PROTOCOL

Placebo-Controlled, Randomized Trial of an Intervention using Probiotics in the Prevention of Neonatal Infection in Community-born Infants

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ABBREVIATIONS AND ACRONYMS

AE	Adverse event
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AWC	Anganwadi center
AWW	Anganwadi worker
BBS	Bhubaneswar
BT	Bacterial translocation
CFU	Colony forming units
CONS	Coagulase negative staphylococci
CRF	Case report form
CSF	Cerebrospinal fluid
DCC	Data coordinating center
DMC	Data monitoring committee
DMS	Data management system
EOS	Early onset sepsis
FDA	Food and Drug Administration
FOS	Fructo-oligosaccaride
GCP	Good clinical practices
GI	Gastrointestinal
GLP	Good laboratory practices
GM	Gram
GMP	Good manufacturing practices
GN	Global Network for Women's and Children's Health Research
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocytes
IGH	Ispat General Hospital
IMR	Infant mortality rate
IRB	Institutional Review Board
JAM	Junctional adhesion molecule
LGG	L. casei
LOS	Late onset sepsis
NEC	Necrotizing enterocolitis

NICHD	National Institute of Child Health and Human Development
NIH	National Institutes of Health
NMR	Neonatal mortality rate
OHRP	Office of Human Research Protection
PFGE	Pulse field gel electrophoresis
PI	Principal Investigator
Probiotics	Health-promoting friendly bacteria
RCT	Randomized controlled trial
RKL	Rourkela
RTI	Research Triangle Institute
RU	Research unit
RUDC	Research unit data center
SAE	Serious adverse event
SCB	Sri Rama Chandra Bhanja
SFI	Senior Foreign Investigator
TBA	Traditional birth attendant
TJ	Tight junctions
TLR	Toll-like receptor
UMB	University of Maryland at Baltimore
US	United States
UTI	Urinary tract infection
VBLW	Very low birth weight
WHO	World Health Organization
ZO-1	Zona Occludens-1

PROTOCOL SUMMARY

Full Title:	Placebo-controlled, double blind, randomized trial of an intervention using probiotics and FOS in the prevention of neonatal infection in community-born Indian Infants
Short Title:	Prevention of infection in Indian neonates using synbiotics
Principal Investigator:	Dr. Pinaki Panigrahi
Senior Foreign Investigator:	Dr. Sailajanandan Parida
Sample Size:	8,842 including 400 in a community pilot for training and establishing the logistics of implementation (8,442 will be included in the analysis)
Study Population:	This will be a population-based study. All infants born in the selected villages will be eligible for enrollment. Neonates will be selected from among 140-200 villages in the four blocks in Khurda and Sundergarh districts. Village/Anganwadi center. Adoption of study villages will be finalized during the community pilot phase.
Participating Sites:	Capital Hospital, Bhubaneswar and Ispat General Hospital, Rourkela
Study Design:	A double-blind, randomized, placebo-controlled study to test the effectiveness of a prophylactic oral administration of a *L. plantarum* + FOS preparation to community-born neonates in reducing the incidence of neonatal sepsis.
Study Intervention:	A probiotic supplement, GastroPlan (*Lactobacillus plantarum,* ATCC 202195 and Fructo-oligosaccharide), will be administered to neonates within the first three days of life and continued for seven days. All infants will be monitored daily during the first 60 days of life.
Primary Outcome:	Death plus Clinical Sepsis (Possible Severe Bacterial Infections-PSBI)
Secondary Outcomes:	Other infections, diarrhea

1. KEY ROLES

Duties of the Research Unit

The research unit is responsible for initial and ongoing training of all personnel and health professionals involved in the study. The research unit is also responsible for implementing the study protocol as approved by the U.S. and Indian Institutional Review Boards (IRBs). Other duties involve data collection, data entry, and data transmission on a timely basis as well as prompt reporting of protocol violations and adverse events to the PI. The research unit also assumes responsibility for disseminating the study findings through publications, conference presentations, and meetings and seminars with stakeholders.

Each site (Bhubaneswar and Rourkela) under the Research Unit, at a minimum, will train one medical officer (pediatrician), several study supervisors, a microbiology officer (microbiologist), a laboratory technician and data entry staff. One community health volunteer from each village, mid level managers, and supervisors will be hired and trained in a three tier structure. In addition, the Anganwadi workers (AWWs) in each participating community will be trained. Each site has an appointed site director who will work in concert with the SFI and take charge of the scientific and administrative activities at their respective sites. They will be assisted by a Program Officer. The senior foreign investigator (SFI) will be responsible for the overall execution of study aims and will supervise the Indian sites. The director of field operations will be in charge of the overall activities in the community (both Rourkela and Bhubaneswar).

Key personnel from the Research Unit include:

- Pinaki Panigrahi, MD, PhD, Principal Investigator – Pediatric Infectious Disease, Microbial pathogenesis, gut microbiota, clinical research
- Ira H Gewolb, MD, Co-Investigator – Clinical Research, Neonatology
- Nigel Paneth, MD, Co-Investigator – Pediatric Epidemiology
- J Glenn Morris, MD, MPH&TM – Infectious Disease, Epidemiology
- Judith A Johnson, PhD – Clinical and Molecular Microbiology
- Hegang Chen, PhD - Biostatistics
- Rama Chaudhry, MD – Clinical and Laboratory Microbiology
- Sailajanandan Parida, MD, Senior Foreign Investigator, Clinical Pediatrics, Pediatric Nutrition
- Nimai Nanda, MD, Department of Pediatrics and Site Investigator – Clinical Pediatrics
- Radhanath Satpathy, MD, Medical Officer/Site Investigator of the Rourkela site, Clinical Pediatrics, Community medicine
- Pravas R. Misra, DVM, Site Diretor, Bhubaneswar site – Laboratory Microbiology
- Lingaraj Pradhan, MD, Medical Officer (Capital Hospital), Bhubaneswar site – Clinical Pediatrics
- S. S. Mohapatra, MD, Director, Field Operations for both community sites, Clinical Pediatrics, Community Medicine

The specific duties of the research personnel are:

Principal Investigator: The U.S.-based Principal Investigator (PI) is responsible for ensuring the proper conduct of the trial at the research sites, accurate collection of data and transmission of information to RTI (Research Triangle Institute managing the contracts with NICHD, Indian sites, and developing publications of study data. Other duties include presenting pre-study education to the staff, applying for and obtaining IRB approval, and informing the IRB of the study progress. The PI also provides other necessary training (GCP, microbiology etc.) to study staff and ensures that personnel are available to carry out the study in an appropriate manner.

Senior Foreign Investigator: The senior foreign investigator (SFI) will be responsible for the overall execution of study aims and will supervise the Indian sites. He will also be responsible for all communication with the Government of Orissa and other central government offices in India and closely work with the principal investigator (PI) in the United States.

Site Directors: The site directors at each of the two research sites will work in concert with the SFI and take charge of the scientific and administrative activities at their respective sites. They will be responsible for managing subcontracts with the parent U.S. institution (UMB). Other faculty and permanent staff members in the departments of pediatrics and microbiology at participating hospitals/medical schools will act as co-investigators and provide service based on their training and expertise.

In addition to the above research team, each site in India will hire the following project staff

Study physician: The study physician is responsible for evaluating and caring for all study infants who are referred to the hospital. The medical officer will be responsible for reporting any adverse events to the site directors.

Study supervisor: The study supervisor is responsible for obtaining consent and enrolling infants in the study. He/she will also be responsible for preparing the study supplement and maintaining the randomization scheme.

Anganwadi worker: This person will be trained by the research unit to administer the study supplement to infants in the home as per the randomization scheme. The AWW will conduct daily home visits to monitor the infant from the time of enrollment until 60 days of life and will be trained to refer the infant to the hospital if there are signs of sepsis or other morbidities.

Microbiology officer: The microbiology officer at each site is a person with postgraduate training in microbiology who is primarily responsible for the processing of all clinical microbiological specimens, interpreting the results, and completing the microbiology data forms. Faculty members and other senior staff will provide assistance to the microbiology officer as needed. This will be a part-time position, as microbiological samples will only be taken from infants with signs of sepsis.

Laboratory technician: The laboratory technician assists the microbiology officer in the handling and processing of clinical samples and other related bench work. He/she will also help the microbiology officer in placing orders (microbiological media, reagents, plastic ware, and glassware with final approval of the site director. This is a part-time position.

Data entry operator/office coordinator: The data entry operator will be responsible for transferring handwritten data from the data forms to the computerized database. He/she will also assist the site director and other investigators in communications, administrative, and organizational aspects of the program, such as ensuring that all forms are accounted for and are appropriately filled out, patient samples are delivered expeditiously, and results are reported accurately.

Study nurse: The study nurse will be responsible for labeling and preparing the boxes of probiotic and placebo according to the randomization scheme.

*The microbiology staff hired for this protocol will not supplant the hospital staff/microbiology labs.

Duties of the NICHD

The NICHD Staff Science Coordinator will serve as the principal representative of the Institute and NIH and, in consultation with relevant NICHD program staff and representatives of the other NIH co-sponsors, will provide overall programmatic oversight, coordination, and assistance to the Global Network. Specifically, the NICHD Staff Science Coordinator will:

- Facilitate communication, cooperation, and the exchange of information among network members and between the network components and other existing programs to support collaborative efforts
- Oversee site participation and performance with the support of the Data Center

Apart from funding the project, the NICHD will facilitate the functioning of the RU by providing scientific input through assistance in the protocol and MOO development, monitoring the implementation of the project though mutually agreed upon site visits along with the DCC, and participating in preparations of publications. NICHD will also provide assistance as needed toward the conduct of the study. NICHD will convene an annual Steering Committee meeting to review site-specific progress and facilitate inter-site collaboration.

Duties of the Data Coordinating Center (RTI International)

Responsibilities of the Data Coordinating Center (at RTI), which is a collaborating center for all Global Network projects, include:

- Provide advice on study design, data collection, data analysis, and publication development.
- Provide technical assistance in the preparation, design, and dissemination of operations manuals, data collection forms, databases, and results reporting summaries.
- Compile for the Network Advisory Group and Steering Committee, the DSMB, the NICHD site visit reports, monthly and quarterly subject enrollment reports, meeting summaries, quarterly research unit performance and progress reports, and other reports as needed.
- Assure maintenance of high-quality databases, supervise all data collection procedures, and arrange for the most efficient transfer of study data where indicated.
- Ensure full compliance with NIH regulatory requirements, including informed consent, reporting of adverse events, human subject safety and welfare provisions, and the requirements of international collaboration.
- Provide training to all research unit site personnel as needed on data management and analysis and quality control and quality assurance.
- In coordination with the NIH co-sponsors, provide periodic onsite monitoring to the research units for those studies being performed at that site.

