To : Chairman
Research Review Committee

From : Dr. Khaleda Haider Khaleda Haider

Subject : My response to the comments offered by two reviewers regarding protocol entitled "Genetic analysis and phenotypic correlation of the plasmids universally present in strains of *S. dysenteriae* type 1.

The protocol has been reviewed by Dr. Glen Morris, Center for Vaccine Development, University of Maryland, School of Medicine, USA, and Dr. Zia Uddin Ahmed, Senior Scientist, ICDDR,B. We have modified the protocol in the light of reviewers' comments and the changes made are summarized as follows:

1. Both reviewers found the subject interesting and relevant, but found it too ambitious and, therefore, lack focus. We have, therefore, reduced the number of parameters which originally were going to be tested.

2. In accordance with both the reviewers suggestions, we have rewritten the research plan so as to study one factor at a time: OMP in the first year and LPS in the following year. During the third year we intend to study the specific regions of plasmid associated with changes in LPS and/or OMP profiles as outlined on page 11. This change has made the experimental design more focused as desired by the reviewers.

O D

Date 7 Aug 90

tachment 1.
ACE SHEET)

ETHICAL REVIEW COMMITTEE, CDDR,B.

incipal Investigator Mohlala-Lois

Trai nvestigator (if any)

plication No. 90-015

Suppo rting Agency (if Non-ICDDR,B)

tle of Study Assessment of the feasibility of the deuterium dilution technique for measurement of breast milk intake

Project status:
( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

rcle the appropriate answer to each of the following (If Not Applicable write NA).

Source of Population:
(a) Ill subjects — Yes (No)
(b) Non-ill subjects — (Yes) No
(c) Minors or persons under guardianship — (Yes) No

Does the study involve:
(a) Physical risks to the subjects — Yes (No)
(b) Social Risks — Yes (No)
(c) Psychological risks to subjects — Yes (No)
(d) Discomfort to subjects — Yes (No)
(e) Invasion of privacy — Yes No marginal
(f) Disclosure of information damaging to subject or others — Yes (No)

Does the study involve:
(a) Use of records, (hospital, medical, death, birth or other) — Yes (No)
(b) Use of fetal tissue or abortus — Yes (No)
(c) Use of organs or body fluids — (Yes) No (urine)

Are subjects clearly informed about:
(a) Nature and purposes of study — (Yes) No
(b) Procedures to be followed including alternatives used — (Yes) No
(c) Physical risks — (Yes) No
(d) Sensitive questions — Yes No NA
(e) Benefits to be derived — (Yes) No
(f) Right to refuse to participate or to withdraw from study — (Yes) No
(g) Confidential handling of data — (Yes) No
(h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure — Yes No NA

5. Will signed consent form be required:
(a) From subjects — Yes (No)
(b) From parent or guardian (if subjects are minors) — (Yes) No

6. Will precautions be taken to protect anonymity of subjects — (Yes) No

7. Check documents being submitted herewith to Committee:
___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
✓ Protocol (Required)
✓ Abstract Summary (Required)
✓ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
___ Informed consent form for subjects
✓ Informed consent form for parent or guardian
✓ Procedure for maintaining confidentiality
___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

gree to obtain approval of the Ethical Review Committee for any changes olving the rights and welfare of subjects before making such change.

Principal Investigator

Trainee

SECTION I - RESEARCH PROTOCOL

Title: Assessment of the feasibility of the doubly labelled water technique for measurement of breast milk intake by Bangladeshi infants in rural communities

Principal Investigator(s): Dilip Mahalanabis, ICDDR,B, Kenneth Brown and K.G.Dewey, University of California, Davis (UCD).

Co-investigator(s): Dr. ASG Faruque, ICDDR,B
William Watkins, UCD

Starting date: September 1, 1990

Completing date: February 28, 1991

Total direct cost:

Scientific Programme Head: Dilip Mahalanabis, Clinical Science Division

ABSTRACT SUMMARY

Field trials will be conducted to assess the possibility of the doubly labelled water technique and the 24-hour test-weighing technique to measure breast milk intake by Bangladeshi infants in rural communities. The doubly labelled water technique relies on a single oral dose of two stable (non-radioactive) isotopes of water, ^{2}H and ^{18}O, preceded by a single sample of urine and followed by interval sampling of urine for seven days. The changing abundance of these two naturally occurring isotopes in excreted urine can be used to calculate the amount of breast milk consumption as well as the energy content of breast milk. At the same time the consumption of non-breast milk foods and liquids will be measured by a locally recruited female field worker. During the final two days of the dilution tests, 24-hour test-

weighing will be completed. For these studies, the diapered infants will be weighed before and after each breastfeeding episode by the same field workers.

