Yes N = No

RS in Cary-Blair _____

RS in PBS _____

Stool _____

16. Data Collector ___ ___ ___

TO BE COMPLETED BY THE LABORATORY

17. Specimens received Y = Yes N = No

RS in Cary-Blair _____

RS in PBS _____

Stool _____

18. Date received ___ / ___ / ___
dd mm yy

19. Time received (hrs.)
00-24, uncertain = ZZ ___ ___

20. Appearance of Stool ___
A = Formed
B = Loose (takes shape of container)
C = Watery (can be poured)
X = No stool collected
Z = Uncertain

21. Blood in Stool
Y = Yes _____
N = No
X = No stool collected
Z = Uncertain

22. Location of frozen RS in PBS.

Mark X here if no RS _____

Box Number RS ___ ___ ___ ___

Row Letter (A-J) _____

Column Number (1-9, 0) _____

23. Location of frozen Stool.

Mark X here, if no stool. ___

Box Number ST ___ ___ ___ ___

Row Letter (A-J) ___

Column Number (1-9, 0) ___

24. Lab Tech handling specimens ___ ___ ___

25. Lab Reviewer initials ___ ___ ___

26. Data Entry 1 ___ ___ ___

27. Data Entry 2

ENCLOSURE(9)

Microbiology Laboratory Results Form (LR)

1. Study Phase LR _____

2. Form Serial Number ___ ___ ___ ___ ___ ___

3. Laboratory Number
****
* *
* *
****

4. Name: _____

5. Childs Regn. No. ___ ___ ___ ___ ___ ___

6. Date Tests Started: ___/___/___
dd mm yy

Sample (circle one): Rectal Swab in CB / Stool
Stool Swab in CB

Pathogens	Type code	Specify
7. E. coli-like	____	
8. Campy	____	
9. Salmonella	____	_____
10. Shigella	____	_____
11. Vibrio	____	_____
12. Other	____	_____

Type codes:

E. coli-like colonies
N = Not found
X = Not tested
Y = Found

Shigella
N = Negative for Shigella
X = Not tested
A = S. dysenteriae
B = S. flexneri
C = S. boydii
D = S. sonnei

Campylobacter
N = Negative for Campy.
X = Not tested
A = C. jejuni
B = Other Campy(specify)

Salmonella
N = Negative for Salmonella
X = Not tested
A = S. enteritidis
B = S. typhi
C = Other Salmonella (specify)

Vibrio
N = Negative for Vibrio
X = Not tested
A = V. cholerae O1
B = V. cholerae O139
C = V. cholerae non-O1
D = Vibrio species (specify)
E = Unspeciated Vibrio

Other pathogens
N = No, not found
X = Not tested
Y = Yes, found (specify)

13. E. coli-like colonies found?
Y = Yes, N = No _____

14. If yes, Number of
colonies saved (circle one) 1 2 3 4 5

15. Box Number EC ___ ___ ___ ___

16. Row = A-J Column 1-9, 0
Colony 1 Location ___ ___
Colony 2 Location ___ ___
Colony 3 Location ___ ___
Colony 4 Location ___ ___
Colony 5 Location ___ ___

17. Review
Laboratory 1 ___ ___ ___
Laboratory 2 ___ ___ ___
Data 1 ___ ___ ___
Data 2 ___ ___ ___

ENCLOSURE(10)

ETEC Results Form (ER)

TEST LOCATION __

C = Cairo, A = Alex., S = Sweden

1. Study Phase ER _____

2. Form Serial # __ __ __ __ __ __

3. Date of Test __ __/__ __/__ __ (dd mm yy)

4. Laboratory Number

5. Child's Registration No. __ __ __ __ __ __

6. CFA Test Results	Colony Number #1	#2	#3	#4	#5
P = Positive N = Negative X = Not done	_____	_____	_____	_____	_____
7. If Q6 = P Enter all CFA from list below:	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____
	_____	_____	_____	_____	_____

CFA1, CFA3, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS17, O159, O166.

8. Toxin Tests Results	Colony Number: #1	#2	#3	#4	#5
LT OD-Background	__.__ __				
LT OD-Colony	__.__ __	__.__ __	__.__ __	__.__ __	__.__ __
Interpretation*	_____	_____	_____	_____	_____
ST OD-Control	__.__ __	__.__ __	__.__ __	__.__ __	__.__ __
ST OD-Colony	__.__ __	__.__ __	__.__ __	__.__ __	__.__ __
	_____	_____	_____	_____	_____

* Interpretation:
P = Positive, N = Negative, X = Missing or not done

9. ETEC Box number: ET __ __ __ __

Location	Colony Number: #1	#2	#3	#4	#5
Row (A-J)	__	__	__	__	__
Column (1-9, 0)	__	__	__	__	__

If no colony, mark X in each space for row, and column and leave space in box empty.

10. Review Lab _____ Lab Supervisor _____

Data 1 _____ Data 2 _____

ENCLOSURE(11)

DETERMINATION OF VIRULENCE FACTORS EXPRESSED BY ENTEROTOXIGENIC *ESCHERICHIA COLI* AND *CAMPYLOBACTER JEJUNI* INFECTING HUMANS RESIDENT IN ABEES, ALEXANDRIA GOVERNORATE, EGYPT

CONSENT FORM

I understand that my child has been asked to voluntarily participate in a research study. The specific purpose of the present study is to determine what is causing individuals in my community to have diarrhea. The overall purposes of the group of studies of which this study is a part is to find better ways to prevent and treat diarrhea.

I understand that participation of my child will involve collection of information on my place of residence, age, duration of pregnancy, mode of delivery, estimated gestational age at birth, and initial diet. I understand that my household will be visited twice per week to determine if my child has developed diarrhea and if so, two rectal swabs and a stool sample will be obtained. If a cause of diarrhea is identified in this stool, additional stool samples may be collected until my child stops excreting the organism which caused the diarrhea.

I further understand that once every month, my child will be visited to complete a brief health questionnaire and that a stool sample and two rectal swabs will be collected. Rectal swabs might cause discomfort. At the time of enrollment of my child and every three months, mid-arm circumference, body length, and weight will be measured. I understand that a very small amount of blood (0.2 ml) equivalent to "a drop of blood" will be collected from my child by a prick in the finger once every four months. This finger prick might cause irritation or infection at the site of puncture.

Because most severe diarrhea occurs in children, this study focuses upon children. My child is asked to continue to participate in the study until he/she reaches 36 months of age.

Although this study may not benefit me or my child directly, the information obtained will help determine new strategies for prevention and treatment of diarrhea.

Volunteering or refusal to participate in this study will have no effect upon the course of my or my child's medical care. I may discontinue my child's participation at any time without any penalty.

ENCLOSURE(12)

Laboratory results and the information I give will be strictly confidential and used only for medical research purposes. Information which identifies me or my child, such as name, will not be disclosed except to authorized personnel.

I agree to allow my child to participate in the study which has been described.

AND

I agree, acting in the position of the responsible adult for a child living in my home, that the child may participate in the study.

If I have any questions regarding this research or if I have any questions regarding my or my child's rights as a patient in this study I may contact Dr. Ali Mourad, Head Department of Microbiology, University of Alexandria School of Medicine, Alexandria OR Head, Committee for Protection of Human Subjects, United States Navy Medical Research Unit Number Three, Cairo; phone 02-284-1375.

Printed Name of Child	Printed Name of Parent or Guardian
Signature of (circle one) [Parent or Guardian]	Date
Signature of Investigator	Date
Witness # 1	Witness # 2

U.S. NAVAL MEDICAL RESEARCH UNIT NO.3

ETEC EPIDEMIOLOGY AND ETEC VACCINE EVALUATION

IN ALEXANDRIA, EGYPT

MANUAL OF STANDARD OPERATING PROCEDURES

FIELD ACTIVITY

OCTOBER 1994

MAPPING

- For the purpose of mapping, each house in the village will be identified with a unique identifier such as the word "Health" and a three digit number. The combination of the word and the numbers is to allow differentiation between our study identifier and other numbers given to the house by other organization or administration.

- In this rural area, a house is defined as a physical building in which one family or an extended family (nuclear family) live.

- All houses are to be numbered in a sequential manner starting from the first house on the main road to the village.

- New houses built after the process of house numbering will follow the sequence of numbers.

- Each house will have a unique identifier and will be prominently displayed at the right side of the main entrance to the house.

- Maps will be drawn for villages participating in the study in the Abees complex, currently known as Abees 8. The maps will include the group of villages known as 800, 801, 802, 803, 804, 805, 806, 807, 808, 809, 810, 811 and 812.

- Maps should include all houses with their unique identifier (three digit number), and all principal topographical and physical elements of the village.

- Maps will be updated annually.

- The team mapping a village should be comprised of:
  * A map specialist
  * Two local health officials, who have been working or living in the area for a reasonable time.
  * A clerk (to paint numbers on houses)

CENSUS

- All households in a village will be visited during the census period. A household is defined as a social unit comprised of individuals of one or an extended family (nuclear family) living together in the same dwelling.

- During the census visit, information will be gathered from a respondent using **CI** and **CH** questionnaires (Appendices 1 and 2). A respondent is the person who will provide the information needed in the form, therefore, identification of this person is of utmost importance. This respondent should be an adult for the majority of the information needed in the form. If a child less than 24 months of age is identified in a household, the mother or the caretaker of the child should supply the information concerning the child. If an adult is not available, the visit should be repeated at the end of the day. If during the second visit an adult is not available, the form is marked for a revisit at the end of the census.

- The **CH** form contains information about the housing condition, type of house, number of rooms, electricity, water supply and other housing and environmental conditions. By contrast, the **CI** form includes information about the subjects resident in the house.

- For all children under 24 months the date of birth should be recorded (estimate age is not acceptable). This should be confirmed through an official record.

- Field workers should ask the questions and report answers and should not assume or conclude the answers from his/her observations. Observations can be written by the interviewer at the end of the form.

- At the end of the day, all forms will be transferred to the data house to be reviewed and prepared for computer data entry.

- Census update will be done annually to allow the creation of an accurate demographic profile of the area.

- Updating the census will be accomplished using **CI** and **CH** forms which were used in the first census.