RTI staff includes:

- Vijaya Rao, PhD, Senior Statistician
- Jay Hemingway-Foday, MPH, MSW, Protocol Manager
- Sarah Taylor, B.S., Assistant Protocol Manager and Biostatistical Programmer
- Suchita Parepalli, M.S., Data Management Specialist

2. INTRODUCTION

As the twenty first century dawns, 10.8 million children younger than age five years die annually. Most of these deaths occur in developing countries. Of the children who die, 4 million die in the neonatal period, defined as the first 28 days of life. [1] India alone accounts for at least 1.3 million neonatal deaths. [2] Neonatal deaths account for about 33% of the total deaths under five years of age around the world; [3] however, in India neonatal deaths make up 64% of all infant deaths. [4] Furthermore, it is estimated that deaths during the second month of life are responsible for the majority (> 60%) of post-neonatal deaths during the first year of life. [5] Thus, more than 85% of all infant deaths in India occur in the first 2 months. With improved maternal and child health services, the infant mortality rate (IMR, defined as death under 1 yr of age per 1000 live births) in India has declined from 78.5/1000 live births in 1991 to 67.6 in 2000. However, during that period there was no significant corresponding decline in the neonatal mortality rate (NMR; death within the first 28 days of life). [6]

Over 60% of births in India take place at home with the assistance of traditional birth attendants and family members; [6] the majority of neonatal deaths occur in the community (rural) setting. Neonatal sepsis accounts for over half of the newborn deaths at the district and sub-district level and continues to be the most common primary diagnosis for admission to Indian hospitals. [7-9] Reduction in sepsis will have a major impact in reducing the NMR and post-NMR in this setting and in other developing countries with similar health problems.

3. BACKGROUND AND SIGNIFICANCE

Pathogenesis of sepsis, current treatment modalities and prevention strategies

Sepsis is a complex disease with multifactorial etiology and pathogenesis [10-13], and no preventive method has been unequivocally established at this time. Various interventions designed to reduce early sepsis, including intravenous immunoglobulin administration [14-16], empiric antibiotic treatment [17, 18], selenium supplementation [19], and breast feeding [20, 21] have been tried with limited success. New experimental therapies such as the use of anti-inflammatory medications [22], anti-endotoxin, and anti-cytokine therapy [23-25], with few exceptions, have failed to demonstrate an improvement in clinical outcome, and some have even increased mortality. [26-28] The 40-year history of therapeutic interventions in clinical trials for sepsis has been referred to as the “graveyard for pharmaceutical companies” [23], since almost none of these strategies have resulted in significantly improved survival of the patients. [24]

Bacterial colonization and gut development

The mammalian gut is sterile in utero and is exposed to bacteria at the time of passage through the birth canal of the mother followed by acquisition from the environment and the care giver(s). Then additional significant changes occur in the intestinal ecology when the infant is weaned from mother’s milk (or introduced to solid food). [29, 30] The resident flora ultimately stabilizes into a mix of obligate anaerobes. [29-31]. These changes in intestinal flora are accompanied by functional and morphological maturation of the gut. Coinciding with the withdrawal of passive maternal immunoglobulins, increased numbers of T and B cells are found in the lamina propria and intra-epithelial spaces [32] and polymeric IgA receptor is expressed. [33] Available data from germ-free mouse models (where these processes are arrested) strongly support the concept that these developmental changes are induced by bacterial flora and NOT just by post-natal age. [33, 35]

Role of gut flora and probiotics in colonic barrier function and bacterial translocation:

Although clinical observations in humans (IBD, celiac disease) have increased interest in this area [48, 49], the majority of the scientific information in this field has come from animal studies. Studies conducted in neonatal rats have shown that the colonic barrier is modulated by commensal flora: *E. coli* and Klebsiella increase gut permeability whereas Lactobacillus brevis decreases gut permeability. [50] Other studies in suckling rats have shown that Lactobacillus reverses cow milk-induced increased intestinal permeability [51] and rotavirus enteritis. [52] Studies from our laboratory have demonstrated the protective role of normal flora (Enterococcus strains) in maintaining the trans-epithelial barrier and blockage of transcytosis of *E. coli* in a Caco-2 cell transwell cluster system. [53] We also have shown that *L. plantarum* blocks bacterial translocation and *E. coli* sepsis in a weanling rabbit ileal loop model. [54] Additional reports in the literature have described similar interactions including the ability of breast milk components to block bacterial translocation in the immature intestine. [55]

Multiple studies have been initiated to clarify the mechanisms by which some but not all bacteria confer increased barrier function in the intestine. These include expression of integral proteins such as TJ proteins; peptides involved in tissue repair (e.g. trefoil factor), mucins, and modulation of other epithelial inflammatory responses in the intestine. [56] Recent data indicate that toll-like receptors (TLR)-2 are involved in responses to cell wall components of the gram-positive bacteria (such as Lactobacilli), while TLR-4 has a role in recognition of gram-negative bacterial compounds. [57] It is intriguing to note in a recent report that TLR-2 enhances ZO-1-associated intestinal epithelial barrier integrity, [58] which supports our earlier observations [59] that L. plantarum mediates protection of ZO-1 proteins during infection with specific Gram negative enteric flora. [59]

Bacterial Translocation (BT) and sepsis

Bacterial translocation (BT) is defined as the passage of viable bacteria from the gastrointestinal tract through the enlarged spaces between the epithelial cells [60] as well as directly through enterocytes [61] to extraintestinal sites including bloodstream. [62] There is increasing evidence in the literature in support the “gut origin of sepsis” where BT plays a critical role. This phenomenon has been shown in a variety of clinical settings in multiple hosts including neonates.

The concept of bacterial translocation from the intestine and sepsis also is strongly supported by evidence from animal studies. In the early 1990s, Runkel et al. showed the significant role played by bacterial translocation, and alterations of intestinal microflora in a rat model of acute pancreatitis and sepsis. [63] A disruption of the intestinal microflora was found in the cecum with significantly higher Gram negative counts in this study. [63] Similar results were later reported by Clements et al. focusing on gut barrier function and Gram negative sepsis [64]. More recently, gut-derived sepsis with other Gram (-) bacteria such as Pseudomonas aeruginosa has been described. [10] Rates of translocation for the various constituents of the indigenous flora vary considerably: Pseudomonas aeruginossa, *Klebsiella pneumoniae*, *E. coli*, and proteus mirabilis have higher translocation rates than Gram positive strains. [65] These results confirm our community data in which Gram negative bacterial translocation from the intestine has been demonstrated to be more prevalent than that with Gram positive organisms such as CONS. (described in detail in preliminary studies section).

BT and sepsis in "adult" human subjects

Reports relating bacterial translocation to adult sepsis have appeared in the literature since the early 1990s and the gastrointestinal tract has been described as the "motor" in driving sepsis. [63, 64, 66-68] In a prospective study of 279 surgical patients, MacFie et al. demonstrated bacterial translocation as a key mechanism and E. coli to be the commonest organism of sepsis [69], in a prospective study of the association between bacterial translocation, gastric microflora and septic morbidity. MacFie published a 13- year experience in 927 patients in 2006, again demonstrating an association between BT and sepsis. [70] In a postoperative case series of 51 patients with schistosomiasis, sepsis was linked with BT; aerobic bacteria were most prevalent in the mesenteric lymph nodes of these patients with E. coli dominating – 26%. [71] Epithelial barrier dysfunction is considered a unifying theme to explain the pathogenesis of multiple organ dysfunction syndrome. [60] New randomized controlled trials (RCTs) to prevent sepsis with probiotic prophylaxis in patients with predicted severe pancreatitis are underway (the Dutch PROPATRIA trial). [72]

BT in neonatal sepsis: Bacterial translocation has also been described as an important mechanism in the development of sepsis in multiple reports involving neonates. [73] There is increasing evidence that translocation of gut flora may be important in preterm infants who have immature intestinal defenses and increased gut permeability. [44, 45, 74, 75] While skin flora are considered the source of Gram positive sepsis (especially coagulase negative Staphylococcus - CONS) in western NICUs, Gram-positive "intestinal bacteria" are also an important source of sepsis in premature neonates. [76] In a prospective study conducted at the University of Maryland Baltimore (UMB), we have shown using molecular techniques that the organisms recovered from blood in our premature population were always identical to the ones cultured from the stool. [77]

In a recent five-year long prospective study of 208 neonatal surgical patients, gut overgrowth with aerobic Gram negative bacilli was shown to be the predominant cause of sepsis and was described as the "missing link in sepsis". These authors commented that "prevention is unlikely to be successful" when the role of gut flora is ignored. [78] Byington et al reported 16% blood culture positivity in 1298 febrile infants < 90 days old. Of the serious bacterial infections in this population, 79% were caused due to Gram negative organisms. [79] While the setting is not identical to that of ours, the age of infants, type of infection, culture positivity rate etc. appear quite analogous to our study population in India (up to 60 days old). Results of our own neonatal surveillance study in 850 neonates (conducted under the NICHD "Global Network", and summarized in Preliminary Studies, below) provide further data in support of the hypothesis that bacterial translocation is the predominant underlying mechanism in late onset sepsis. [80, 81]

Acid blockers, bacterial overgrowth and sepsis - indirect evidence in favor of gut derived sepsis in the neonatal population: In a study of 371 VLBW infants participating in a NICHD U.S. Neonatal Research Network placebo-controlled dexamethasone RCT, Stoll et al. found H2-blocker therapy before study entry was associated with increased risk of sepsis: 46.8% were attributable to Gram positive organisms, 26.6% to Gram negative, and 26.6% to fungi. [82] At the 2006 PAS/SPR meeting, Bianconi and colleagues reported that ranitidine resulted in a 7.6-fold increase in risk of sepsis among 574 neonates. [83] In this study 33.8% isolates were Gram negative organisms. Also this year, Guillet et al. demonstrated an association of H2-blocker therapy and increased risk of necrotizing enterocolitis in a cohort of 11,072 VLBW infants. [84] While these studies fell short of providing a direct link between the organisms in the intestine and blood (where other risk factors were adjusted in regression models), bacterial overgrowth under reduced acid conditions appears to be the common underlying factor that could have led

to translocation and sepsis.