1. Because the project will assess techniques to measure breast milk intake, nursing infants and young children between 4 and 18 months of age will be invited to participate. Consent for participation will be requested from the infants' mothers.

2. There are no physical or other risks associated with the study. ^{2}H and ^{18}O are naturally occurring stable (non-radioactive) isotopes. These substances have been used in many studies of premature infants, infants, and children and are considered completely safe in the doses proposed.

^{18}O is relatively abundant, forming approximately 0.2% of the oxygen found in the environment. The relative abundance of ^{2}H is 0.015%. The dosage proposed for our study is extremely low (0.28 g $H_2{}^{18}O$/kg body weight and 0.10 g $^{2}H_2O$/kg body weight). With these doses, ^{18}O concentration is increased by approximately 20% over natural abundance and ^{2}H concentration is increased by 100%.

The level of enrichment of ^{18}O is similar to the range of variation in the environment: seasonal changes in the ^{18}O content of rain water in Chicago, for example, are approximately 2% of natural abundance, while differences in polar versus equatorial water are about 5%. Furthermore, elevation in the plasma levels of doubly-labelled water after ingestion of the isotopes by the

subjects is relatively short-lived, as the isotopes are progressively diluted out by subsequent water ingestion. The biological half life of these isotopes is approximately 6 days.

The safety of deuterium in human studies has been demonstrated in studies of total body water over the past two decades. This method has been used repeatedly in children and infants both in developed and developing countries with no adverse consequences.

The safety of ^{18}O has been extensively studied in mice, to which exceedingly high levels of ^{18}O were given for three successive generations. These mice were raised on almost pure $H_2{}^{18}O$ and ^{18}O. These techniques achieved an average body water $^{18}O/^{16}O$ ratio of 38 to 54%. No histological abnormalities were found and the mice were observed to live, grow and reproduce normally.

The use of ^{18}O in human subjects has begun only in the last decade, due to the cost of the enriched ^{18}O isotope and the relatively new capability of mass spectometers to analyze low levels of enrichment. Several investigators have reported using doubly-labelled water to determine energy expenditure in adults and infants with no adverse effects being noted.

Reviews:

Research Review Committee: _____
(approved/not approved)

Ethical review Committee: _____
(approved/not approved)

Director' signature: _____

Research Plan

1. OBJECTIVE

To develop accurate, acceptable methods to measure breast milk consumption by infants and young children in rural Bangladeshi communities.

2. BACKGROUND & RATIONALE

Proposed future studies of the relations between infant feeding, nutrient intake, risk of infection and physical and behavioural development will require accurate, non-invasive measurements of the children's breast milk consumption. Available techniques include (1) around-the-clock test-weighing of infants before and after each nursing episode, (2) manual or mechanical expression of all milk for measurement prior to feeding, and (3) newly developed stable isotope dilution techniques. In addition, measurement of the amounts of nutrients consumed from breast milk requires collection of representative milk samples for analysis of milk nutrient composition.

Previous studies in rural Bangladesh have relied on 12-hour test-weighing and extrapolation of the observed 12-hour intake to estimate total 24-hour consumption (Brown, 1982a). Although this method can provide reasonably accurate and precise estimates for groups of children, it is not able to measure individual intake sufficiently accurately because of the substantial variability in the proportion of 24-hour intake that individual children consume during the 12 daytime

hours. Moreover, test-weighing is disadvantageous because of the need to manipulate the baby before and after each feeding, thereby interfering with the usual nursing practices. Expression of milk is likewise undesirable because of the interruption of customary feeding patterns and the uncertainty whether the amount expressed truly reflects the amount that the infant would have ordinarily consumed. Thus, use of the isotope dilution technique would be potentially advantageous if it proves to be feasible and acceptable for application in Bangladeshi households.

The deuterium dilution technique is based on the rationale that a fixed oral dose of this stable (non-radioactive) isotope of hydrogen will equilibrate with total body water. The deuterium label will be excreted quantitatively in relation to the amount of water intake, following the kinetics of an open, one-compartment pharmacologic model. Thus, water intake during specific intervals post-dosing can be estimated from knowledge of the original dose and the concentration of deuterium in body fluids, such as urine, saliva, or blood, at intervals following the initial dose. If all sources of non breast milk water intake are known and the amount of total water intake can be estimated from the deuterium dilution techniques, then the amount of breast milk intake can be calculated as the difference.