- In updating the census, original census will be printed, taken to the field and verified for accuracy by house-to-house visit. Any modification, deletion or addition will be noted directly on the print out of the census.

Individuals who have departed the household will be noted, along with their present location, if known. Individuals who have newly arrived or that had previously been censused and have relocated within the study area will be identified on the appropriate form.

- During census updating, information will be supplied by an adult. Mothers or caretakers will supply information concerning infants and children under their care.

- The census team for each village should be comprised of:
  * Two trained social workers.
  * Two experienced local health officials.

ENROLLMENT

- At the beginning of the enrollment phase, a list of children under 24 months of age will be provided to the field team (a physician should be included in the team to decide the eligibility of the children).

- Screening of subjects and filling of data forms for enrollment will be done by a physician.

- The parents or guardians of each potential participant will be approached about the study, and written informed consent for participation will be solicited.

- Children will then be evaluated for eligibility. Eligibility criteria are:
  * No congenital abnormalities (e.g., cyanotic congenital heart disease).
  * No chronic illness that requires continuous medication (lifetime disease).

- Children will be enrolled by the physician using the Enrollment (**EN**) form (Appendix 3). **EN** forms will be provided to the field team from the data house. Each **EN** form will have a computer pre-printed label containing the village no., the house no., child name, child sex, child's age in mos, child registration no (RID), caretaker name and caretaker RID.

- The **EN** forms will then be assigned a form serial number in the data house. The set of the **EN** forms will be logged in a log book and then sent to the field teams.

- After filling the **EN** form the physician will inform the mother or the caretaker of the child about the time she and her child should present themselves to either the clinic (The Rural Health Unit) or a convenient place (a house in the village) for performing the baseline anthropometric measurements (height, weight and mid-arm circumference) of the child.

- At the clinic or the designated house, the baseline anthropometric measurements will be obtained and recorded on the **EN** form. Children who have been scheduled for this exam, but who do not appear will be revisited at their home and rescheduled for the clinic. If they fail to appear at the clinic for the 2nd time, anthropometric measurements will be taken at the home.

- All births occurring in a participating study village (in which enrollment phase has begun) will be continuously

monitored and eligible newborns will be enrolled on an ongoing basis following the same previously described procedures and methods.

- The occurrence of new births can be determined by reviewing of birth registrations every week (Thursday) from the government health office present in the Rural Health Unit and through means of active surveillance visits (the weekly survey which inquire about new birth in the family) as will be mentioned later.

- The information about newborns, which includes newborn's name, date of birth, his mother's name, village number and household number, will be sent to the data house (computer unit) where census records will be updated.

- The field manager will be actively involved with the project and will ensure the field activities are done on schedule and correctly.

- Newborns will be enrolled in the study using (Newborn) **NB** form. This form is identical to the **EN** form except that the header will be printed on it (in the **EN** form the header comes from pre-printed labels) having the information corresponding to the **EN** labels.

- Immediately following enrollment, the field manager will review the consent and enrollment forms and ensure the following are accomplished:
  * Twice-weekly surveillance will be commenced.
  * Monthly surveillance will be performed.
  * Anthropometric measurement will be obtained and then every three months.
  * A finger-prick blood sample will be collected and every four months thereafter.
  * Environmental hygiene survey will be conducted and every six months during the project.
  * Newborns in the study area should be identified and enrolled.

Enrollment Phases

- Phase 0: Enroll village 800, about 35 children in November 93.
- Phase A: Enroll village 806, about 55 children in December 93.
- Phase B: Enroll village 807, about 35 children in June 94.
- Phase C: Enroll village 801, about 50 children in December 94.
- Phase D: Enroll village 804, about 45 children in June 95.

- Phase E: Enroll village 805, about 50 children in December 95.
- Phase F: Enroll village 808, about 40 children in June 96.

WEEKLY SURVEILLANCE

- Children enrolled in the study will be visited twice weekly.

- At the twice weekly visits, interviewers will inquire about diarrheal symptoms over the past four days (or since the last visit, if this comes sooner) using the Weekly Surveillance (**WS**) form (Appendix 4).

- Computerized pre-printed identification labels (stickers) for each enrolled child will be affixed onto a blank **WS** form. The identification sticker includes data about the number of the week of the visit, the number of the village, the number of the house, the child name, sex, date of birth, age in mos, RID number, caretaker name and caretaker RID number.

- Each **WS** form will be assigned a form serial number in the data house. The set of **WS** forms will be logged in a log book and then sent to the field teams.

- Children in each village will be divided into three groups (I, II, III) by the field manager, according to the location of their residence. Each child will be visited twice weekly according to the following schedule:
  Group I: Saturday and Tuesday
  Group II: Sunday and Wednesday
  Group III: Monday and Thursday

- In case of a one-day holiday, the visits of that day will be postponed to the next day. In case of a two-day holiday, the visits of the whole week will be divided over the remaining four days. But in case of three consecutive days holiday, one visit in that week will be canceled.

- The field worker will interview the mother or the caretaker of the child and inquire if the child has had liquid or loose stools since the last visit.

- If the child has had liquid or loose stool, then two rectal swabs will be obtained from the child. Each swab should be inserted separately into the rectum. The field worker should ensure that the rectal swab has evidence of fecal soiling.

- The duplicate rectal swab specimens will be placed; one in Cary-Blair transport medium and the other in phosphate-buffered saline (PBS) in the field and transported to the laboratory. On extremely rare

occasions, if only one rectal swab is obtained, it should be placed in Cary-Blair medium.

- A stool cup with a specimen number sticker will also be provided to the mother to collect a stool sample from the child. If a house has two children with diarrhea, the field worker should write the name of the child on the label. Stool specimens will be collected on the same day or the next day and transported in an ice box to the laboratory.

- The field monitor will place a WS yellow specimen label on:
  1. rectal swab in Cary-Blair
  2. rectal swab in PBS
  3. stool specimen cup
  4. **WS** form
  5. **LT** form (Laboratory Transmittal form - Appendix 5)

- If the specimens collected on the date of visit are rectal swabs (stools are to be collected on revisits) do the following:
  a. place a yellow Lab Accession Number (LAN) sticker on a stool cup to be given to the mother
  b. answer 'Y' to the question 'Revisit for Stool' on the **LT** form, staple the rest of the color stickers to the **LT** form
  c. in the data house fill out a new **LT** form with all the header information copied
  d. affix a yellow sticker onto this new blank **LT** form and fill in 'Y' in Q11 (is this a revisit?)
  e. log and submit this data form to the field manager, who will send it out the next day

- Information about diet, with a particular focus on breast-feeding status, will also be obtained on the **WS** form by the field worker.

- The field worker will refer children sick with diarrhea, to the Abees Health Unit for diagnosis and treatment.

- The physician will then be notified to examine children with diarrhea and to obtain further information about the severity of the illness, oral rehydration solution will be supplied, and referral will be offered as appropriate.

- If the interviewer was not able to fill out the **WS** questionnaire form due to absence of the mother (or caretaker), the unfilled questionnaire form is sent back to the data house and the child will be revisited the

next day.

- If, during the revisit, the interviewer failed to collect the required data or the stool sample for any reason, the visit will not be repeated and the questionnaire form is sent to the data house.

- At the data house, the clerk should check the continuity of serial numbers of the different forms after receiving them from the field.

- These twice-weekly visits will continue until the child reaches 36 months of age or until the end of the study period, whichever occurs first. All children will be followed for at least 12 months.

- Ongoing monitoring for newborns, deaths and out-migration will be accomplished as part of these follow-up visits.

- Two field teams, headed by a physician, should be responsible for the field activity in each village. Each field team should consist of two social workers or one social worker and one Ministry of Health nurse.

MONTHLY SURVEILLANCE

- In addition to the twice weekly visits to the participating children, every fourth week of surveillance - another survey (the monthly surveillance) will take place.

- Every 4th week, monthly and weekly surveillance will be done by the same team on the same day. Start the interview by first filling out the Monthly Surveillance (**MS**) form (Appendix 6).

- A pre-printed label for the **MS** form corresponding to this individual will be peeled out from the list of labels and affixed onto a blank **MS** form. The **MS** forms will then be assigned a form serial number, logged in a log book and then sent to the field teams.

- During the monthly surveillance visit, if a child has any loose stools since the last visit (last three days), i.e. the answer to Q17d. in the MS form is Yes, then rectal swabs in Cary Blair and PBS and stool specimens should be obtained and yellow WS stickers will be applied to:
  1- rectal swab in Cary Blair
  2- rectal swab in PBS
  3- stool specimen
  4- WS form (this form should be filled out in the usual way)
  5- MS form (Q19)
  6- LT form

- If the child has no diarrhea, the answer to Q17 d. is No or uncertain; then:

  a. Collect two rectal swabs (CB and PBS), and stool specimens
  b. Attach Blue specimen labels to the specimens and Q19 of this form
  c. Fill out the **WS** form for today (this form should be filled out in the usual way); no label is required for this form
  d. Attach Blue label to **LT** form

- For the monthly surveillance, a stool cup will be left after obtaining the rectal swabs, to be collected the next day. If there is no stool specimen the next morning, a stool sample is no longer required, and is thus not collected from that child and is noted on the form.

- If the specimens collected (in **WS** as well as **MS**) on the date of visit are rectal swabs (stools are to be collected on revisits) do the following:
    a. place a LAN sticker on a stool cup to be given to the mother
    b. answer 'Y' to the question 'Revisit for Stool' on the **LT** form, staple the rest of the color stickers to the **LT** form
    c. in the data house fill out a new **LT** form with all the header information copied
    d. affix a colored sticker onto this new blank **LT** form and fill in 'Y' in Q11 (is this a revisit?)
    e. log and submit this data form to the field manager, who will send it out the next day

- After completing the Monthly Surveillance form, the form will be checked for completeness and correctness by the data manager in the data house.

ANTHROPOMETRY

- The anthropometric measurements (height, weight and mid-arm circumference) will be taken every 4 months.

- A pre-printed list of children from whom anthropometric measurements will be obtained will be sent to the field team responsible for this activity. The list contains child name, age, village number, household number, individual number, child registration number (RID) and mother's name. It also contains three blank spaces for weight, height and mid-arm circumference.