Probiotics in gut development, immunomodulation and barrier function

Significant structural and functional development of the human gut starts upon exposure to the bacterial world via regulatory signals that condition the development and function of the gut. [62] In human volunteers, probiotics have been consumed to enhance natural immune function (interferon production, phagocytic activity etc.). [85] Other reports point toward the immunologic basis of probiotics [86-88] including their role in the development of innate immunity via NK-cell stimulation. [89] Distinct regulatory effects of specific probiotic strains are associated with induction of local immunoglobulins, cytokines (TH-1 vs. TH-2), and other mediators of inflammation.[90] Multiple *in vitro* murine and human tissue culture and animal models have been utilized to study the beneficial role of probiotics in increasing barrier function. [49, 91-93] Swedish reports showed reduction of BT in Sprague-Dawley rats using *Lactobacillus plantarum.* [94] In another study Mao et al described *L. plantarum* to be more effective than *L. reuteri* when used with arginine in methotrexate-induced enterocolitis in rats. [95] Kasravi et al. showed almost identical results where mucosal proliferation was induced by both *L. reuteri* and *L. plantarum*; but bacterial translocation was reduced by only L. plantarum. [96] In early 2000, an Italian study by Mangiante and colleagues showed reduction in intestinal permeability and experimentally-induced pancreatic necrosis by *L. plantarum.* [97] Similar results of reduction in intestinal permeability were reported in an Irish study this year utilizing *L. plantarum* in an experimental biliary obstruction model. [98]

Lee et al have utilized rabbit pups to examine the effect of Lactobacillus on bacterial translocation in a newborn model and they demonstrated a 46%, 61% and 23% reduction in bacterial translocation to mesenteric lymph nodes, spleen, and liver. [99] In a weanling rabbit ileal loop model, we have demonstrated significant reduction in the level of *E. coli* septicemia with *L. plantarum*. [54]

Use of probiotics to improve "human" gut barrier function

The results from animal studies and virtual lack of toxicity has helped the probiotic field move from simple GI disturbances such as diarrhea in healthy adults and children [92, 100-109], to atopy/allergy in children [109-114], and to critically ill patients [72, 115-117] where gut barrier function is compromised. These include sepsis, multiple organ dysfunction and necrotizing enterocolitis. [60, 117-119] Use of live *L. plantarum* in the adult ICU setting reduced septic morbidity in the ICU setting in a study of parents. [120] Probiotics have also been used in experimental models and clinical trials of inflammatory bowel disease, irritable bowel syndrome, and celiac disease where gut barrier is considered to be sub-optimal. [121-125]

Probiotics in "neonatal sepsis" and other "best practice" recommendations

Recent reviews have used the probiotic concept as "germ warfare" in the defense of the premature gut. [126] With few options available to prevent nosocomial sepsis in the neonatal units, experts are now suggesting approaches to strengthen gut barrier function using probiotics [119]. In the adult intensive care setting, probiotics are recommended among "best practices" to limit mortality from sepsis. [116, 120, 127]

Lessons learned from veterinary medicine

The experience with germ-free animals may be analogous to the newborn situation. If germ-free animals are infected with a single strain of organism, that organism, even if not indigenous or pathogenic to the animal, can populate the gut in very high concentrations. Germ-free pigs and guinea-pigs can develop severe enteritis when removed from the germ-free environment or

when contaminated with a single species; however, protection can be afforded by prior seeding with their normal stool flora. [128] the potential importance of gut microbial ecology and the role of normal gut flora is also emphasized by several other examples including “mucoid enteropathy" in rabbits and ailments in rodents and chickens. [129, 130] "Schaedler's Cocktail," a combination of harmless bacteria, is used routinely in raising specific pathogen-free rodents. [129] These kinds of data suggest that similar therapeutic maneuvers may be applicable in the human infants.

Dysbacteriosis and probiotics in Russia

In many parts of Russia, probiotic preparations are used for up to 30 days in all infants in an attempt to create “benign” stool microfloral patterns to prevent/cure “dysbacteriosis”. [131] Russian physicians describe gastrointestinal dysfunctions that are accompanied by an alteration in the functional characteristics of the gut flora or “intestinal dysbacteriosis.” The entire neonatal population in much of Tatarstan receives probiotics in an attempt to prevent/cure dysbacteriosis in early infancy. Since no blinded, controlled studies of this therapy have been performed, the level of colonization obtained and its efficacy in preventing such dysfunctions cannot be ascertained. However, since thousands of infants have been treated, the risks involved in such treatment appear minimal, and the observation of a relatively low incidence of infections during infancy in Tatarstan (an Eastern European country with high infant mortality) would argue in favor of its safety and some degree of efficacy. (Personal communication, A. Kuznetsova, Kazan Institute for Advanced Medical Studies, Tatarstan, Russia; via UMB-Kazan exchange program 1991-2001)

Clinical trials of probiotics in neonates

Hoyos et al. reported a clinical trial of Colombian preterm infants using a probiotic preparation of Lactobacillus acidophilus and Bifidobacterium infantis (n = 1,237); they showed a reduction of neonatal necrotizing enterocolitis (NEC) and improved oral feeding tolerance. [132] Improved feeding tolerance was reported with similar probiotic treatment (Bifidobacterium breve) in preterm Japanese infants. [133] However, a prospective double-blind Italian study of Lactobacillus casei (n = 489 preterm infants) did not demonstrate a significant reduction in NEC or urinary tract infection (UTI). [118] A Finnish trial randomized mothers with a family history of atopic disease to prenatal administration of Lactobacillus rhamnosus vs. administration of the same preparation to their infants for six months after birth. The incidence of atopic eczema was reduced by approximately one-half compared to a placebo group. [113] In a randomized study, Bifidobacterium lactis was administered to 128 preterm infants during the first 6 weeks of life. There was no impact on nosocomial infections that were recorded after the first week of life. [134] A small trial of Bifidobactrium breve in 40 VLBW infants demonstrated colonization and a trend toward reduced sepsis, earlier achievement of full feeds, and better weight gain. [135] In another study from Taiwan, Lin et al. showed reduction of sepsis and necrotizing enterocolitis in infants treated with a combination of Lactobacillus acidophilus and Bifidobacterium infantis. [118] However, a very recent study by Bin-Nun and colleagues demonstrated a reduction in the incidence of necrotizing enterocolitis, but not sepsis, using a Bifidobacteria and Streptococcus combination. [136]

Although promising, the variable results in the above studies suggest that probiotics may have limited effectiveness in preventing neonatal sepsis. However, none of the above studies analyzed stool cultures of the infants to examine the ability of the strain to colonize, nor was the selection of the probiotic based on demonstrated ability of the probiotic preparation to normalize gut colonization and block translocation. Several studies used combination of strains (an approach taken by food supplement industry with no scientific basis), that makes it impossible to evaluate the effect of individual strains. A clear justification against this approach is the

withdrawal of Lactinex [a combination product of *L. acidophilus* and *L. bulgaricus* (common yogurt strains)] from the U.S. drug market many years ago. Highly variable results with Lactinex were seen in clinical trials that were attributed, in part, to the inhibitory effect of bulgaricus strains on acidophilus. Our current study was designed after careful evaluation of the Lactobacillus strain in experimental *in vitro* and animal models, followed by preliminary safety studies in hospitalized newborns.

Probiotic colonization studies by our group

In a study of low birth weight Indian infants using two commercially available probiotics, we observed that *Lactobacillus acidophilus* had a stool colonization rate of 60% compared to 0% for *L. sporogenes,* with twice daily administration over a 4-week period. (80) In a second study using an *L. casei* (LGG) preparation in two groups of hospitalized Indian infants, colonization by LGG was $\leq$ 50% in infants of 1500-1999 gm birth weight (over a 1-week period) and < 25% in a VLBW cohort after administration for 3 weeks. (81) In both studies, Lactobacilli/ Bifidobacteria were isolated from only a handful of control infants (< 5 percent of the population) at an age of 3 to 4 weeks.

Recently, we have completed a series of in vitro and in vivo experiments to identify a suitable probiotic preparation, using the same preparation in further clinical studies in newborn infants in the hospital setting. The Lactobacillus plantarum strain used in our studies has shown excellent protective effects in the experimental models and colonized in 80-90% of the infants up to 60 days after one-week administration.

Available data from our own studies suggest that administration of probiotics to community-born newborn infants may reduce late- onset sepsis.

Recommendations of international agencies/expert working groups

The concept of “germ warfare” in the defense of the premature gut has been accepted and expanded for the newborn population. [126] Similarly, strengthening of the gut barrier function by probiotics to prevent nosocomial sepsis in the neonatal units is being suggested by experts in the field. [119] There is a consensus to move away from single center small trials to large scale interventions with adequate power that can address the true utility of probiotics in neonatal sepsis (primarily Gram negative). [119]

Although not in direct relevance to neonatal sepsis, many international bodies have recommended the use of probiotics in other infections. A clinical review entitled “Probiotics for the developing world” published last year summarized the opinion of the meeting of the International Scientific Association for Probiotics held in London. [137] This report reviewed the evidence in favor of the use of probiotics, examined several models in sub-Saharan Africa and recommended the use of probiotics for diarrheal diseases in the developing countries especially for communities not reached by governmental agencies. [137] In an effort to reduce the use of antibiotics in the face of increasing antibiotic resistance bacteria, the WHO has advocated, where possible, a policy of microbial interference. [138] The Food and Agricultural Organization (FAO) of the United Nations published an expert panel report in 2001 stating that “adequate scientific evidence exists to indicate that there is a potential for the derivation of health benefits from foods containing probiotics”.[139]

4. PRELIMINARY STUDIES

Population-based surveillance of neonatal sepsis:

In an effort to identify the most appropriate intervention against neonatal sepsis, we established a sepsis surveillance program in the community setting and conducted detailed microbiology of neonatal infection in India for the first time. All pregnant mothers in 223 participating villages were registered at seventh month of their pregnancy and their pregnancy was followed by lady government workers called Anganwadi workers - AWW. All live born babies were followed daily for 60 days after birth by AWWs. These workers and study personnel were trained to identify sign/symptoms of sepsis using a modified WHO model and records maintained by AWW and study staff every baby with suspect sepsis was referred to study hospitals where they were evaluated by study pediatricians. Complete septic workup was done when the study pediatrician considered the baby as case of "clinical sepsis" deemed appropriate.