As stated, the application of the deuterium dilution technique

requires knowledge of all non-breast milk sources of water intake. These sources include so-called "preformed" water given as liquids or included in foods, as well as the water derived from oxidation of foods (including breast milk). Also, non-labeled atmospheric water may enter the body through the skin and lungs. Thus, each of these other sources of water intake must be either measured or estimated during the period of the deuterium dilution study.

The major advantage of the deuterium dilution technique is its ability to measure breast milk intake under natural conditions for relatively long (4-7 days) periods of time. Its disadvantage is the need to measure other sources of water intake, the need to employ incompletely validated assumptions regarding biologic isotopic fractionation (for example,with transcutaneous water exchange), and the cost of the isotope itself and the necessary analytic procedures. In addition to the deuterium dilution technique, which can accurately estimate the volume of milk intake but not its nutrient content, the use of a second isotope has recently been proposed to assess indirectly the energy consumption from breast milk (Lucas,1990). By using doubly-labeled water (that is, water containing a stable isotope of oxygen, ^{18}O, as well as a stable isotope of hydrogen, ^{2}H)it is possible to measure total energy expenditure. The rationale for this technique is explained below:

As described above, labelled hydrogen (deuterium) exits the

body as water in direct proportion to the intake of unlabelled water. The isotopic flux of ^{18}O is somewhat different because the labelled oxygen in water can also exchange with the oxygen in CO_2 and thereby be excreted in expired air as well as in water. Thus, the difference in the rate of isotopic turnover of ^{18}O and ^{2}H is directly proportional to the amount of CO_2 production and excretion. CO_2 production, in turn, is directly related to oxygen consumption, which for a given dietary respiratory quotient is proportional to energy expenditure.

Once the total energy expenditure is known, total energy intake can be calculated as the sum of energy expenditure and energy stored. Energy stored can be estimated from the change in body weight during the interval of observation and the energy content of the tissue gained. Energy intake, in turn, can be used to calculate the energy intake from breast milk, which is the difference between total energy intake and intake from non-breast milk sources. Thus, by using this doubly labelled water technique it is possible to estimate both the volume of breast milk intake as well as its energy content.

Before it will be possible to decide which measurement technique is most appropriate to use in future studies of breast milk intake in rural Bangladesh, it will be necessary to assess the feasibility of the doubly labelled water technique during preliminary field work. This preliminary

experience will provide the investigators with the opportunity to modify the proposed techniques as necessary to suit local conditions. Moreover, the data derived should provide potential funding agencies with the confidence that the future studies can be successfully implemented.

B. Specific Aims

1. To conduct ten doubly labelled water studies to measure breast milk intake under realistic field conditions in rural Bangladeshi homes.

2. To conduct a limited number of 24-hour test-weighing studies of the same children to compare the relative feasibility of the two techniques.

C. Methods

1. Deuterium dilution technique:

a. Subjects: 10 non-ill male or female infants between 4 and 18 months of age.

b. Site: rural households selected for convenience to ICDDR,B facilities and for willingness of families to collaborate.

c. Procedures:

On the first morning of the isotope study, a baseline urine sample will be collected from the infant to

determine the naturally occurring concentrations of ^{2}H and ^{18}O. Samples will be obtained either with plastic urine collection bags or by placing cotton balls inside clean disposable diapers and expressing the urine from the cotton with a plastic syringe. After collecting this sample, and approximately one hour after the previous feeding, the infant will be weighed to the nearest gram and an accurately weighed dose of sterile isotope providing approximately 0.28 g $H_2{}^{18}O$ and 0.1 g $H_2{}^{2}O$ per kilogram of body weight will be administered by mouth in a volume of about 13 ml of water, using a plastic syringe and tubing. The dose will be flushed through with an additional 2 ml of sterile water. The next feeding will be withheld for approximately 1 hour to allow rapid mixing and absorption of the dose and to avoid any regurgitation. Each infant will be observed for 1 1/2 hours after dosing in order to weigh any regurgitated fluid using pre-weighed tissues. Timed urine samples will be collected at 4-5 hours post-dose and subsequently once a day (in the morning) for the next seven days, using a new plastic syringe each time. Samples will be placed in a cold box in the home and transported to the lab for storage at -20°C. On the morning of the seventh day, infants will again be weighed to the nearest gram approximately one hour after a feeding.

Throughout the study it will be necessary to measure all non-breast milk liquids and foods that are consumed. Initially, this will be accomplished by weighing all items at the time of preparation and at the time of feeding. Detailed descriptions of these methods have been published previously from studies in rural Bangladesh (Brown, 1982b). During the first day of observation the dietitian will inquire whether any foods or liquids are usually offered to the child at night. If so, attempts will be made to pre-weigh these items and place them in special containers so that they can be reweighed the following morning to determine the amounts consumed. Our previous experience in this setting suggests that night time consumption of non-breast milk items by children in this age group is very unlikely.