- At the clinic (The Rural Health Unit) or a convenient place (a house in the village), the baseline anthropometric measurements will be obtained and recorded on the list. Children who have been scheduled for this exam, but who do not appear will be revisited at their home and rescheduled for the clinic. If they fail to appear at the clinic for the 2nd time, anthropometric measurements will be taken at the home.

- The anthropometric team is a different field team which consists of an experienced physician and a nurse.

BLOOD COLLECTION

- Every 4 months blood will be collected from children greater than or equal to 6 months of age. If the child is less than 6 months of age (ascertained by the age printed on the label), no blood will be collected.

- This activity will be recorded on the blood collection and storage **AB** Form (Appendix 8).

- A pre-printed label for the **AB** form corresponding to this individual will be peeled out from the list of labels and affixed onto a blank **AB** form. The **AB** forms will then be assigned a form serial number, logged in a log book and then sent to the field team responsible for blood collection.

- A sheet of stickers of the BL series (red color) with 5 stickers per page should be made available to the clinic. If blood is collected, a BL (red) sticker should be placed on the **AB** form, and one each aliquot of blood specimen and the remaining stapled to the **AB** form.

- The **AB** form along with the blood specimen is then sent to the Alexandria University laboratory where the blood is to be spun and the serum separated from the cells.

- The blood collection team is a different field team and only does blood collection. This team consists of a trained physician and a nurse.

Finger-stick Blood Drawing Procedure

Materials Required

1. 100 microliter heparinized capillary pipettes
2. sterile 2.5 ml conical polypropylene micro-centrifuge tubes
3. sterile phosphate buffered saline (PBS), pH 7.0
4. capillary pipette bulb
5. sterile, disposable, single-use lancets
6. ABO blood typing antiserum and materials for test
7. alcohol swabs (70% ethanol in water)
8. rack for micro-centrifuge tubes
9. refrigerated (cool-pack) specimen carrier

Procedure

1. Decontaminate the finger (or heel) of the subject with a cotton swab saturated with 70% ethanol. Allow the finger to dry thoroughly while holding it to prevent re-contamination.
2. Using the disposable, sterile lancet, deftly prick the decontaminated end of the subjects finger or heel.
3. Apply gentle pressure to the digit or the heel in order to express a drop of blood. Do not vigorously "milk" the site, but apply gentle pressure only.
4. While holding the site, place a 100 microliter heparinized capillary pipette to the drop of blood and allow it to fill to the graduated mark (100 microliter mark).
5. When full to the mark, place the capillary tube into a 2.5 ml conical polypropylene micro-centrifuge tube containing 0.9 ml sterile PBS and having been labeled with the subject's specimen sticker.
   As two hundred microliters of blood is the desired specimen volume to be obtained, two duplicate 100 microliter specimens should be taken from each subject.
6. Using a bulb, expel the blood from the capillary tube into the PBS. Close the top of the microcentrifuge tube and place it into the rack in the specimen carrier. NOTE: The blood is now diluted 1:10 by the PBS, giving an effective 1:20 dilution of the serum. The field workers must ensure that the label (lab sticker) is on each tube.

PASSIVE SURVEILLANCE

- Rationale:
  * Passive surveillance will be performed in the same catchment area as active surveillance in order to identify diarrheal cases in individuals who are not enrolled in the protocol.
  * Medical care provided through passive surveillance should help to foster community acceptance of the surveillance program in Abees.

- Individuals under 10 years of age presenting to Abees Health Unit suffering from diarrhea will be directed to the special clinic designated for the project, the "Diarrhea Clinic", in the Health Unit.

- At the Diarrhea Clinic patients' information will be recorded on the Passive Surveillance (**PS**) Form (Appendix 9) and they will be classified according to residence.

- If the residence is in a study village in Abees, the village number and the name of the household will be recorded on the **PS** form. Additional information will be added to the **PS** form later by Data Management, such as house number, individual number, child registration number (CID) and the exact date of birth.

- Medical history and physical examination will be done by the study physician.

- A stool cup with a specimen number sticker will be provided to the caretaker to immediately collect a stool sample from the child.

- Two stool swabs will be taken from the stool sample; one is placed in Cary-Blair transport medium, and the other in phosphate-buffered saline (PBS).

- Stool specimens will be transported in a refrigerated box to the laboratory in Alexandria.

- If the patient fails to provide a stool sample, two rectal swabs will be obtained from him/her, one will be placed in Cary-Blair transport medium and the other in the PBS. The physician/nurse should ensure that the rectal swabs have evidence of fecal soiling.

- The physician/nurse will fill the Passive Surveillance Specimen Transmittal (**LP**) Form (Appendix 10).

- The Diarrhea Clinic physician's responsibility is to ensure that the specimen number sticker is placed on:
  * **PS** Form
  * **LP** Form
  * Stool specimen cup
  * Stool swab in Cary-Blair
  * Stool swab in PBS

- The physician will prescribe the proper treatment for the patient who will receive it from the Health Unit pharmacy free of charge.

- After completing the **PS** and **LP** forms, the forms will be sent to the Data Manager in the data house.

- At the data house, each form will be assigned a form serial number and will be logged in a log book.

- If the patient's residence is in a study village, the Data Manager will complete Q8 - Q10 on the **PS** form using the computer census data.

U.S. NAVAL MEDICAL RESEARCH UNIT NO.3

ETEC EPIDEMIOLOGY AND ETEC VACCINE EVALUATION

IN ALEXANDRIA, EGYPT

MANUAL OF STANDARD OPERATING PROCEDURES

LABORATORY ACTIVITY

OCTOBER 1994

STANDARD OPERATING PROCEDURES FOR THE MICROBIOLOGY LABORATORY

General considerations

The following procedures have been established for use in the enteric disease protocols of the University of Alexandria, Department of Microbiology with the Naval Medical Research Unit Number Three.

I. SPECIMEN COLLECTION

A. Field personnel should carry to the homes, a small, portable insulated box, containing frozen cool-packs, into which specimens can be placed. Specimens are obtained from the patient and placed in the appropriate primary container (test-tube, stool container, etc.) which has been labeled with the patient's name and registration number and appropriate colored laboratory sticker. Specimens are then transferred to a larger reusable, leak-proof, closed transport container kept in the vehicle that are easily decontaminated. Plastic insulated coolers are ideal. The top of the cooler should be marked in Arabic with the University address and the telephone number of the lab. Also the cooler should be marked with the international biohazard symbol and the words, in Arabic, "Caution, Hazardous Medical Materials".

B. Specimens are to be refrigerated (4°C or below) while in transit or storage. Frozen (-20°C) reusable "cool packs" should be placed in the bottom of each transport cooler prior to its use by field personnel. Cool packs should be used throughout the year, regardless of the ambient temperature.

C. Specimens for analysis for bacteria must be preserved in the field in Cary-Blair transport media. If specimens are needed for parasitology, they must be placed in an appropriate preservative, such as Polyvinyl Alcohol (PVA), Merthiolate-Iodine-Formalin (MIF), or formalin-saline. Unpreserved stool specimens will be considered to be unsatisfactory for bacterial culture and for examination for parasites, but satisfactory for virus culture.

D. Specimens must come to the laboratory with complete documentation (ie. an LT form must be completed and accompany the specimen). When laboratory personnel receive specimens from the field they should first check that the required information is given on the laboratory transmittal form. Next, specimens should be checked for the following:

1. physically accounted for
2. properly labeled and with required forms
3. in the appropriate transport medium
4. refrigerated (arriving in the cool box)

If any of the previous criteria are not met, it should be noted on the LT form, and the laboratory supervisor should be notified.

II. Types of specimens and usage

Laboratory studies will be performed only on specimens that meet the acceptance criteria for each type of specimen. Specimens that arrive at the laboratory without proper handling, or documentation, or specimens of uncertain origin will be rejected by the laboratory supervisor with

written documentation made on the Laboratory Transmittal form as to why the specimen was not accepted. The laboratory supervisor should notify the Field Manager and the Data Manager of the reasons for the rejection, in order for immediate action to be taken, and to allow for a follow-up specimen to be obtained.

The following specimen types are used for the purpose indicated.

A. **Rectal Swab in Cary-Blair:** bacterial culture
B. **Rectal Swab in PBS:** Detection of rotaviral antigens
C. **Unpreserved stool (frozen):** Detection of viral antigens

III. Methods of collection

A. Rectal Swabs

Moisten two cotton-tipped swabs with Cary-Blair transport medium. Gently pass the tip of one swab approximately one to one and a half inches beyond the anal sphincter. Slowly rotate the swab and remove. Repeat with the second swab. Insert only one swab at a time. Check carefully for adequate fecal soiling of the swabs. Immediately place one swab into Cary-Blair transport medium and break off the end of the swab stick, being careful to break off the stick <u>below</u> the portion touched by the fingers. Replace and tighten the cap of the Cary-Blair tube. Place the second swab in a cryovial containing 1.8 ml of phosphate-buffered saline (PBS). Swirl (mix) the swab in the PBS to inoculate the PBS with the stool. Remove and discard the swab and tightly cap the tube. Label both tubes with the patient's registration number, date and name, and a colored laboratory sticker and place them in the transport cooler. At the lab, the PBS vial should be frozen at -40°C or colder. **Field Note:** The desired concentration of stool in PBS is 10%. Visually inspect rectal swabs to ensure that they are inoculated with sufficient stool, if no stool is visible, a second specimen may be required.

B. Stool specimens

Obtain stool specimens by passing stool directly into a clean, dry, wide-mouth, leak-proof container with a tight-fitting lid. Alternatively, stool can be passed into a clean, dry receptacle and transferred to a stool cup container. Stool contaminated with either urine or sewer water is not acceptable. Record the patient's name, registration number, and the date and time the stool sample was produced on the container.

1. Unpreserved stool specimens

Stool, collected as described above, is refrigerated in the field (cool box) and transported to the laboratory.