Our trained village-level personnel identify cases of suspected sepsis based on the UNICEF/ WHO model of PSBI (sign/symptoms of clinical sepsis) and refer them to our study hospitals where the babies are examined by study physicians. A screening form is completed by the physician for each baby. Based on the clinical evaluation of the physician, the baby is either enrolled in the study with clinical sepsis, referred to the general pediatric ward in the hospital (for other illness not related to sepsis), or returned home with advice. **Table 1** below gives a quantitative measure of our triage (village level), screening (hospital) and final assignment of diagnosis for the purpose of calculation of sepsis rates during a three year period.

Table 1. Referral and determination of clinical sepsis cases

Total births	# of babies referred to hospital	Final clinical sepsis cases
12,622	1,973	842

Definition of clinical sepsis: Infants were recorded as having clinical sepsis when "ANY ONE" of the following criterion was met:

- Clinically sick with signs of PSBI to warrant hospitalization and IV antibiotics for five or more days, and recovery upon treatment (or death due to severity of disease)
- Culture positive (blood or cerebrospinal fluid)
- Positive chest x-ray (pneumonia)

We collected samples from the baby (axillary skin, umbilicus, stool, and any other specific sepsis foci) and the mother (vaginal tract and nail bed). This population-based study provided insight into the timing of infection (EOS vs. LOS). Results of the microbiological analysis provided the critical information on the source of infection, which led us to believe that the probiotic approach is the most appropriate intervention to prevent sepsis in the majority of infants.

Table 2. Disease burden of clinical sepsis in the surveillance study (April 2002-March 2005)

Site	Total # of births	Clinical sepsis cases enrolled in the study hospitals
BBS	5,720	384
RKL	6,902	458
TOTAL	12,622	842

During the study period (April 2002 to March 2005), there were 12,622 births.

Timing of sepsis: We had originally speculated that EOS in the community would be quite common because of lack of clean water, poor hygiene practices during delivery and cord care, and practices by traditional birth attendants during delivery. However, of the infants born at home and enrolled in the study with suspected sepsis, only 5% were admitted within 72 hours of birth. Previous Indian studies have also indicated higher incidence of neonatal sepsis during the late neonatal (8-28 day) period. (69, 70)

Table 3. Timing of presentation of clinical sepsis in the community setting

Time of presentation	Infants enrolled for clinical sepsis
Early Onset (%)	38 (5%)
Late Onset (%)	804(95%)
Total	842 (100%)

Infecting organisms: Predominant organisms were Klebsiella spp., *E. coli*, coagulase negative staphylococci (CONS), and *S. aureus*. However, Gram-negative organisms were the predominant organisms associated with LOS infection, especially Klebsiella spp. CONS were considered controversial and a decision was made by the research group to examine them on a case-by-case basis for clinical outcome before discarding them as skin contaminants.

Concordance of blood culture with other body sites: Analysis of concordance between blood isolates and isolates from other sites was done in an effort to identify possible transmission routes. When a blood culture was positive, the same bacterial species was first looked for in other cultures (i.e. other body sites of the baby and the mother). If the same organism was found at any other site, a second line of match was performed by comparing the antibiogram. Organisms showing the exact same antibiogram (sensitivity and resistance to same set of antibiotics) were subjected to a third level of confirmation by pulse field gel electrophoresis (PFGE). The results revealed several important phenomena. Staphylococci were found in all colonized sites, suggesting that transmission could occur through environmental exposure (breaks in the infant's skin or maternal handling) or via intestinal translocation. In contrast, Gram-negative LOS had a strong concordance with enteric bacterial colonization, suggesting that bacterial translocation from the infant's gut may be important in Gram-negative LOS (72).

Hospital-based safety pilot of GastroPlan (L. plantarum and FOS blend)

This double-blind, placebo-controlled study was conducted at two of our participating hospitals. To maintain blinding, trained OB study nurses not involved in the care of the newborn administered the study supplements at our attached hospitals. Randomization schedule was created by RTI and study sites were provided with sealed envelopes with treatment assignment to be opened sequentially for eligible infants. Training was conducted by the PI and RTI

personnel. Thirty-three infants were randomized to control and probiotic groups in 1:2 proportion; 30 infants completed the study. Treatment groups received *L. plantarum* preparations (contents of one capsule containing 10^9 organisms – ATCC 202195 and 150 mg FOS) or placebo preparations (5% dextrose saline) orally once a day starting on day one to three of life and continuing for seven days thereafter. Stools were collected at baseline (before administration) and on days 3 ± 1, 7 ± 2, 14 ± 3, 21± 3, 28 ± 3, months 2 through 6 ± 7 days. A minimum of four doses were given in the hospital for the infant to stay eligible in the study and rest of the doses were allowed to be administered at home. Daily clinical events were recorded and a detailed microbiological culture of stool (for all organisms) was conducted.

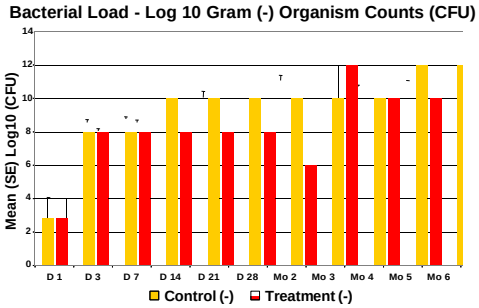
No serious adverse reactions were recorded during the 28-day active follow-up. All babies were followed for 180 days at home for general health. Monthly stool collection was done for examination of *L. plantarum* and change in colonization pattern. Starting day 3, a high level of colonization (80%) was observed in the treated group that continued up to four months after the one-week administration (**Table 4** below).

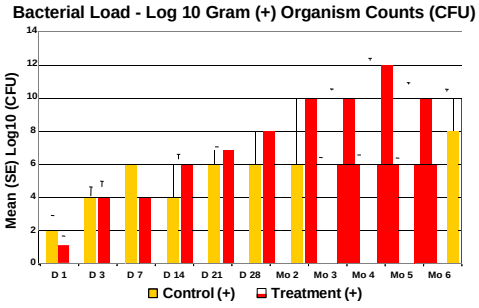
Table 4. Number of stool samples positive (%) for Lactobacillus plantarum

Group Assignment	Baseline*	Day 3	Day 7	Day 14	Day 21	Day 28	Month 2	Month 3	Month 4	Month 5	Month 6
Placebo	0/8 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/12 (0%)
L. Plantarum	0/13 (0%)	16/19 (84%)	14/19 (74%)	17/19 (89%)	15/18 (83%)	18/19 (95%)	18/18 (100%)	17/18 (94%)	15/17 (88%)	10/18 (56%)	6/19 (32%)

*pre-treatment. The denimonator is not same for each day since all samples could not be collected w hen baby did not stool

As seen in the graphs below, mean log Gram negative colony counts were significantly lower in the probiotic group on days 3, 14, 21, and 28, and also on months 2, 5, and 6 (p=0.02 at 3 months and p<0.01 at all other time points). The mean log Gram positive counts were significantly higher in the probiotic group than in the placebo group on days 14 (p=0.001) and 28 (p=0.04) and at each of the follow-up months (p<0.001 at mo 2-6).





Apart from the changes in the bacterial load that are directly linked to translocation and sepsis, there was also a change is species diversity upon treatment. The mean number of Gram positive species including anaerobes and microaerophilics (that are considered normal flora and typically do not translocate from the colon) was higher in the *L. plantarum* group than in the placebo group on days 21 (p=0.001) and 28 (p=0.04) and at each of the 2-6 month examination (p<0.001 at each time point). While both groups acquired a mean of only one Gram negative species during the first month, the mean number was lower in the *L. plantarum* group on months

2-6. These results demonstrate that probiotic treatment created and maintained a microbial milieu with a reduced load of Gram negative organisms. This, in turn, should reduce bacterial translocation of Gram negative sepsis during at least during the first two months in the newborn period that may extend up to 4-6 months in the infants with continued colonization.

Long term follow-up of pilot study: The first five months of the six month follow-up period were uneventful for both groups. However, during the 6th month, 4 of the 11 controls were brought to the hospital for diarrhea; one stool was positive for rotavirus antigen. None of the 19 treated infants had any health problems. Because of the small sample size, analysis of the groups was done by Fisher's exact test revealing a significant difference between the groups (p=0.012).

Main Hospital-based Clinical Trial: After completion of the pilot, a larger randomized controlled, hospital-based study was performed in three of our participating hospitals using the same study design in order to estimate the colonizing ability and safety of the probiotic Lactobacillus plantarum in the healthy newborn gut. The hospital-based clinical trial completed enrollment in August 2006 with a total of 284 patients. The final dataset is currently being analyzed.

The hospital-based trial cannot test the effectiveness of probiotics, as that would require a much larger sample size as planned in the current study. However, that will generate robust data on the "colonizing ability" of the strain, the most critical aspect that has been historically ignored in almost all prior clinical trials in this field. This also provides additional assurance on the safety and tolerability of the probiotic preparation in the newborn population.

Potential Risks and Benefits of Probiotic

Apart from the long history of health benefits, probiotics in general, also have an excellent record of safety in human use [140-142]. However, with the use of such live organisms, there exists a risk of blood stream infection and there are reports of opportunistic blood stream infection with Lactobacilli [143-149].

The highest frequency of probiotic-related sepsis has been reported with the use of the yeast strain *Saccharomyces boulardi* [150-159], and Lactobacillus *rhamnosus* (also called Lactobacillus GG). [160-166] Sporadic cases of lactobacillemia have also been reported with *Lactobacillus acidophilus.* [167-169] Lactobacillemia with *L. plantarum* has not been reported in the literature.

All cases of probiotic bacteriemia or fungemia (Saccharomyces) have been reported in patients with underlying immune compromise, chronic disease, or debilitation, and no reports have described sepsis related to probiotic use in otherwise healthy persons . [170] Analyzing the current European guidelines for safety assessment of Lactobacilli in food and food supplement, Bernardeau et al. concluded that the Lactobacillus genus essentially posed negligible biological risk. [171]

We have done a comprehensive antibiogram of the strain (See Appendix) and will make injectable antibiotics available at study sites to attend to any unforeseen events associated with our therapy.