Baseline and enrichment levels of deuterium and ^{18}O will be determined by isotope ratio mass spectometry. Linear regression equations obtained from the log transformed enrichments of ^{2}H and ^{18}O versus time will be used to calculate the disappearance rates of each isotope. In growing infants, it is also necessary to adjust for the change in isotope dilution space over the period of the study. Baseline dilution spaces will be determined from the intercept of each regression line. The dilution spaces at the end of the isotope study will be estimated by assuming that they change in proportion to body weight.

Milk intake using the deuterium dilution method will be calculated using the method of Lucas and Davies(1990)and Fjeld et al (1989). Apparent water influx will be corrected for isotopic fractionation using several approaches; the calculations of insensible water loss made for each infant (described below) will permit an estimate of the proportion of water output that is subject to fractionation in each case. Environmental water influx via respiratory and cutaneous surfaces will be estimated using the technique described by Fjeld et al based on direct measures of ambient temperature and humidity, and will be substracted from total water influx to obtain net water influx via milk.

The infants' energy expenditure will be calculated from the disappearance rates of both isotopes using the methods of Lucas and Davies, assuming an RQ of 0.87 for breast-fed infants. To calculate total energy intake, energy stored in new tissues during the period of the study must be added to energy expenditure. Energy stored will be determined as follows: a) total body water (TBW) at the beginning of the study will be calculated from ^{18}O dilution space, and TBW at the end of the 7-day study will be estimated assuming that it represents the same proportion of body weight, b) increase in lean body mass and protein during the study will be determined from the change in TBW, using published values for the water and protein

content of lean tissues at this age, c) increase in body fat will be calculated as the difference between body fat (body weight - lean body mass) on day 0 and body fat on day 7, and d) conversion factors of 9.25 kcal/g of fat and 5.6 kcal/g of protein stored will be used to determine total energy stored (carbohydrate storage will be assumed to be negligible).

2. Twenty-four hour test-weighing:

a. Subjects and site: same as above

b. Procedures:

If possible, the same subjects included in the doubly-labelled water studies will be included in the 24-hour test-weighing trials. This will permit an assessment of the relative acceptability of the two techniques in the same individuals. (Although a comparison of the estimates of breastmilk intake provided by the two techniques would be interesting, the small number of subjects in this feasibility trial will not provide sufficient statistical power for these comparisons).

The test-weighing procedures have been described previously in detail (Brown, 1982a). Briefly, diapered infants will be placed on a battery-operated electronic balance sensitive to 1 gram (Sartorious model 3826). The infant will be weighed again immediately after the feeding (without changing the diaper or clothes) and the time

interval will be recorded to correct for the insensible water loss (IWL) that will have occurred during that period of time. IWL will be estimated for each infant by weighing the child before and after two 30-minute intervals each day. During these half-hour periods no food, breast milk or liquid will be given. The average IWL per minute will be calculated for each infant and multiplied by the total time elapsed during feedings in the test-weighing record. This estimate of total IWL during feedings will be added to the milk intake measurement by test-weighing to obtain corrected milk intake data. The complete set of 24-hour test-weighing studies will be conducted for two days (if possible) to assess day-to-day variability in intake. This will permit the calculation of the appropriate number of days of observation that will be required if this technique is used in future studies.

3. Interpretation of data

These preliminary trials will be used to assess the feasibility of the proposed techniques for implementation in the field setting. As such, no specific scientific hypotheses are being tested and no formal estimate of sample size has been computed. If the procedures can be completed without difficulty, fewer than the stated number of studies may be conducted. On the other hand, any procedures found to be unacceptable may be modified in order to make them more appropriate for field application.

In either case, the laboratory analyses and descriptive statistical analyses will be completed to help in the assessments of the quality of the procedures and to assist with future sample size determinations.

4. Schedule of Studies

Each set of studies will last 7 days. In order to complete the effort within one month, three teams will work simultaneously. Each team will be composed of two locally recruited female workers who will share responsibilities for weighing of food preparation and consumption, collection of urine specimens, and test-weighing. The first day of each set of studies will be staggered so the field supervisors can be present to weigh the children and administer the isotopes in each case. The start-days have been further staggered because only one electronic balance is currently available (see table).

A total of two months' time has been budgeted for each of the field personnel to allow for training, initial contact with study families, and other contingencies.