2. Preserved stool specimens

Stool for parasitology should be placed in preservative within two hours of passage. Stool should be mixed in approximately one part stool to three parts preservative. Mix well in order to get good preservation of all portions of the sample. When sampling the stool, select portions from each of the following areas that apply:

a. Outer portion
b. Inner portion
c. Bloody portion
d. Mucus-containing portion

IV. Specimen processing

A. Rectal swabs in Cary-Blair

Rectal swabs in Cary-Blair transport medium should be kept refrigerated at 4°C or below (never frozen) until used for inoculation of bacteriological media. Specimens may be held for up to three days in refrigeration prior to use. Caps should be on tight so that no evaporation occurs.

B. Rectal swabs in PBS

Rectal swabs in PBS are used for virus culture. After inoculation and during transport, PBS should be refrigerated. The PBS should be frozen (-40°C or colder) as soon after inoculation as possible. Once frozen, PBS **should not be allowed to thaw**, as each freeze-thaw cycle reduces the titer of replicative virus.

C. Stool (unpreserved)

Unpreserved stool is refrigerated in the field, as soon after collection as possible. It should be transported to the laboratory under refrigeration. Once in the laboratory, the specimen should be aliquoted into cryovials and frozen at -20°C or colder (the colder, the better). Stool handled in this way is acceptable for viral antigen detection studies.

STANDARD OPERATING PROCEDURES FOR THE MICROBIOLOGY LABORATORY

I. PROCESSING OF SAMPLES ARRIVING FROM THE FIELD

For each patient, three samples should arrive from the field, although not necessarily on the same day: a rectal swab in Cary-Blair transport medium (refrigerated), a rectal swab in PBS (refrigerated), and a cardboard stool cup (refrigerated). Specimens should be accompanied by a Specimen Transmittal Form (LT) of which the left-hand side should already have been filled out in the field. The right-hand side of this form, headed by "TO BE COMPLETED BY THE LABORATORY" should be completed at the time the specimens are processed. Process the specimens as follows:

A. RECTAL SWAB IN CARY-BLAIR

1. Inoculate one MacConkey's agar and one Salmonella-Shigella agar plate by rotating the rectal swab across one quadrant of each plate. Set plates aside to be streaked.

2. Place swab in tube containing alkaline peptone water and inoculate by twirling swab. Remove swab and return to original Cary-Blair transport medium. Incubate alkaline peptone water 6-8 hours at 35-37°C.

3. Twist swab in original Cary-Blair transport medium to re-inoculate.

4. Inoculate a 1 ml vial of Brucella broth by twirling rectal swab in the vial. Remove swab and return to original Cary-Blair transport medium. Mix vial of Brucella broth well, then pour contents onto 0.65 μm pore-size cellulose acetate membrane filter placed on a sheep's blood agar plate (BAP), being careful not to allow the liquid to flow over the edge of the filter. Place the lid on the petri plate and allow to stand on a flat surface for 30 minutes. Remove filter carefully and discard. Place plate in candle jar and create a microaerophilic environment (5% O_2, 10% CO_2, 85% N_2) as follows. Tear off top of one Campy gaspack envelope. Add 10 ml of water to Campy gaspack envelope and place upright in candle jar with plate. Immediately close tightly and incubate at 42°C overnight.

5. Twist swab in original Cary-Blair transport medium to reinoculate.

6. Place rectal swab into tube containing Selenite F broth and inoculate by twirling swab. Break off top of swab against side of tube and incubate 18-24 hours at 35-37°C.

7. Fill out the right-hand portion of the Specimen Transmittal Form (LT) under "TO BE COMPLETED BY THE LABORATORY"

B. RECTAL SWAB IN PBS

1. The second rectal swab will arrive in a vial containing 1.8 ml of phosphate-buffered saline (PBS). The PBS vial should be frozen immediately upon arrival at -70°C or lower for subsequent virus culture.

2. Fill out the right-hand portion of the Specimen Transmittal Form (LT) under "TO BE COMPLETED BY THE LABORATORY"

C. STOOL

1. Condition of the stool is recorded according to the following criteria on Specimen Transmittal Form (LT)
   - Formed
   - Soft: takes shape of container
   - Watery: can be poured

   Also note the presence of: blood, mucus, etc.

2. A 1-ml sample (pea-size portion) of stool is placed in each of two 2 ml cryovials, labeled with the person's number, and frozen at -20°C or lower. Care should be taken to sample all parts of the specimen (external and internal), especially the parts with mucus or blood. Stool is used only for subsequent antigen detection of rotavirus.

3. Fill out the right-hand portion of the Specimen Transmittal Form (LT) under "TO BE COMPLETED BY THE LABORATORY"

D. FILLING OUT THE SPECIMEN TRANSMITTAL FORM (LT)

The rectal swabs and stool sample will arrive with a Specimen Transmittal Form (LT) of which the left-hand section should already be filled out. When processing these

specimens, the laboratory should fill out the right-hand side of this form, titled "TO BE COMPLETED BY THE LABORATORY". Upon completion, this form should be sent to the data house. Complete the form as follows.

General: This form is used to record information for specimens being collected in the field and transported to the laboratory. The form is filled out by three different individuals. Parts 1-15 are filled out in the field by the field worker responsible for obtaining the specimens. Parts 16-25 are filled out in the laboratory by laboratory personnel receiving the specimens. Parts 26-27 are filled out in the data house by the data entry personnel who receive the completed form from the laboratory.

1. Study Phase: Major phases of the study are designated by a letter. The current phase may be obtained from the Data Manager. Field workers should have this information prior to going to the field.

2. Form Serial Number: Each form is assigned a unique serial number by the data house. The numbering is consecutive and is usually stamped on the form.

3. Date of Collection: Date specimen was produced by the patient.

4. Lab Number (Sticker): Each specimen will be assigned a unique laboratory number. Field workers are to select the next consecutive number for the appropriate type of specimen. Passive surveillance numbers begin with the letters "PS" and are pink in color. Weekly surveillance numbers begin with the letters "WS" and are yellow in color. Monthly surveillance numbers begin with the letters "MS" and are blue in color. Field workers must have a supply of each type of these numbers with them, and apply the appropriate number the specimen. The sheet of unused numbering labels must be attached by the field worker to the form so that the lab can use the labels.

5. Serial Number of Visit/Passive Surveillance Number: This is the number assigned to the Visit Form, or with the case of Passive Surveillance, it is the number assigned in the clinic.

6. Name of Subject: Please write the English equivalent of the individual's name.

7. Village Number:

8. Household Number: This is the unique number each household received when the village was mapped and censused. This number never changes for the house.

9. Individual Code: This is a two digit code for each member of a family or home group. This number never changes for the individual.

10. Child's Registration number: This is the unique registration number that each child receives when enrolled in the study. This number never changes for that individual.

11. Is this a revisit? Indicate if the field visit is a follow-up visit (a revisit) or the first visit. An X in this space indicates it is a Passive Surveillance (clinic) specimen.

12. Source of Specimens: Indicate if the specimens were part of the weekly (W), monthly (M), Clinic (P), or combined weekly and monthly (C) visit. Choose only one answer.

13. Specimens Collected: Write Y or N to indicate the presence or absence of each type of specimen. Leave nothing blank.

14. Specimens to be collected on a revisit: If needed specimens must be collected at a later date, indicate that here by placing a Y in those specimens that must be obtained. Place a N by specimens not needed. Leave nothing blank.

15. Data Collector: The field worker should place her/his three initials in this block in order to identify who collected the specimen and filled out the form.

16. Specimens Received: This part if filled out by laboratory personnel. They will confirm that the specimens listed in part 13 arrive in the laboratory.

17. Date received: Today's date as first day of the month, the month, and the last two digits of the year.

18. Record the time the specimen was received at the lab. Use the 24 hour clock, with midnight being 00, noon being 12, etc. If the hour the specimen arrived in the lab is unknown, place "ZZ" in the spaces provided.

19. Appearance of Stool: This only applies to a stool specimen in a container, not to specimens in Cary-Blair transport medium or a swab in PBS. Select the appropriate response.

20. Blood in Stool: If gross blood is seen in a stool specimen, mark the appropriate response.

21. Location of frozen Rectal Swab in PBS: If no rectal swab in PBS was obtained, mark X, otherwise, fill in the box number, then the row (A-J) and then the column (1-9) with 0 being equal to 10.

22. Location of frozen Stool: If no stool was obtained, mark an X in the location. If stool was obtained, fill in the correct box information.

23. Location of frozen Serum: If no serum was obtained, mark an X in the location. If serum was obtained, fill in the correct box information.

24. Lab Specimen handler: The individual handling the specimen should place their three initials in the space provided.

25. Lab Reviewer initials: The laboratory supervisor that reviews the specimen information on the LT form places her/his initials here. Placing the initials indicates that the reviewer agrees with the information, and the reviewer

assures that no blank spaces have been left open. Every space must have a response.

26. Data Entry 1. These are the initials of the first individual to enter the data into the computer.

27. Data Entry 2. These are the initials of the second individual to enter the data into the computer. Initialing the form here indicates that all the data requested on the form has been filled in, and it has been entered into the computer as given.

II. WORK-UP AFTER 6-8 HOURS:

ALKALINE PEPTONE WATER

Take alkaline peptone water from incubator and with flamed and cooled loop, take two loopfulls from the top two centimeters and streak onto a TCBS plate. Streak for isolation and incubate plate at 37°C overnight.

III. WORK-UP OF PRIMARY ISOLATES AFTER 24 HOURS:

As specimens are worked up, all inoculated plates and biochemicals, as well as results of those tests should be noted on the Microbiology Laboratory Results Form (LR) belonging to that specimen. Instructions on filling out this form can be found under heading E of this section. Make sure that all tubes which are inoculated are marked with the patient number, the date, and from which plate the colony came: direct or subculture.

A. SELENITE F BROTH

After 18-24 hours, remove selenite F Broth from the incubator, shake well, and streak a Salmonella-Shigella agar plate for isolation. Incubate 18-24 hours at 35-37°C and work up using the method described below for the Salmonella-Shigella plate.