Safety of probiotics in the pediatric and neonatal population

Multiple studies in the literature have used probiotic preparations for various disease states in the pediatric and neonatal population (described in the background section earlier). So far in

children less than one year old, only a few reports have directly linked the cases of Lactobacillus sepsis to the ingestion of the same probiotic supplement. Kunz and colleagues [172] described 2 cases of LGG sepsis in premature infants (10 and 12 weeks old) with short-gut syndrome who were being fed *Lactobacillus rhamnosus* GG supplement via gastrostomy or jejunostomy tube feeding. In one of the two cases, the blood isolate was indistinguishable from the probiotic strain by pulse field gel electrophoresis. De Groote et al. reported a similar case in an 11 month child. [162] In another case report, a 6-weeks-old infant with antibiotic-associated diarrhea after cardiac surgery developed LGG endocarditis three weeks after treatment with 10x9 CFU of *Lactobacillus rhamnosus* GG daily, and was successfully treated with penicillin G. [173] The second case reported in the same paper, described a six year-old girl with cerebral palsy and microcephaly, who developed sepsis after taking *Lactobacillus rhamnosus* for 44 days with her feeding via gastrojejunostomy tube. She was also treated successfully with ampicillin. The authors concluded that this report should not discourage the appropriate use of Lactobacillus or other probiotic agents but should serve as a reminder that these agents can cause invasive disease in certain populations. [173]

Blood stream infection with other Lactobacillus-like species

Careful evaluation of the literature reveals three other cases of blood stream infection with Lactobacillus-like organisms in the pediatric population. All three relate to septicemia with Pediococus (a member of the lactic acid-producing family of bacteria), that were not linked to a bacterial (probiotic) intake. These include a two-week-old infant with congenital jejunoileal atresia [174], a two-month-old infant with gastroschisis [175] and a three-month-old, also with gastroschisis, corrected by two surgical interventions at two hours and two days of age, that had two episodes of staphylococcal sepsis and was treated with multiple antibiotics. [176] It is evident in these case reports that these events occurred only when concomitant severe anomalies of the GI tract was present and invasive procedures were being carried out in these young infants. Justifiably, the authors of recent reports have urged caution, while using commercially available probiotics in children and adults with gastrointestinal anomalies, pending additional study. They have added that such warnings for this particular category of GI patients should not be applicable to the overwhelming majority of children. [176]

Safety and tolerance of Lactobacillus plantarum in vulnerable patients

As noted above, there has not been any report of *L. plantarum* sepsis in the adult or pediatric population. *L. plantarum* was first used in debilitated elderly persons in Swedish nursing homes to reduce infections that are now used in acutely ill patients as a preventive therapy against sepsis. In a study involving 45 patients with severe acute pancreatitis, Kecskes et al. [177] observed significant reduction of infected necrosis and abscesses patients receiving live *L. plantarum* (4.5% compared to 30% in the control group receiving heat killed *L. plantarum).* Further evaluations of the patients revealed that the length of stay was 13.7 days in the treatment group vs. 21.4 days in controls. The only patient who developed sepsis in the treatment group did so eight days after the treatment had been discontinued. The authors concluded *L. plantarum* supplementation to be an effective tool to prevent pancreatic sepsis, to reduce the number of operations and length of stay and recommended at least two or more weeks of treatment or even more appropriately, as long as the patients are treated with antibiotics or have signs of GI colonization. [178] In a prospective randomized trial involving 172 patients following major abdominal surgery, the incidence of bacterial infections was compared in patients receiving either conventional parenteral or enteral nutrition, or enteral nutrition with *Lactobacillus*. The incidence of bacterial infections after liver, gastric or pancreas resection was 31% in the conventional group compared to 4 % in the *L. plantarum* group. [67] In the analysis of 95 liver transplant recipients, 13% of patients developed infections in

Lactobacillus group compared to 48% in controls. In addition, the duration of antibiotic therapy was significantly shorter in the lactobacillus-group. Cholangitis and pneumonia were the most frequent infections and enterococci the most frequently isolated bacteria. It was recommended that such eco-immunonutrition be already started while patients are on the waiting list for transplantation [68,179]. These studies have prompted other multicenter trials of *L. plantarum* prophylaxis in patients with predicted severe pancreatitis. [72]

Last but not the least, different Lactobacillus strains have been used to treat/prevent HIV-associated diarrhea and boost immune function in HIV patients. [180-184] In a study using *L. plantarum*, Rundles et al. showed positive colonization, improved nutrient status and growth in a cohort of children exposed to HIV. The authors also demonstrated the ability of these children to elicit specific systemic immune response after oral supplementation. [185]

5. OBJECTIVES

Feasibility testing in a community pilot: While we are not able to set any gold standard (due to lack of knowledge in conducting RCTs in the community setting of a totally different culture with rural and tribal population), we will evaluate several parameters before we move forward with the large RCT enrolling 8,442 infants. We expect high rates in the following parameters to be recorded and examined in the community pilot of 400 infants. These infants will undergo all procedures starting with screening, consenting, intervention at home, 60-day followup, and transport and clinical care in case of suspicion of sepsis.

- Percentage of infants consented - 90%
- Percentage of babies ineligible - 25-50%
- Randomization rate among eligible infants - 90%
- Adherence to intervention (all seven doses) – 80%
- Adherence to five or more doses – 90%
- 60-day followup – 90%
- Ability of CHV and AWWs to administer the intervention at home – 95%
- Collection of all data forms and transport to the data center at the study hospital within collect clinical data.

Primary outcome: Sepsis plus Death, the former composed of septicemia, meningitis, culture negative sepsis/inflammation, and lower respiratory tract infection (LRTI). Modified WHO/UNICEF guidelines (http://www.unicef.org/sowc08/ docs/sowc08_ panel_2_4.pdf) for neonatal sepsis will be used to diagnose PSBI.

Sepsis (PSBI) - Septicemia/meningitis: Positive for bacterial or fungal culture of blood or cerebrospinal fluid, or culture positive sepsis.

Sepsis (PSBI) - Culture negative sepsis/inflammation: Presence of any of the following signs when warranting hospitalization and use of antibiotics for five or more days based on the decision of the pediatrician indicated sepsis.

- Refusal to feed
- Lethargy (absent movement, or movement with stimulation only)
- Respiratory rate > 60/min, nasal flaring

- Axillary temperature ≥ 37.5°C/99.5°F
- Axillary temperature ≤ 35.5°C/95.9°F
- Convulsion
- Severe lower chest wall in-drawing

Sepsis (PSBI) - LRTI (includes pneumonia): Presence of reduced breath sounds, uneven breath sounds, expiratory grunting, or crackles on auscultation by the pediatrician in conjunction with any of the sign/symptoms mentioned above.

Secondary outcomes:

- Other infections including omphalitis, impetigo, diarrhea
- Any other condition deemed infectious by the pediatrician
- Weight gain
- Allergic and atopic episodes

WHO definitions will be used for omphalitis, abscess, acute otitis media, pustules, and diarrhea.

These objectives will be measured using an established surveillance and referral system in the study communities. We will utilize the services of trained Anganwadi (government) workers (AWWs) in each village managed and supervised by study personnel and physicians. Infants will be randomized to receive the supplement or placebo within the first three days of life and continued for six more days, for a total of seven doses. All infants will be followed daily for 60 days of life by study staff, and will be referred to study hospitals immediately upon suspicion of sepsis or any other major morbidity. Demographic and treatment-related data will be collected at home and additional clinical and microbiology culture data obtained from hospital records if the infant is referred to study hospital or becomes hospitalized.

6. STUDY DESIGN

This is a prospective, double-blind, randomized, placebo-controlled study that will test the effectiveness of prophylactic oral administration of a *L. plantarum* probiotic preparation in reducing the incidence of neonatal sepsis in community-born infants in India.

Newborn infants will receive once daily dosing of the probiotic supplement for seven days and will be monitored daily for the first 60 days of life. Infants of consenting parents will be randomized 1:1 into treatment or placebo groups. The primary outcome of interest is the feasibility of implementing the intervention. All infants exhibiting signs of sepsis or other health problems requiring consultation with a physician will be referred to the study hospitals for evaluation, sepsis workup and treatment.

We will utilize our well-established village-level network of personnel trained to identify signs of major morbidities including sepsis, as well as study hospitals and attached microbiology laboratories to carry out this study. Based on our power analysis, we plan to enroll 4,221 infants in each arm of the study.

This population-based study will be carried out in the selected Anganwadi centers (AWC) in the districts of Khurda and Sundargad in the state of Orissa, India. One AWC is equivalent to about one village of 1000 people – a big village may have two AWCs, and two small villages/hamlets may form one AWC. Capital hospital, Bhubaneswar (BBS) and Ispat General Hospital, Rourkela

(RKL) will serve as the study hospitals for patient referral and care.

7. STUDY POPULATION

Selection of Study Population

All community-born infants will be screened for eligibility. >2000g, and <72 hours of age will be screened by the Anganwadi worker. Infants who meet eligibility criteria and whose parent(s) provide consent prior to delivery will be enrolled into the trial and randomized to receive either the placebo or probiotic supplement.

Our surveillance study has provided valuable data on annual births that has been used to design this study. **Table 5** summarizes the annual births from 2002-2005 in our target areas.