D. SIGNIFICANCE

The experience gained through the feasibility trials will permit rational planning of subsequent studies relating infant's nutrition, risk of morbidity, and physical and

behavioural development. The same information will be useful to develop field studies of women's lactational performance. Because the proposed techniques have not been used previously in field settings in Bangladesh, it is unlikely that funding agencies would be willing to support the larger studies without preliminary evidence of successful implementation of the field techniques. Thus, this initial effort is critical to the development of these later community-based investigations.

E. FACILITIES REQUIRED

1. Access to families in rural villages (transportation to field, introduction to local authorities and individual families).
2. Freezer space for sample storage.
3. Administrative support for form development, purchasing of supplies, personnel management.
4. Data analysis (access to computer).

F. COLLABORATIVE ARRANGEMENTS

The studies will be completed as a collaborative project between investigators at ICDDR,B and members of the Program in International Nutrition at University of California, Davis.

REFERENCES

1. Brown KH, Black RE, Robertson AD, Akhtar NA, Ahmed G, Becker S. Clinical and field studies of human lactation: methodological considerations. The Am J of Clin Nutr 35: pp 745-756. April 1982(a).

2. Brown K, Black RE, Becker S, Nahar S, Sawyer T. Consumption of foods and nutrients by weanlings in rural Bangladesh. The Am J of Clin Nutr 36: pp 878-889. November 1982 (b).

3. Fjeld CR, Brown KH, Schoeller DA. Validation of the deuterium oxide method for measuring average daily milk intake in infants. Am J Clin Nutr 48: pp 671=9. 1988.

4. Lucas A and Davies PSW. Physiologic energy content of human milk. In: Atkinson SA, Hanson LA and Chandra RK (eds), Breastfeeding, Nutrition, Infection and Infant Growth in developed and Emerging Countries. Nrefoundland, Canada: ARTS Biomedical Publishers and Distributors, 1990, pp 337-357.

CONSENT FORM

Title of Project: Feasibility of labelled water technique to measure breastmilk intake by Bangladeshi children in rural communities.

Purpose: Staff member of the ICDDR,B request you and your breast feeding child to participate in a study to measure the amount of breast milk that he/she consumes.

Procedures: If you decide to volunteer, the following procedures will be completed.

1. A local female worker will come to your home to weigh your child and to collect a specimen of urine using a diaper or a collection bag. Then a small dose (1 tbs.) of "heavy" water will be given to the child by mouth. This form of water can be mesured in the urine and permits an estimate of how much breast milk is consumed. This type of water occurs naturally and is not harmful.

2. During the next seven days the female worker will come to your home to measure all foods and liquids that your child consumes. On the morning of each visit an additional sample of urine will be collected.

3. During the last two days of the study, the worker will weigh your child before and after every nursing. This will provide an alternative means of estimating milk intake.

Risks: There are no physical or other risks imposed by these studies.

Benefits: This study will not be of immediate direct benefit to you or your child. However, when the results of the dietary intake studies are available, they will be shared with you, and any necessary dietary counselling will be provided. The studies are of benefit to society as a whole because the procedures that are developed will permit more extensive evaluation of infant feeding in rural Bangladesh.

Confidentiality: Information from the study will be used only by the investigators at ICDDR,B and will not be shared with others.

Right to refuse or withdraw: You may refuse to participate in the study at any time. You may change your mind about being in the study and withdraw once it has started.

If you agree to participate in this study, please indicate that you understand the purpose and methods and that you are willing to participate by signing below. If you have any questions now or later please ask us at any time.

_____ _____

Date Signature of participant

_____ _____

Date Signature of witness

SUMMARY - BUDGET

Personnel Cost	#	% of time	# months	US$
1. Dr. D. Mahalanabis		10%	3	-
2. Dr. ASG Faruque		100%	3	3,486
3. Field supervisor	1	100%	2	788
4. Field attendant	1	100%	2	428
5. Community H.Workers	6	100%	2	720
6. Local consultant	1	30%	2	600
				6022

Supplies	260
Printing & Communication,rent etc	100
Transportation	1,227
Training cost for staff members	60
Weighing scales (3)	900
Guest House Charges for William Watkins	1,800
Total	10,369

Table 1: Proposed schedule of Feasibility

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Team 1	W_1	-	-	-	-	TW_1	TW_1					W_4	-	-	-	-	TW_4	TW_4					W_7	-	-	-	-	TW_7	TW_7
Team 2			W_2	-	-	-	-	TW_2	TW_2					W_5	-	-	-	-	TW_5	TW_5										
Team 3					W_3	-	-	-	-	TW_3	TW_3					W_6	-	-	-	-	TW_6	TW_6							

W = Initial weighing of infant and dosing with stable isotope

TW = 24-hour test-weighing studies