B. MACCONKEY'S AGAR PLATE

Lactose fermenters (pink colonies) are probably *E. coli* and are subcultured for subsequent toxin testing:

Pick five separate and distinct lactose fermenting *E. coli*-like colonies at random and stab them into five separate cryotubes with nutrient agar. Incubate the stabs overnight at 37°C, then transfer them to a 4°C refrigerator, for later LT/ST toxin testing.

Non-lactose fermenters (clear or non-pink colonies).
Choose a well-isolated colony and subculture by inoculating one set of biochemicals, including TSI, MIO, and urea agar as follows:

1. Touch top of colony using a sterilized inoculation needle and stab to bottom of TSI tube with single up-and-down motion. After stabbing, immediately streak slanted surface of agar, remove needle, and loosely cap tube. Set aside.

2. Following TSI, inoculate MIO by stabbing in a single up-and-down motion in the center of the agar three-fourths of the way down the tube, keeping the needle as vertical as possible. Replace cap (loose) and set aside.

3. Following MIO, inoculate urea agar tube by placing needle in tube and rapidly shaking needle back and forth. Cap loosely.

4. Flame needle and allow to cool.

5. Place inoculated TSI, MIO, and urea agar in a rack and incubate at 35-37°C overnight.

Inoculate one set of the above biochemicals for each morphologically different well-isolated colony found on the plate.

C. SALMONELLA-SHIGELLA AGAR PLATE

Only non-lactose fermenters (clear or non-red colonies) are subcultured

Choose a well-isolated colony and subculture by inoculating one set of biochemicals, including TSI, MIO, and urea agar as follows:

1. Touch top of colony using a sterilized inoculation needle and stab to bottom of TSI tube with single up-and-down motion. After stabbing, immediately streak slanted surface of agar, remove needle, and loosely cap tube. Set aside.

2. Following TSI, inoculate MIO by stabbing in a single up-and-down motion in the center of the agar three-fourths of the way down the tube, keeping the needle as vertical as possible. Replace cap and set aside.

3. Following MIO, inoculate urea agar tube by placing needle in tube and rapidly shaking needle back and forth. Cap loosely.

4. Flame needle and allow to cool.

5. Place inoculated TSI, MIO, and urea agar in a rack and incubate at 35-37°C overnight.

Inoculate one set of the above biochemicals for each morphologically different well-isolated colony found on the plate.

D. BLOOD AGAR PLATE IN CANDLE JAR

Remove BAP plate from the microaerophilic after overnight incubation and check for growth and typical *Campylobacter* morphology: colonies are non-hemolytic and grey or colorless. They may be flat and watery with irregular edges, or round and convex with entire edges. Colonies may be pinpoint in size or spreading over large areas of the plate. Suspect colonies should be screened by the following tests: oxidase test (see below) and gram stain (see below).

Oxidase Test: Touch the pure culture and obtain a large amount of pure growth with a

cotton swab. Drop a drop of oxidase reagent onto the bacteria on the cotton swab and observe for a color change in 10 to 30 seconds. **Kovac's modification:** Place 3-4 drops of oxidase reagent on a piece of filter paper. Using a wooden applicator stick, immediately mix a few colonies from a plate or slant into the reagent. The color change occurs in a positive test as in the routine procedure. Read for color change within 10 seconds.

positive: development of deep blue or purple color within 10 to 30 seconds. (i.e. *Campylobacter*)

negative: no color change. (i.e. *E. coli*)

Gram Stain for *Campylobacter*: Do routine gram stain, but allow safranin counterstain to remain on slide for up to 3 minutes, or use 0.1% basic fuchsin as a counterstain and allow to remain on slide for up to 3 minutes. *Campylobacter* is seen as small, slightly curved, gram-negative rods with an "S" or seagull shape.

E. TCBS AGAR PLATE

Check TCBS agar for growth and typical yellow or blue-green colonies. If there is no growth, incubate for an additional 24 hours, before discarding. Some coliforms may grow, but colonies are typically small and translucent. Sucrose fermenting *Proteus* spp. produce yellow colonies and must be carefully differentiated from *Vibrio* spp. If yellow colonies are present, screen w~ oxidase (*Vibrio* spp. should be positive). However, if the oxidase test is negative with yellow colonies, the colony must be subcultured to TSA, BHIA, Mueller-Hinton agar or BAP and retested with oxidase within 24 hours. Presumptively identified *Vibrio* spp. should be further identified by serotyping and API-20 E. Blue-green colonies may be *V. parahaemolyticus*. These colonies should be inoculated into nutrient broth containing 0%, 1%, and 3% NaCl. *V. parahemolyticus* will only grow in 3% NaCl and should subsequently be confirmed by API. The procedure is described in Chapter 37 of Balows: Manual of Clinical Microbiology 5th Edition.

F. FILLING OUT THE MICROBIOLOGY RESULTS FORM (LR)

When specimens arrive in the microbiology laboratory accompanied by a Specimen Transmittal Form (LT), a Microbiology Laboratory Results Form (LR) needs to be filled out for each Rectal Swab in Cary-Blair specimen received. Use the information on the LT form to fill out questions 1-5 on the LR form. Since questions 1 through 17 will be transferred to the computer, answers should be written clearly.

1. Copy the Study Phase from # 1 on form LT to # 1 on form LR.
2. Ensure the serial number is stamped on the form.
3. Place the appropriate sticker for this person in the box next to number 3.
4. Copy the name of the child from question 6 on the LT form to question 4 on the LR form.
5. Copy the child's registration number from question 10 on the LT form to question 5 on the LR form.
6. Write the current date.
7. Indicate whether the specimen is a rectal swab or a stool swab by circling the appropriate choice.

8-13. These questions are not answered until the specimen is thoroughly worked up and any pathogens have been identified.

14. After streaking the specimen on a MAC plate and incubating overnight, five lactose fermenting colonies should be saved for subsequent toxin testing. If this was done, mark **Y** for Yes in the space. If no lactose fermenting colonies are found, mark **N** for No in this space.
15. If lactose fermenting colonies are found, indicate the how many were saved by circling the appropriate number (1, 2, 3, 4, or 5).
16. If lactose fermenting colonies were saved, write the number of the box in which they can be found.
17. For each lactose fermenting colony saved, write the location within the box. This should be written as a combination of a row letter (A, B, C, D, E, F, G, H, I, or J) and a column number (1, 2, 3, 4, 5, 6, 7, 8, 9, or 0). For example, If the cryovial containing Colony 1 was placed in the left-top space within the box, one would write **A1** in the space after **Colony 1 Location**.
18. When completed, the space after **Laboratory 1** should be signed with first, middle, and last initials of the laboratory person who has performed the work. The space after **Laboratory 2** should be signed by the laboratory person who has checked that this form was properly filled out. After arriving in the data house, the first computer person who has entered this form into the computer should initial after **Data 1**, and the computer person who has checked that entry was done properly should initial after **Data 2**.

Bottom half of LR form:

This portion of the form is for use by laboratory technicians while a rectal swab in Cary-Blair is being cultured. On initial inoculation of media, those media which are inoculated are checked off on the form. If a non-lactose fermenter (NLF) is found, this is written in the appropriate space after the name of the media on which the NLF was isolated. For example, if a selenite broth was inoculated, selenite is check-marked; if a NLF was found on the SS inoculated from this selenite, "NLF" is marked after **SS Sub**. When the TSI, MIO, and Urease are inoculated with this NLF colony, the results are written in the section containing Biochemical reactions (below the second thick black line) under **Colony 1** and after **TSI Subculture** (or **MIO Subculture**, or **Urease Subculture** respectively. If another pathogen is found, its reactions are written under **Colony 2**. When final confirmation has been done by API, the API code number is written in the box with the number of the appropriate colony (1 - 7). When a Mueller-Hinton plate is inoculated, Y is circled. Results of antibiotic sensitivity testing are written on side 2 of the form. The zone of inhibition around the appropriate paper disk is measured in millimeters and written under Diameter next to the appropriate antibiotic and under **Agent 1** if this plate contains colony 1 (or **Agent 2** if this plate contains colony 2, etc.). Shigella serotyping results (if applicable) are noted in the **Shigella** section on side 2 (reverse) of the form. The correct serogroup is circled and if serotype is determined as well, the appropriate serotype is also circled. If only the serogroup has been determined, but serotyping has not been done, write "ND" (not done) after **Serotype** on the form.

After final confirmation of the pathogen(s) by biochemicals, API, and serogrouping and/or typing, the name(s) of the organism(s) is/are found in the "Type codes:" section on side 1 of the LR form. If *E. coli*-like colonies (lactose fermenters: LF) were found and saved, write "Y" under **Type code** after question 8. As an example, if *Shigella boydii* was isolated from the sample, write "C" under **Type code** after question 9. If no other pathogens were isolated, write "N" after questions 10, 11, 12, and 13.

The storage location of pathogens isolated should be noted in the section **Location of frozen isolates:** at the bottom of side 2 of the form. Write the species of the isolate, then the box number, as well as the row within that box (A, B, C, D, E, F, G, H, I, or J), and the column within the box (number 1, 2, 3, 4, 5, 6, 7, 8, 9, or 0)

IV. WORK-UP OF PRIMARY ISOLATES AFTER 48 HOURS:

A. BLOOD AGAR PLATE: READ FOR CAMPYLOBACTER

Remove BAP plate from 42°C incubator and microaerophilic candlejar and check for growth and typical *Campylobacter* morphology: colonies are non-hemolytic and grey or colorless. They may be flat and watery with irregular edges, or round and convex with entire edges. Colonies may be pinpoint in size or spreading over large areas of the plate. Suspect colonies should be screened by the following tests: gram stain (see below) and the oxidase test (see below). If no growth is detected, discard plate.

Gram Stain for *Campylobacter*: Do routine gram stain, but allow safranin counterstain to remain on slide for up to 3 minutes, or use 0.1% basic fuchsin as a counterstain and allow to remain on slide for up to 3 minutes. *Campylobacter* is seen as small, slightly curved, gram-negative rods with an "S" or seagull shape.