Table 5. Annual number of births during surveillance study period

Site	Births in our field Sites during 2002-2005			
	Year 1	Year 2	Year 3	Total Years (1-3)
BBS (96 AW centers)	1864	2148	1708	5,720
RKL (127 AW centers)	2300	2381	2221	6,902
12,622 Total combined births in 3 years, Mean 4,207 births per year				12,622

A. Inclusion Criteria

- Neonates between 24 hours and 72 hours of age in the community
- Weight > 2000 g at time of screening
- Breastfeeding begun by 24 hrs of life
- Able to tolerate oral feeds
- Willingness of parent or guardian to provide informed consent

B. Exclusion Criteria

- Informed consent not available
- Difficulty in carrying out study (e.g. maternal sickness)
- Mother unlikely to stay in the village for 60 days
- Evidence or suspicion of clinical sepsis before the baby is randomized
- Baby is on antibiotics
- Breast feeding not established by 24 hr
- Inability to establish oral feeds (in case of maternal death or ailment)
- Gestational age reported voluntarily by mother to be < 35 weeks
- Infant > 72 hr old
- Baby did not cry immediately after birth
- Mother had fever (> 38 0C) within 2 days of delivery
- Mother had foul smelling amniotic discharge within two days of delivery
- Mother had abdominal tenderness within 2 days of delivery
- Amniotic fluid was meconium stained

- Presence of major congenital anomalies*

8. STUDY INTERVENTION

Regimens, Administration, and Duration

CHVs, Anganwadi workers (as well as supervisors in the field) will be trained to administer the intervention material. The probiotics will be prepared at the Baby's house. The CHV will examine the baby, record vital signs, and confirm that breastfeeding has been initiated. She will then prepare the intervention material. The CHV will not touch the study agent by hand at any time. She will apply alcohol based handwashing solution to her hands and let is dry completely before opening the capsule bottle. She will take out one capsule (placebo or study supplement), and open it into the wide mouth plastic re-suspension container by pulling the two sides, thereby letting the powder drop into the container. She will then open a dextrose saline vial and pour the contents into the re-suspension container without touching the mouth of the vial. After putting the cap on, the container will then be shaken gently by swirling action (vigorous shaking causes froth formation and loss of material) until the powder goes into solution. The mixture will then be drawn into a disposable syringe without the needle. The CHV will slowly pushed/pour the 2 ml contents into the baby's mouth. The CHV will be required to monitor the baby for 30 minutes for vomiting. If vomiting occurs, she will prepare and administer a second. In case of vomiting after second dosing, no third dose will be given. The supervisor and study physician will examine the baby before the next dose is administered. A total of seven doses will be given to infants starting day 2 or 3 of life.

All units used (plastic containers for re-suspension, dextrose saline vials, and syringes) will be used one time only and discarded. Below is the clinical trial material box from the Phase II hospital trial. Identical packaging will be used. The container with probiotic capsules will be kept at -20 ^{0}C at study hospitals and taken by the supervisor every week to the field office, where it will be kept under refrigeration.

Product Formulation and Preparation

The study supplement used in this protocol is a *L. plantarum* probiotic preparation registered with the U.S. FDA (Dietary Supplement Section).

GastroPlan is a preparation that contains *L. plantarum* 5 mg 1-2 X 10^9 colony forming units of

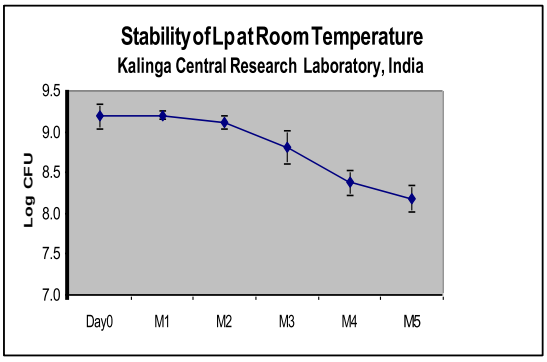
ATCC 202195 in lyophilized form, fructooligosaccharides (FOS) (150 mg), maltodextrin 345 mg as excipient). Placebo in this study will be comprised of only maltodextrin.

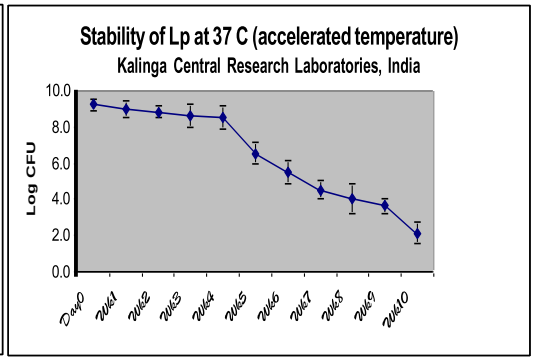
Product Supply and Accountability

Capsules containing the probiotic supplement and placebo will be packaged into bottles labeled with a unique randomization code. Two mls of 5% dextrose saline in vials containing will be used for reconstitution of the powder in separate small plastic mixing containers. Plastic syringes (without needles) will be used to draw the probiotic mixture from the mixing containers and to feed to babies. Although 7 doses are required, 10 units of each will be provided in a box for each infant to allow room for repeat doses (in case of vomiting within 30 minutes of feeding) and accidental loss during mixing etc.

Stability/viability of *L. plantarum* in the probiotic preparation: Stability can be a major factor for all probiotic preparations, since there is loss of viability upon exposure to heat or moisture. We had conducted viability studies at the PI lab in the U.S. and observed minimal loss of viability during the first 3-4 months at room temperature. At both 4^{0}C and –20^{0}C there was only a half log drop in viability over a one year period. This level of stability is considered excellent. According to a U.S. Food FDA report; many preparations undergo 4-5 log drop under such storage conditions (see appendix 6). Our clinical trial material will be stored under refrigeration at 4-6^{0}C and stay in the field for a maximum period of two weeks, and hence, is expected to have minimal/no loss in viability.

Test samples were kept in storage rooms (non air-conditioned) and tested monthly by colony counts. To simulate the high temperature of Summer months, duplicate samples were tested weekly at 37^{0}C (laboratory incubator). Viability testing was also done after reconstitution of the lyophilized product in 5% dextrose saline. Please note that 98^{0}F is close to the high temperatures in Orissa in May/June. Although the daytime high may cross 100^{0}F, morning and nights are much cooler (in the 60s and 70s) resulting in an overall average temperature lower than 98^{0}F.





There was no drop in viability in the first two months at room temp, followed by a gradual minimal drop during the subsequent 3 months resulting in less than one log final drop. Under accelerated temperature (98^{0}F), there was no drop in the first 3-4 weeks, followed by a linear but much more drastic drop (about half a log every week) of about 6 logs in 10 weeks.

There was no drop in viability of the reconstituted supplement at 3, 6, or 12 hr at room temp or at 37^{0}C.

We have made plans to store our clinical trial material under refrigeration (with 24 hr power

backup) at study hospitals. They will be distributed via study supervisors to the field every week (based on new births and screening in their area) and the 7-day course be used up within the following week. Hence the maximum time it will be out of refrigeration is two weeks. Even in the worst hot periods of the year the *L. plantarum* supplement will have no drop in viability assuring us the quality of the clinical trial material. Extreme care will be taken with the inventory, and we will make sure that the 7th dose for a given infant is not beyond 15 days of dispatch of the supplement from study hospitals.

Re-constitution will be done right before administration (the AWW lives in the same village within minutes of walking distance from any house).

Our clinical trial material will be stored under refrigeration and stay in the field for a maximum period of two weeks, and hence, is expected to have minimal/no loss in viability.

Assessment of Participant Adherence with intervention

Infants will be monitored for 30 minutes after the supplement is given to ensure that the doses are swallowed. If a dose is spit up or vomited within 30 minutes, a second dose will be given. If the dose is vomited or spit up again, no more doses will be given. The infant will resume dosing the following day. The date and time of each dose will be recorded in the Daily Home Monitoring and Dosing Log.

Concomitant Medications and Procedures

All medications will be given by a study physician and will be recorded on the appropriate study forms (Daily Hospital Exam Form and Hospital Antibiotic and Antifungal Treatment Form). It is expected that no medication will be administered during the study period unless clinically indicated. In case of suspected infection, antibiotics will be given only after the collection of the appropriate cultures. If an infant is taken to a non-study hospital, information on diagnosis and treatment will be captured on the Hospital Referral Form.

Newborns and infants in this study will receive routine vaccination for HepB, DTaP, and IPV as provided by the health department. Pneumococcal or *Haemophilus influenzae* (Hib) are not routinely available. We will not change the practice in the study area.

9. STUDY PROCEDURES AND EVALUATIONS

Clinical Evaluations and Procedures

Clinical evaluation of the infant will occur at the following time points:

- Screening: The CHV will do a brief clinical assessment to determine if the baby has any signs of sepsis, congenital anomalies or health conditions that would exclude participation in the study
- Enrollment: The CHV will measure weight, assess feeding practices
- Daily Monitoring: The AWW will measure temperature, assess feeding patterns, and assess whether there are symptoms of illness.
- Hospital Referral: The AWW will assess whether there are symptoms of illness that necessitate hospital referral. If referred, the study physician will conduct a complete physical exam and assessment for sepsis. The physician will also assess signs of illness.
- Hospitalization: If the infant is hospitalized, the physician will conduct a complete clinical evaluation on a daily basis and will record results and treatments given, if appropriate,

on study forms.

Clinical sepsis/PSBI will be defined using criteria described earlier.
All sick infants are expected to be referred to the study hospital, examined and treated with antibiotics, as determined by a blood culture.

Laboratory Evaluations

Blood and stool samples will be collected from all infants that are hospitalized with signs of clinical sepsis during the study period.

Specimen Collection, Preparation, Handling and Shipping

Stool cultures: Stool cultures will be collected on infants who receive a septic workup as clinically indicated during the study period (60 days) at the discretion of the treating physician. A commercial culturette containing a gel medium will be used to scoop a swab full of stool to put into the medium. The swab and diaper (with remaining amount of stool) will be immediately transported to the laboratory and stored under refrigeration temperature. In case of collection at night, samples will be stored at 4° C.

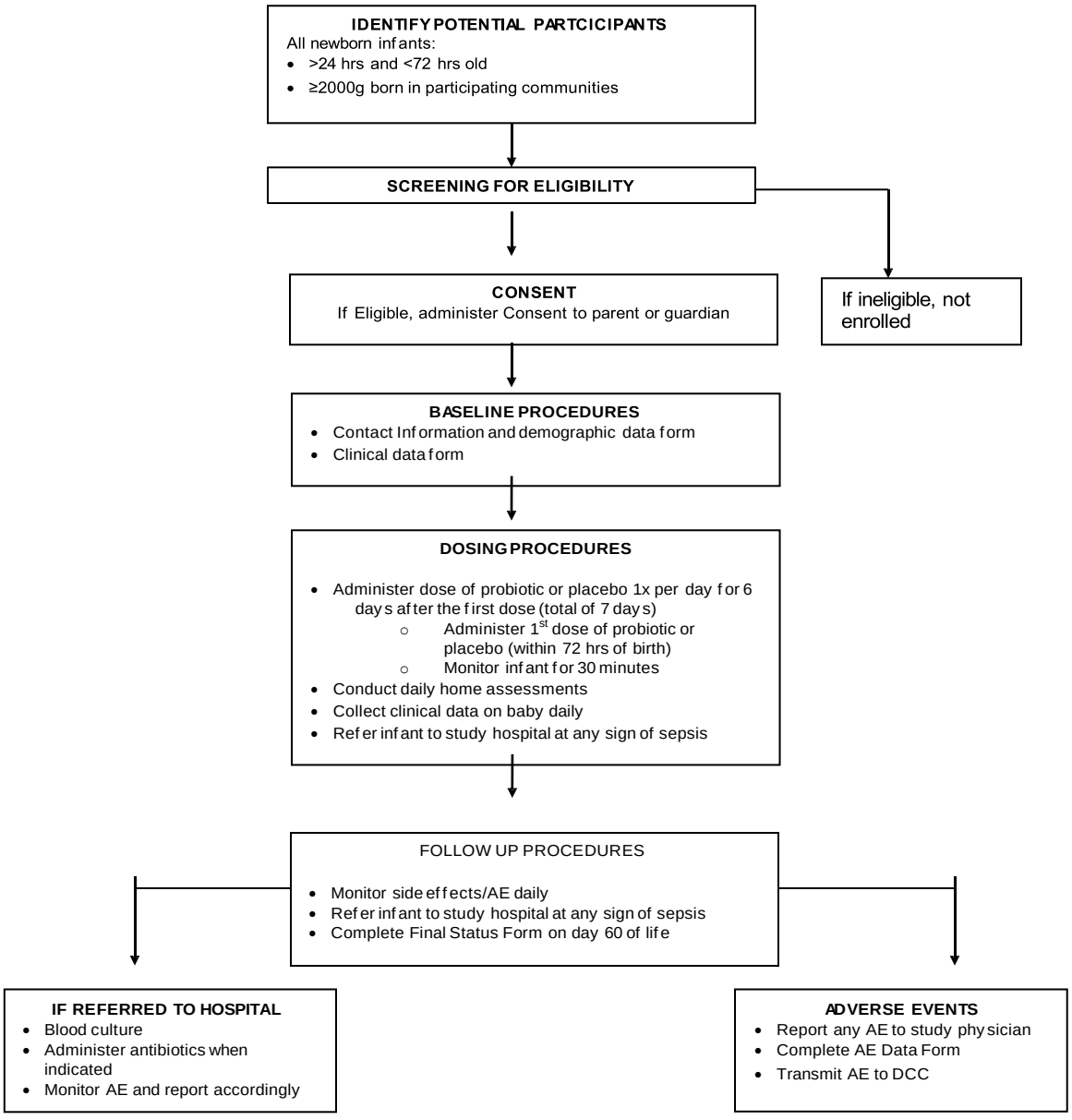
Blood/CSF cultures: Blood/CSF culture data will be collected on all infants who receive a septic workup when clinically indicated during the study period (60 days).

All samples will be sent to the attached microbiology laboratory in the hospital for processing.

The microbiology officer will be responsible for:

- Detailed aerobic and anaerobic processing of stool and blood specimens.
- Identification of bacterial isolates by conventional microbiology and API rapid diagnostic system.
- All clinical isolates will be stored frozen at -70 ° C in the study laboratory.

Sequence of Procedures



Definitions

Identify Potential Participants
Infants who are >24 hours and <72 hours of age are eligible to be screened for participation. In addition, infants must be >2000g at the time of screening (inclusion criteria).

Screening for Eligible Participants
Infants will be screened for eligibility by screening for inclusion and exclusion criteria listed earlier in this document.

Consent
Infants will need the consent of at least one parent or guardian in order to participate.

Baseline Procedures
The CHV will conduct a home visit to all newborn infants of consenting women. Infants will be screened by the CHV. If eligible, the infant will be enrolled in the study and randomized to receive placebo or probiotic supplement.

The CHV will conduct a clinical assessment of the infant, including temperature, feeding practices and the presence of health concerns. The CHV will then administer the supplement to the infant.

Dosing Procedures
CHV will administer a dose of probiotic or placebo to the infant everyday for six days after the first dose is given, for a total of seven days. Before dosing, the CHV will evaluate the infant to make sure there are no signs of sepsis or symptoms of other illnesses.

Follow Up Procedures
The CHV will conduct daily visits to all enrolled infants for the first sixty days of life in order to monitor the infant's health and development. CHVs have been trained to recognize signs of sepsis and will refer infants with suspected sepsis to the study hospital sites. Infants will also be referred to the study hospital if they have signs of any other major morbidity. Apart from birth weight, the CHV will also record weight on day 7 ($\pm$1), 28 ($\pm$2), and 60 ($\pm$3) in a hanging scale.

Mothers and other ladies (grand mothers etc.) have been educated via focus group meetings and video movies on neonatal sepsis and how to recognize sign symptoms of sepsis and what to do when sepsis is suspected in a baby (how to go to the hospital and what happens in the hospital when a baby arrives there). We have seen that many parents now bring their babies on their own without the CHV.

While we have full confidence that mothers will bring their babies with the smallest possibility of ailment, because this is a research study and an interventional study, we consider it is our duty to tightly monitor the population and not leave any baby unattended in case of an adverse event.

10. ASSESSMENT OF SAFETY

Definition of an Adverse Event (AE)

An adverse event (AE) is any untoward medical occurrence that may arise during the study period.

A serious adverse event (SAE) is an adverse event that results in any of the following:

- Death
- Life-threatening illness including sepsis
- Hospitalization
- Medical/surgical care to prevent permanent impairment or damage
- Persistent or significant disability or incapacity
- Other events the investigator considers serious

Specific events to be monitored in the study include:

- Fever: Sustained axillary >37.5^{0}C measured for 3 minutes
- Hypothermia: Sustained axillary < 35 ^{0}C measured for 3 minutes
- Convulsion: Visible convulsion (reported by parents) but confirmed by study personnel
- Lethargy: listlessness, inactivity, apathy, or decreased level of consciousness or alertness compared to the previous day
- Feeding poorly: lack of interest in feeding, or an inability to take in adequate nutrition observed for more than 6 hours
- Vomiting: three or more episodes a day, any projectile, bloody or bilious vomiting
- Diarrhea: Change in consistency of stool with more water content as reported by the mother
- Bloody stools: If blood is macroscopically visible in stool, no test is needed. In suspected cases, Guaiac testing should be done
- Abdominal distention: distension of abdomen above the thoracic cage, glossiness of abdominal wall, loss of superficial creases over the abdominal skin or visible loops.

There is a small possibility of adverse events arising from the supplement itself. It is expected that these adverse events will manifest as diarrhea, vomiting, sepsis and, in severe cases, lactobacillemia. Our already established network in the communities now assures us that every patient will be tracked and brought to the hospital within 12-18 hours. All cases of sepsis will be confirmed by blood culture and treated with IV antibiotics. A list of antibiotics the ATCC strain of *L. plantarum* (in the current GastroPlan preparation) is sensitive to is attached at the end of this document. This includes a list of common antibiotics that will always be made available for the patients. Any other supportive treatment required will also be provided.

Adverse Event Procedures and Reporting Requirements

The PI will be notified of the occurrence of SAEs and AEs within specific time frames:

- Fatal or life-threatening events (SAEs) must be reported within 48 hours.
- Other serious and unexpected events (AEs) must be reported within 7 days.

Written notification of the SAE or AE will be e-mailed as PDF attachment (or faxed) within the stated time frames to the PI, who will forward to NICHD program officer on a regular basis. These events will be reported to the local IRB in Orissa and the U.S. IRB following local guidelines.

Local Regulatory Requirements

Approval of the study has been obtained from the Indian Council of Medical Research, and the Health Ministry Screening Committee apart from local institutional IRBs. Approval for import of GastroPlan has also been obtained from the Drugs Controller General of India.

11. CLINICAL MANAGEMENT

Toxicity Management

There is no known toxicity related to the probiotic supplement. *L. Plantarum* has been administered to extremely sick patients, including post-operative patients, without any reported adverse events. However there is always the possibility of lactobacillemia because the supplement contains a live microbial product. If this or any other adverse event is experienced, the infant will be referred to the study hospital for further examination and will be managed with antibiotics as needed.

Other Disease Events

Any infant with signs or symptoms of a major morbidity will be referred to the study hospital for further examination, whether or not it is related to the study.

Criteria for Permanent Treatment Discontinuation for an Individual Participant

Discontinuation of study treatment will occur for the following reasons:

- Treating physician advises against further administration of the intervention. The study physician may decide to discontinue study treatment for an individual participant in the case of sepsis (any organism), or other major morbidity
- The mother of the infant relocates outside of the study area or withdraws from the study

Criteria for Premature Study Discontinuation

The study will be discontinued under the following circumstances:

- A large number of cases of lactobacillemia due to *L. plantarum* are recorded during the study period
- The DSMB recommends discontinuation of the study.

12. STATISTICAL CONSIDERATIONS

Sample Size Calculation

Based on the surveillance data and a conservative approach, we are estimating that over the coming five years the incidence of sepsis and death will be between 8-9%. Assuming a two-tailed test with 5% type I error rate and 80% power, the attached table describes the number of infants that will be required when we want to show a 20% and 25% relative difference.

Relative drop in sepsis incidence	9% Base Rate	8% Base Rate
20%	3716	4221
25%	2337	2653

Based on these scenarios we propose to enroll 4,221 infants per group (8,442 total) that will assure detection of a 20% drop even if the combined sepsis/death goes down to 8% during the study.

Statistical Analyses

The primary outcome in this study is the incidence of sepsis and death of infants during the first sixty days of life. The null hypothesis of the study is that treatment with the *L. plantarum* preparation versus placebo for the first week of life will have no effect on the rate of clinical sepsis and/or death. The proposed study sample is of adequate size to detect a relative reduction of 20% in the outcome measure. The proposed randomization procedure is expected to allocate subjects into groups with comparable baseline measures. Moreover, the randomization procedure is also expected to equally distribute potential confounders across both study groups.

This will be an intent-to-treat analysis.