Oxidase Test: Touch the pure culture with a cotton swab. Drop a drop of oxidase reagent onto the bacteria on the cotton swab and observe for a color change in 10 to 30 seconds.
positive: development of deep blue or purple color within 10 to 30 seconds. (i.e. *Campylobacter*)
negative: no color change. (i.e. *E. coli*)

B. SUBCULTURE E. COLI STABS FOR LT/ST TOXIN AND CFA TESTING

Remove *E. coli* nutrient agar stabs from the refrigerator and streak the growth from each stab for isolation on a MAC plate. Incubate overnight at 37°C. The next day, divide a CFA plate into five sections and number the sections 1, 2, 3, 4, and 5. Transfer a single colony from the MAC plate to a section on a CFA plate and inoculate one well containing Luria Bertani broth of a microtiter plate. This microtiter plate will be used for LT/ST toxin testing by GM1 ELISA. Each *E. coli* colony, numbered 1 to 5 on the CFA plate is used to inoculate a well with Luria Bertani broth correspondingly numbered with the colony number 1, 2, 3, 4, or 5 and the specimen number. Wells on the outside of the plate are filled with Luria Bertani broth, but not inoculated. The plate is incubated while shaking at 37°C overnight. Because positives on the GM1 ELISA performed later will be tested for colonization factor antigens, the CFA agar plate should not be discarded. The CFA agar plate is placed in a plastic bag to prevent drying and can be stored at room temperature for storage of 7-10 days maximum.

C. SUBCULTURES: READ BIOCHEMICALS & IDENTIFY

Remove biochemicals (TSI, MIO, urea agar) from 37°C incubator after overnight incubation and read reactions as follows. Never incubate more than 24 hrs as readings change.

1. **Triple Sugar Iron Agar (TSI):** caps must be loose. Examples of reactions:

slant: yellow (A = acid) butt: yellow (A = acid): the organism ferments glucose, lactose and/or sucrose, written as A/A.
(i.e. *E. coli*: A/A, + Gas, no H_2S)

slant: red (K = alkaline) butt: yellow (A = acid): the organism ferments glucose only, written as K/A.
(i.e. *Salmonella, Shigella*: K/A)

slant: red (K = alkaline) butt: red (K = alkaline): the organism cannot ferment glucose, sucrose, or lactose, written as K/K.
(i.e. *Pseudomonas*: K/K, -Gas, no H_2S)

The presence of bubbles or splitting in the agar indicates gas production by the organism (written as + Gas). (i.e. most *E. coli*: + Gas)

A black precipitate in agar, seen as black streaks, or blackening of the entire tube indicates the organism produces H_2S, written as $+H_2S$.
(i.e. *Proteus mirabilis*: $+H_2S$)

3. **Motility Indole Ornithine Agar (MIO):**

This semisolid medium indicates the motility of the organism, seen by the formation of cloudiness throughout the medium (motile), or cloudiness only along the stab-line (non-motile). Ornithine decarboxylase is read in the butt of the tube. The development of a turbid purple to faded yellow-purple color in the media indicates a positive test (i.e. *Shigella sonnei, Salmonella* spp.). development of a bright yellow color in the media indicates a negative test (i.e. *Shigella boydii, Shigella dysenteriae, Shigella flexneri, Salmonella typhi*). This medium is also used to detect the production of indole by Kovac's reagent, which should be done after reading the motility and ornithine decarboxylase tests.

V. WORK-UP OF PRIMARY ISOLATES AFTER 72 HOURS:

A. LURIA BERTANI BROTH: TESTING FOR LT AND ST TOXINS

After incubating the Luria Bertani broth for 18-24 hours at 37°C, test each isolate for the presence of LT and/or ST toxins using the GM1 ELISA. Results should be recorded on the ETEC Results Form (ER). Positives on the GM1 ELISA will be tested for colonization factor antigens, using the colonies from the original CFA agar plate, which was saved in a plastic bag. See GM-1 ELISA directions below.

B. INOCULATE MICROBANK TUBES

Collect a thick amount of bacteria with a sterile cotton swab from the TSI tube and inoculate into a 1 ml tube of glycerol broth. The swab is twirled well to disperse the bacteria wiped against the side of the tube and discarded. The glycerol broth is added to duplicate "microbank" vials, which are labeled with the specimen number. The glycerol broth is left in the "microbank" vials for 30 minutes, after which the fluid is removed from the microbank vials using a sterile Pasteur pipette, so that only the glass beads remain. Vials are capped tightly and immediately frozen at -70°C. One aliquot will be shipped to NAMRU-

3, the second aliquot is kept at the University of Alexandria. Fill out the location of the vials on the ***E. coli*** Storage Form (EC). Make three copies of the filled-out ***E. coli*** **Storage Form (EC)**, include one copy in each of the two boxes, and mark boxes clearly with sample numbers, type of samples, and destination. Send the third copy to Stans and send the original ***E. coli*** **Storage Form (EC)** to NAMRU-3.

VI. FURTHER TESTING

A. IDENTIFICATION OF PRESUMPTIVE PATHOGENS BY API 20E

Make a bacterial suspension from bacterial growth on the TSI slant using a cotton swab equivalent to a no. 0.5 McFarlan standard in 1.25 ml of 0.85% NaCl. Place the API strip in the appropriate plastic holder, which contains several drops of distilled H_2O for humidification. Using a sterile Pasteur pipette, fill the tube section of each cupule with the suspension. Overlay the LDC (lysine decarboxylase), ODC (ornithine decarboxylase), and urea cupules with mineral oil. Incubate overnight at 37°C. Add 1 drop of Kovacs reagent to the IND (indole) cupule. Read the reaction within three minutes. Add 1 drop of KOH and 1 drop alpha-naphthol to the VP (Voges-Proskauer) cupule. Wait 5-10 minutes before reading. Add 1 drop of oxidase to the ESC (esculin) or PPA (phenylalanine deaminase) cupule. Read the reaction within 5 min. Read all reactions as described in the package insert and derive a code by scoring each set of three reactions on the strip. Refer to the API-20E Codebook for identification.

B. SEROTYPING OF SALMONELLA ISOLATES

After presumptive biochemical identification of an isolate as *Salmonella*, the isolate must be serotyped by agglutination reaction on a glass microscope slide. Make a dense suspension of bacteria by taking a good loop-full of growth from the TSI slant and emulsifying it in a drop or two of water placed on a slide. Add one drop of the appropriate antisera, being careful not to allow the dropper to touch the slide. Mix the bacterial suspension and antisera very well and observe under a bright light, by looking through the slide. If agglutination is present, the organism is positive for that group.

C. SEROTYPING OF SHIGELLA ISOLATES

After presumptive biochemical identification of an isolate as *Shigella* the isolate must be serotyped by agglutination reaction on a glass microscope slide. Make a dense suspension of bacteria by taking a good loop-full of growth from the TSI slant and emulsifying it in a drop or two of water placed on a slide. Add one drop of the appropriate antisera, being careful not to allow the dropper to touch the slide. Mix the bacterial suspension and antisera very well and observe under a bright light, by looking through the slide. If agglutination is present, the organism is positive for that group.

D. SEROTYPING OF VIBRIO ISOLATES

After presumptive biochemical identification of an isolate as *Vibrio cholerae* the isolate must be serotyped. Make a dense suspension of bacteria by taking a good loop-full of growth from the TSI slant and emulsifying it in a drop or two of water placed on a slide. Add one drop of the appropriate antisera, being careful not to allow the dropper to touch the slide. Mix the bacterial suspension and antisera very well and observe under a bright light, by looking through the slide. If agglutination is present, the organism is positive for that group.

E. ANTIBIOTIC SENSITIVITY TESTING

The standardized disc susceptibility test described here is a modification of the procedure described by Bauer, Kirby, Sherris, and Turck. The test is rapid, practical, and reproducible for detecting antimicrobial susceptibility of rapidly growing pathogens. The test is done using 15 x 100 mm sterile plastic Petri dishes filled with 60-80 ml of Mueller-Hinton agar.

1. Preparation of the Inoculum:

a. Select 4 to 5 similar-appearing colonies of the organism to be tested with a wire loop from a pure culture or the primary isolation plate.

b. Transfer these colonies (by touching the top of each colony) to a tube of 3 to 5 ml Muller-Hinton or typticase soy broth.

c. Incubate the tube at 35°C long enough (2 to 8 hours) to produce an organism suspension with moderate cloudiness. Dilute the broth culture with sterile saline or broth to obtain a turbidity equivalent to that of a turbidity standard obtained by adding 0.5 ml of 1.175% barium chloride dihydrate ($BaCl_2$ $2H_2O$) solution to 99.5 ml If 0.36 N (1%) sulfuric acid (0.5 McFarlan standard). The turbidity standard can be stored in the dark at room temperature for 6 months or more, provided the tube is sealed to prevent evaporation. The standard must be thoroughly mixed just before use, preferably on a vortex mixer.

2. Streaking plates.

a. Dip a sterile cotton swab into the inoculum, which should be barely turbid.

b. Rotate swab around inside of tube to squeeze out as much excess inoculum as possible.

c. Streak the swab over the agar surface evenly in three directions.

d. Allow inoculum to dry for 3-5 minutes with plate closed.

e. Place discs on the agar with a dispenser or sterile forceps. Press discs down gently on the agar with sterile forceps to insure even contact.

f. Place no more than about six discs evenly spaced on the plate.

g. Place antibiotics which diffuse well in the outer circle, and discs which produce smaller inhibition zones (such as vancomycin, colistin, and polymyxin B) in the central area of the plate.

3. Incubate plates immediately, or within 30 minutes at 35°C. Do not use higher temperatures because some methicillin-resistant staphylococci may be missed. Do not incubate in a candle jar.

4. Read plates after 18-24 hours incubation as follows:

a. Read the zone size around each disc against a dark background under reflected light. Measure zone diameters (including the 6 mm disc) with a ruler on the under-surface of the Petri dish without removing the cover. Carefully prepared templates may also be used to read zone diameters. (A reading of 6 mm indicates no zone.) If blood agar is used, measure the zones from the surface with the cover removed from the plate. End point: Complete inhibition of growth as judged by the naked eye, except for sulfonamides and

Proteus species.

b. Sulfonamides: Slight growth (80% or more inhibition) may occur throughout the zone of inhibition because some organisms grow through several generations before sulfonamide takes effect, and because some Mueller-Hinton agar contains thymidine which inhibits sulfonamide activity.

c. Strains of Proteus mirabilis and Proteus vulgaris may swarm into areas of inhibited growth around certain antimicrobics. The zones of inhibition are usually clearly outlined and this veil of swarming growth is ignored.

d. If rapid results are desired, zone diameters are often readable after 6-8 hours incubation but should be confirmed after overnight incubation.

e. Interpret zone sizes as shown in the table below.