Primary methods of analyses will be chi-square and Fisher's exact tests for binary outcomes and t-tests and analysis of variance (ANOVA) for continuous variables. All these univariate tests will be done at a significant level 0.05. These analyses will also determine potential factors which confound the clinical outcomes. If there is an observed tendency for any of these factors to be unevenly distributed between the treatment and placebo groups, multiple logistic and linear regression will be used to adjust for these confounders. Stepwise regression techniques will be used to select variables to include the most parsimonious model.

Interim Analyses

This study is expected to be monitored very carefully. Apart from any evident trends (clustered adverse events, epidemics etc.) that will be tracked during our monthly monitoring, it is expected that the DSMB would like to examine data sets at least once a year. This can be done blinded and the study continued at the decision of the DSMB. On the other hand, if any large treatment change (improvement or deterioration) is visible between the two groups, the DSMB may opt to do an unblinded interim analysis. Typically, we will expect the DSMB to recommend two interim analyses, one at the end of year 1, one at the end of year 2 and one at the end of the study (year 3). This will be done using the O'Brien-Flemming boundary rules. Based on Jennison and Turnbull, and assuming a two-tailed test with 5% type I error and 80% power, the maximum sample size per group of the O'Brien-Flemming test is 1.017 times the number of infants needed per group without interim analysis. For example, we will need 4293 (in place of 4221) infants per group to have 80% power to detect a 20% relative difference. Although there is a slight increase in the sample size, our proposed sample size is large enough to facilitate interim analyses and have adequate power (we also have the ability to increase our enrollment number by additional 144 (72 per group x 2). The nominal significance levels to be used in these analyses are 0.00052, 0.014 and 0.045. If any of the interim analyses demonstrates significant changes in any group, consideration will be given to early termination of the study. For example, if at year 1 a z-test of clinical sepsis and/or death rate between treatment and placebo group gives a normal p-value of 0.00052 or less, a significant difference at a 0.05 level can be declared. These scenarios are for discussion purpose only. The final determination will be made by the U.S. DSMB.

13. RANDOMIZATION, ENROLLMENT, and BLINDING

Eligible infants will be randomly assigned to receive the probiotic supplement or placebo preparation. A sample allocation of 1:1 for controls vs. treatments will be used. To ensure balanced randomization in each village/Anganwadi center, a computer generated randomization list with a block size of 4 will be developed by the UMB BioCore.

Participant Identification/Study IDs

We will create unique 7 digit IDs to be assigned to each infants. The following scheme will be used for this purpose:

1st four digits: ID number of the village starting with one letter for the site, B (for Bhubaneswar) and R (for Rourkela), followed by three numbers for the village codes.

5th , 6th and 7th digits: Serial number of the participating infant (Starting from 001)

Examples

B001001: is the first infant enrolled in the first Anganwadi center in Bhubaneswar
B096025: is the 25th infant enrolled in Anganwadi center # 96 in Bhubaneswar
R127038: is the 38th infant enrolled in Anganwadi center # 127 in Rourkela

Other than the BioCore and clinical trial supplier, no other study personnel will have access to the assignment: P (placebo) or T (treatment)

14. DATA HANDLING AND RECORDKEEPING

Data Management Responsibilities

Data will be collected on paper forms at the home and entered into computers at participating hospitals using the Data Management System (DMS) developed for this study. Electronic data will be transferred from each hospital to a single Research Unit Data Center (RUDC) in Kalinga. The Kalinga Hospital Data Center will transmit the data to the Data Coordinating Center (DCC) on a weekly basis.

The research sites are responsible for keying the data in a timely fashion. It is the responsibility of the DCC (RTI) to assure the quality of computerized data for each site.

Source Documents and Access to Source Data/Documents

In addition to the data collection instruments designed for this study, the following source documents may be used:

- Sixty day card
- Hospital records

15. CLINICAL SITE MONITORING

Site visits
The PI, other U.S.-based faculty working on this trial and Program officials from the National Institute of Child Health and Human Development (NICHD) and RTI staff will visit participating clinical research sites to review the individual subject records, including consent forms, CRFs, supporting data, laboratory specimen records, and medical records (physicians' progress notes, nurses' notes, individuals' hospital charts), to ensure protection of study subjects, compliance with the protocol, and accuracy and completeness of records.

Microbiology
An overall system of quality control documentation and certification will be maintained at each site, with central oversight from the reference laboratory at AIIMS to ensure that the microbiology results meet high standards, including reproducibility and uniformity across all sites. This mayl include molecular typing and detailed stool analysis (aerobic and anaerobic) of control and treated babies.

16. HUMAN SUBJECTS PROTECTION

Institutional Review Board
This protocol and the informed consent document and any subsequent modifications will be reviewed and approved by the IRBs or ethics committees responsible for oversight of the study. A signed consent form will be obtained from the parent or legal guardian of the eligible infant. The consent form will describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation. A copy of the consent form will be given to the subject's parent, or legal guardian.

Protocol Registration
The protocol is registered on clinicaltrials.gov. The identifier is NCT00518596.

Informed Consent Process
Infants will need the consent of at least one parent or guardian in order to participate. Mothers will be asked for verbal consent at the time they receive the sixty day card, around the seventh month of pregnancy. Infants born to all mothers who have provided verbal consent during pregnancy will be screened for eligibility within 24 hours of birth. Full written consent will be obtained at this time.

Participant Confidentiality
All laboratory specimens, evaluation forms, reports, and other records that leave the site will be identified by coded number only to maintain subject confidentiality. All records will be kept locked. All computer entry and networking programs will be done with coded numbers only. Clinical information will not be released without written permission of the subject, except as necessary for monitoring by IRB, the FDA, the NICHD, the OHRP, or the pharmac eutical supporter(s) or the supporter's designee.

17. PUBLICATION POLICY

The authorship of manuscripts, posters or oral presentations, or other reports of the results of this study will be guided by the criteria for authorship formulated by the International Committee of Medical Journal Editors and published in its Uniform Requirements for Manuscripts Submitted to Biomedical Journals (updated October, 2001; available at http://www.icmje.org). According to these criteria, each author should have participated sufficiently in the work to take public responsibility for the content. Authorship credit should be based only on substantial contributions to (a) conception and design, or analysis and interpretation of data; and to (b) drafting the article or revising it critically for important intellectual content; and on (c) final approval of the version to be published. Conditions (a), (b), and (c) must all be met.

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Appendix A. Antibiotics sensitivity pattern of L. plantarum to antibiotics commonly used in neonates

Antibiotic	**Sensitivity (zone size in mm)**
Penicillin (CIP 5)	S (20)
Augmentin (SAM 20)	S (30)
Amikacin (AN 30)	S (23)
Cefotaxime (CTX 30)	S (34)
Ceftadizime (CAZ 30)	S (25)
Ceftriaxone (CRO 30)	S (35)
Cefpodoxime (CPD 10)	S (30)
Cephalothin (CF 30)	S (15)
Erythromycin (E 15)	S (34)
Gentamicin (GM 10)	S (26)
Ciprofloxacin (CIP 5)	I (6)
Vancomycin (VAN 30)	R (0)
S: sensitive, **R**: resistant, **I**: intermediate sensitive	

All Lactobacilli possess an atypical peptidoglycan in their cell wall which makes them inherently resistant to Vancomycin. However, they are highly sensitive to commonly used antibiotics. The above table describes a list of antibiotics tested against *L. plantarum*; most of them are safe to use in neonates. The proposed *L. plantarum* strain does not contain plasmids, a major concern of transmissible of bacterial el

July 20, 2007

Amendment # 1 to

Placebo-Controlled, Randomized Trial of an Intervention using Probiotics in the Prevention of Neonatal Infection in Community-born Infants

1. UMB has received the first NGA of the R0-1 grant (HD 053719-01A1).

2. RTI Intl will not be responsible for data management-related issues. GCRC will handle data management needs for the current grant.

June 21, 2008

Amendment # 2 to

Placebo-Controlled, Randomized Trial of an Intervention using Probiotics in the Prevention of Neonatal Infection in Community-born Infants

1. The field feasibility pilot phase with the proposed enrollment of 400 infants is brought to an end after enrollment of 250 subjects at RKL and BBS site.

2. The main field RCT should begin.

3. Enrollment window is extended to day 4 (in place of 3). DSMB approval has been obtained (BRAAN amendment letter attached)

BRAAN Amendment

In this amendment we are requesting two changes. Neither one changes the risk-benefit ratio.

1. Change in enrollment period: In our current protocol, we are approved to enroll newborn infants in the community >24 hours and <72 hours of age. During our pilot phase, we have not been able to enroll a significant population (about 25%) of eligible infants due to the fact that their mothers gave birth in local hospitals. In our previous surveillance study conducted during 2002-2006, we had noted that bulk of deliveries in our study villages were at home. However, there is a very recent change in the practice, where instead of opting to deliver at home or in the village nursing center, many women are going to the hospitals. This change is due to implementation of several new incentives by the government where the mothers are given monetary help for attending a hospital for delivery. These mothers are typically being discharged on the third day after delivery. Keeping travel time etc. in the picture, we are missing many eligible infants that could be enrolled if the enrollment time is extended to fourth day. This extension does not pose any risk to the infant and does not change the scientific integrity of the study. The recently convened DSMB has allowed this change (letter attached).

To reflect this change, we have modified the inclusion criteria (1-4 days) in the Braan protocol and consent forms (BRAAN consent form and local language consent form).

2. Initiation of enrollment in the main trial: In our original protocol (total sample 8,842), we had proposed to enroll a convenience sample of 400 infants in a pilot feasibility phase before launching the main trial with enough power (8,442). The purpose was to primarily examine logistics of operations in the villages. To examine safety, prior to launching this field pilot trial, we had earlier completed hospital-based clinical and had noted reduced infections and other adverse events without any adverse events related to study intervention.

We have now enrolled 250 infants in this pilot phase and completed their 60-day followup. Unblinded results have been reviewed and approved by the DSMB. Based on our experience (smooth operation during the pilot phase), and discussions with NICHD program staff, we would like to start the main RCT (8,442 infants) now, in an effort to conserve time and expense and expose the optimal number of infants to this intervention.

Hence, we feel that it is quite appropriate to start enrollment in the main trial.

Attached please find the DSMB note to IRBs and modified local language consent form.

Pinaki Panigrahi,MD,PhD
PI