ZONE SIZE INTERPRETATIVE CHART

Antibiotic or Chemotherapeutic agent	Disc potency	Diameter of zone of inhibition (mm) Resistant	Inter-mediate	Susceptible
Ampicillin when testing: Gram Negative enteric organisms and enterococci	10 μg	11 or less	12 - 13	14 or more
Cephalothin	30 μg	14 or less	15-17	18 or more
Chloramphenicol	30 μg	12 or less	13-17	18 or more
Erythromycin	15 μg	13 or less	14-17	18 or more
Nalidixic acid	30 μg	13 or less	14-18	19 or more
Streptomycin	10 μg	11 or less	12-14	15 or more
Tetracycline	30 μg	14 or less	15-18	19 or more
Trimethoprim-Sulfamethoxazole	1.25 μg 23.75 μg	10 or less	11-15	16 or more

G. TESTING FOR ST/LT TOXINS BY GM1 ELISA

1. Prepare GM1 in a concentration of 0.3 nmol/ml in sterile PBS (vials are supplied in concentrations of 120 nmol/ml). Coat ELISA plates with GM1. Add 100 ul of 0.3 nmol/ml to each well and incubate plates either at room temperature overnight, or at 37°C for 4 hours. Plates should be incubated stacked on top of one another in a humid chamber (such as a polyethylene bag lined with wet tissues).
Extra plates can be stored in the same humid chamber at 4°C for a maximum of 2 weeks.

2. LT TOXIN
Wash GM1-coated plates twice with sterile PBS. Block by adding 200 ul of 0.1% BSA diluted in sterile PBS to each well for 30 mins at 37°C. Incubate and cover each plate individually.

3. After incubation plates are washed once with sterile PBS. LB is prepared and immediately before use 45 ug of lincomycin and 2.5 mg of glucose are added per ml of the media. 100 ul of LB/ lincomycin/ glucose are then added to each well.

4. Pick one bacterial colony using a sterile wooden stick and inoculate into each well without scratching the bottom of the plate.
Use the same stick to inoculate a CFA agar plate (agar plates are divided into 6 parts, so 6 colonies can be inoculated in one plate). Three colonies are picked and used from each patient. Avoid inoculating columns number 1,12 and wells A and H since background readings are often attained when they are used.

5. Stack plates on top of one another and incubate them in a humid chamber in a shaking incubator at 37°C at 180 RPM overnight.

6. Next morning check for bacterial growth in each inoculated well by signs of turbidity. Also note turbidity in any of the uninoculated wells. Evidence of turbidity in uninoculated wells would indicate contamination which results in false background readings.
This plate represents the LT assay.

7. ST TOXIN

For ST toxin determination a new GM1-coated plate is washed twice in PBS. Block by adding 200 ul of 0.1% BSA in PBS to each well. Incubate as outlined above for the LT assay.

8. Wash the ST-plate once with PBS. Add 100 ul of ST-CTB conjugate diluted 1:300 in 0.1% BSA-PBS to each well. Stack plates and incubate at room temperature in a humid chamber for 60-120 mins.

9. Wash the ST plates 3 times in PBS. Transfer 50 ul of the culture medium from the LT plate to the corresponding wells in the ST plate. Immediately add 50 ul of the ST monoclonal antibody (culture medium 1:3) diluted 1:400 in 0.1% BSA-PBS to each well. Tap plates lightly to insure mixing and incubate plates stacked in a humid chamber at room temperature for 60-120 mins.

10. Wash the LT plate 3 times in PBS-Tween 0.05% and autoclave the discarded material. Add 100 ul of LT 39 monoclonal antibody diluted 1:100 in 0.1% BSA-PBS-Tween to each well.
Tap plates lightly and incubate as outlined above for the LT assay.

11. Wash all plates 4 times with PBS-Tween. Add 100 ul of anti-mouse horse radish peroxidase conjugate, diluted in 0.1% BSA-PBS-Tween to each well. (use Jackson no 115035062, lot 23494 is diluted 1:3500) Stack plates on top of one another and incubate for 90-120 mins at room temperature.

12. Prepare the developing substrate by dissolving 10 mg orthophenylene diamine (OPD) in 10 ml of 0.1 M sodium citrate buffer (pH 4.5) to which you add 4 ul of 30% hydrogen peroxide immediately before use.

13. Wash plates 4 times with PBS-Tween. Add 100 ul of freshly prepared OPD to each well. Wait 2 mins between each plate and the next. Wait 15-20 mins and read the first ST plate at 450 nm in a Titertek spectrophotometer. Read first all ST plates then the LT plates in the order of OPD addition.

14. The LT reaction represents a direct ELISA. The LT positive result equals absorbance value of 0.1 or higher above background. The ST reaction represents a competitive ELISA.
In non-toxin producing (negative) samples the monoclonal antibody is free to react with the ST-CTB conjugate to form a complex and a yellow color develops upon addition of the enzyme conjugate and the substrate. In toxin producing (positive) samples the toxin produced reacts with the monoclonal antibody added, no monoclonal antibody is free to react with the ST- CTB conjugate and so no color develops.
ST positives are taken to be 50% or more inhibition of absorbance of the ST background averaged with ST negative control cultures.

DETERMINATION OF BACTERIAL PATHOGENS
BY QUICK DOTBLOT TEST

1. Mark a sheet of nitrocellulose membrane (NC) into 5 mm square grid. Cut the grid into 20 cm strips and soak the strips in PBS.

2. Lay the strips on a paper towel and allow them to air dry for 5-10 mins.

3. Prepare bacterial suspensions from CFA agar plates using a sterile cotton swab and emulsifying a swab-full of bacteria in 200 ul PBS. Adjust the turbidity of the suspension to 4 on the McFarland standard turbidity scale.

4. Add 2 ul of each bacterial suspension to a marked position on the NC strips. Fifteen samples can be added on each strip and twelve duplicate strips are needed. Repeat the process for the reference strains, adding them in a fixed order to another set of twelve duplicate strips.

5. Allow samples to air dry for 5-15 mins, then place each strip in a separate well in an alternating form between sample and reference strips in the reaction trays provided.

6. Block all strips in 1% BSA in PBS for 15-30 mins with gentle rocking.

7. Discard the blocking solution then add 1.5 ml of monoclonal antibodies to each well. The same monoclonal is added to both sample and reference wells. Twelve monoclonals are available. CFA 1 and CS6 are diluted 1:20 in 0.1% BSA-PBS-Tween, while CS1, CS2, CS3, CS4, CS5, CS7, CS17, PCF0159, PCF0166 and CFA 3 are diluted 1:30. Incubate trays at room temperature for 90-120 mins with gentle rocking.

8. Wash 3 times each for 5 mins with PBS-Tween while shaking. Add 1.5 ml of anti-mouse IgG horse radish peroxidase(Jackson no 115035062) diluted 1:2000 in 0.1% BSA-PBS-Tween to each well. Incubate trays at room temperature for 90-120 mins with gentle rocking.

9. Wash 3 times each for 5 mins with PBS-Tween while rocking. Wash once more in TBS and develop with 4-chloro-1-naphthol hydrogen peroxide for 10 mins. Prepare substrate immediately before use by adding 1.7 ml of 4CN (3 mg dil in 99.9% methanol) to 8.3 ml TBS and 5 ul of 30% H_2O_2.

10. A positive reaction is represented by a blue dot graded +1 to +4 at the corresponding monoclonal.

11. Wash the membrane in tap water and air dry. Photocopy immediately as the membrane turns pale after long time storage.

H. TESTING FOR COLONIZATION FACTOR ANTIGENS (CFA'S)

E. coli colonies which test positive for the presence of LT and/or ST toxin on the GM1 ELISA, are subsequently tested for colonization factor antigens (CFA's) from the appropriate section of the original CFA agar plate which was stored in a plastic bag at 4°C. Results should be recorded on the **ETEC Results Form (ER)**.

I. REPORTING OF RESULTS

All test results should be noted on the **Microbiology Laboratory Results Form (LR)** belonging to that specimen. When completed, this form should be sent to the data house.

VII. MEDIA USED:

1. **Modified Cary-Blair Transport Medium** is an oxygen-reduced medium containing 0.16% agar which is provided in tubes containing a reduced O_2/increased CO_2 atmosphere. Cary-Blair preserves the rectal swab, prevents it from drying or changing pH for several days, so that the sample will not deteriorate on its way to the laboratory.

2. **MacConkey's Agar** (MAC) is a differential, mildly selective medium, which selects for gram-negative enteric bacilli by inhibiting gram-positive organisms with bile salts. After 24-hour incubation, lactose fermenters will appear pink, whereas non-lactose fermenting colonies will be colorless, grey, or white. Non-lactose fermenting colonies are potential pathogens and will be subcultured to biochemicals for further identification. MacConkey selects for *E. coli, Aeromonas, Plesiomonas, Shigella* and *Salmonella* spp. Some *V. cholerae* will grow as small, non-lactose fermenting colonies on this agar.

3. **Salmonella-Shigella Agar** (SS) is a differential, moderately selective medium which inhibits gram-positive organisms and most of the family Enterobacteriaceae with bile salts and selects for *Salmonella*. It is somewhat inhibitory to *Shigella* spp. After 24-hour incubation, lactose fermenters will appear red-pink, whereas non-lactose fermenting colonies will be colorless, grey, or white with or without black centers. Non-lactose fermenting colonies should be subcultured to biochemicals for further identification. SS agar may inhibit some *Shigella* species.

4. **Alkaline Peptone Water** (APW) is a non-selective enrichment medium for Vibrio. Incubate at 35-37°C for 6-8 hours, then subculture to TCBS agar. If incubation continues beyond 8 hours (it should not), take two loop-fulls from the top two centimeter-layer of the APW and subculture to a fresh tube of APW for 2-4 hours. Then plate out to TCBS.

5. **Thiosulfate Citrate Bile Salts Sucrose Agar** (pH 8.6) is a selective medium for *Vibrio* which requires heavy inoculation and incubation at 35°C for 18-24 h (use a minimum of 2-3 loopfulls of specimen or heavy streaking with a swab from Cary-Blair). TCBS can also be inoculated with two loopfulls from the surface of an enrichment medium such as alkaline peptone water after overnight incubation. *Vibrio cholerae* appears as a medium-sized, smooth, yellow colonies with opaque centers and translucent periphery. *Vibrio parahaemolyticus* appears as blue-green colonies on this agar.

6. **Brucella Broth** is used as a nutritive broth into which the rectal swab is stirred. The brucella broth is then poured onto a 0.65 μm pore-size cellulose acetate membrane filter placed on a sheep's blood agar plate for isolation of *Campylobacter*. *Campylobacter* will pass through this filter, due to its small size, whereas other bacteria will not be able to pass through, allowing selection for this organism without the use of antibiotics.

7. **Blood Agar Plate** is used with a 0.65 μm pore-size cellulose acetate membrane filter for isolation of *Campylobacter*. A fresh 0.65 μm pore-size cellulose acetate membrane filter is placed on the plate and 1 ml of Brucella broth with a rectal swab suspension is poured onto the filter (not the agar itself). Incubate at 42°C in a microaerophilic environment (5% O_2, 10% CO_2, 85% N_2). Remove filter after overnight incubation and continue to incubate at 42°C in a microaerophilic environment for another 24 hours.

8. **Selenite F Broth** is for selective enrichment used to improve the recovery of *Salmonella* and *Shigella*, but mainly *Salmonella*. Growth of other bacteria is suppressed. By subculturing during the early incubation period, the chances of detecting low numbers of pathogens are increased. Approximately 1 g of stool specimen is inoculated into the broth (10 ml), mixed well and incubated at 37°C for 12-18 hours. The growth is streaked on SS agar.

9. **Phosphate Buffered Saline** (PBS): One of the rectal swabs is placed in PBS and frozen at -70°C or lower for subsequent virus culture. Due to the presence of a buffer, pH changes which may kill sensitive pathogens are minimized.

10. **Triple Sugar Iron Agar** (TSI): A differential medium for gram negative rods on the basis of their abilities to use carbohydrates fermentatively and to liberate H_2S. This orange-red agar medium contains 0.1% glucose, 1.0% lactose, and 1.0% sucrose. The medium contains phenol red to indicate changes in pH and ferrous sulfate to detect the formation of H_2S. The presence bubbles or splitting in the agar indicates gas production by the organism. A black precipitate in agar, seen as black streaks, or blackening of the entire tube indicates the organism produces H_2S. Examples of reactions (never incubate more than 24 hrs, as readings change):

slant: yellow (A = acid) butt: yellow (A = acid): the organism ferments glucose, lactose and/or sucrose, written as A/A. (i.e. *E. coli*: A/A, + Gas, -H_2S)

slant: red (K = alkaline) butt: yellow (acid): the organism ferments glucose, but no lactose or sucrose, written as K/A. (i.e. *Salmonella typhimurium*: K/A, + Gas, + H_2S; or *Shigella flexneri*: K/A, -Gas, -H_2S)

slant: red (K = alkaline) butt: red (K = alkaline): the organism cannot ferment glucose, sucrose, or lactose ?, written as K/K.
(i.e. *Pseudomonas*: K/K, -Gas, -H_2S)

11. **Motility Indole Ornithine** (MIO): This semisolid medium tests motility by the formation of cloudiness along the stab-line/H_2S ppt. (in positive cultures) and is suitable for detecting indole by Kovac's. The indole test is done by adding 0.5 ml of Kovac's reagent down the side of the tube. The ability of the organism to decarboxylate ornithine is indicated by the color of the medium.

Motility test:

positive (motile): presence of cloudiness throughout the medium. (i.e. *E. coli*)
negative (nonmotile): cloudiness present only along the stab-line. (i.e. *Shigella sonnei*)

Indole test:

positive: development of a bright fuchsia-red color at the interface of the reagent and the broth within a minute of addition of Kovac's reagent. (i.e. *E. coli*)
negative: no color development within a minute of addition of Kovac's reagent. (i.e. *Shigella sonnei, Salmonella typhi*)

Ornithine decarboxylase test:

positive: development of a turbid purple to faded yellow-purple color in the media. (i.e. *Shigella sonnei, Salmonella* spp.)
negative: development of a bright yellow color in the media. (i.e. *Shigella boydii, Shigella dysenteriae, Shigella flexneri, Salmonella typhi*)

12. **Urea agar/ Urea broth.**
positive: bright pink
negative: straw color, no change

13. **Colonization Factor Antigen agar** (CFA): Used for detecting colonization factor antigens in *E. coli*.

14. **Microbank Tubes** are used for the conservation of stock strains or bacteria which need to be subcultured repeatedly. Collect a thick amount of bacteria with a sterile cotton swab from the TSI tube and inoculate into a 1 ml tube of glycerol broth. The swab is twirled well to disperse the bacteria wiped against the side of the tube and discarded. The glycerol broth is added to duplicate "microbank" vials, which are labeled with the specimen number. The glycerol broth is left in the "microbank" vials for 30 minutes, after which the fluid is removed from the microbank vials using a sterile Pasteur pipette, so that only the glass beads remain. Vials are capped tightly and immediately frozen at -70°C. One aliquot will be shipped to NAMRU-3, the second aliquot is kept at the University of Alexandria.

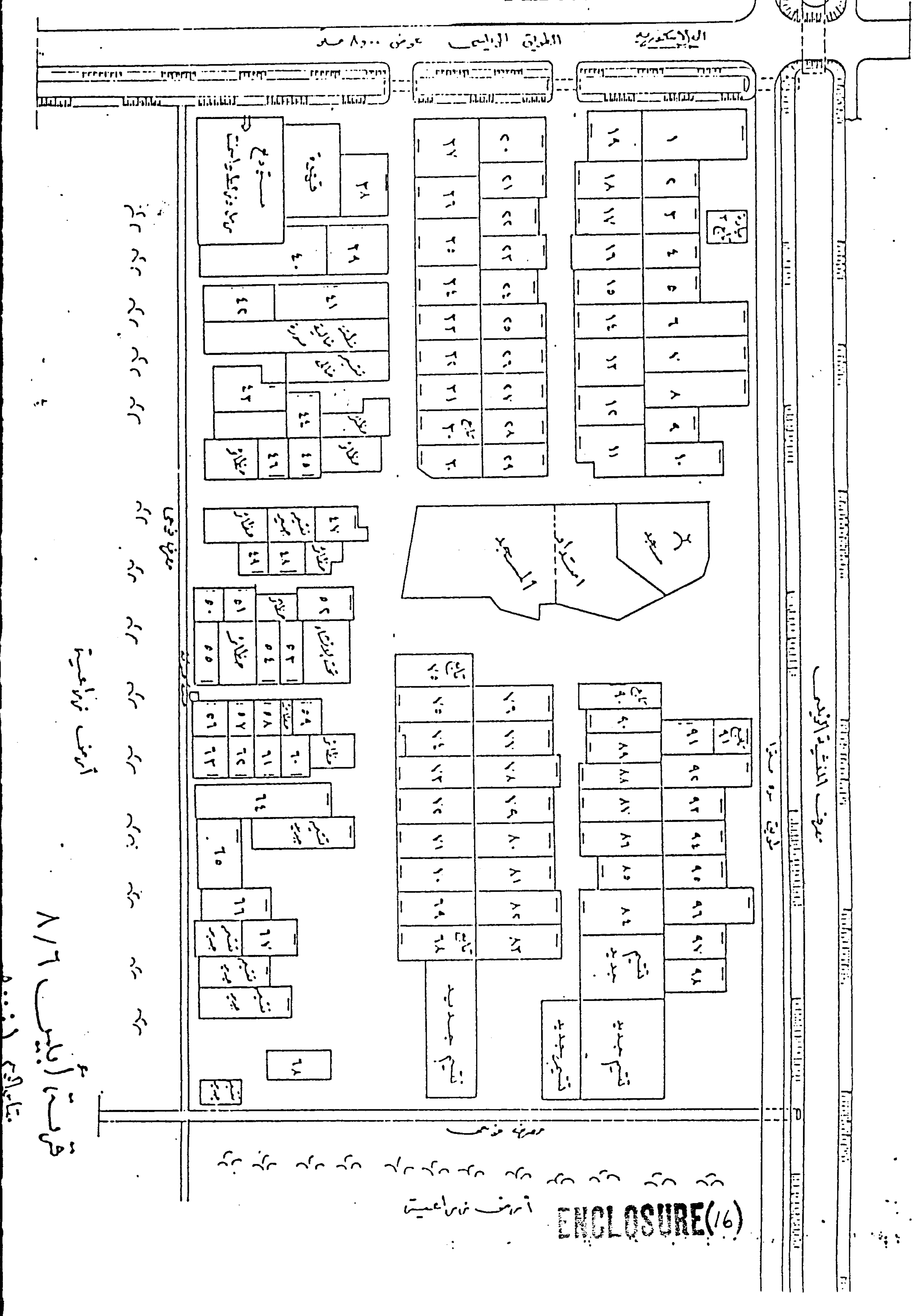
15. **Luria Bertani Broth** (LB broth): Used in ST/ LT toxin testing. See procedure for GM-1 ELISA.

VIII. STORAGE OF ISOLATED PATHOGENS

Isolated pathogens should be stored in cryotubes deeps with a non-carbohydrate containing medium, such as tryptic soy agar, brain heart infusion agar, or sheep's blood agar. Don't use nutrient agar, which does not contain any salt.


معرف المنشية - الرئيسي
الدرجة بين الرئيسية ـــ طريق رئيسي
ABEES 800
مسجد
مضيفة
حظائر
بدون ترقيم
تابع
بديد
أرض زراعية

ABEES 806



ENCLOSURE(16)

ABEES 807

(٦) ENCLOSURE