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Effect of bovine lactoferrin on seroconversion following polio vaccine administration in children: protocol for a double-blinded randomised controlled trial

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ABSTRACT

Introduction The oral polio vaccine (OPV) has substantial results in eliminating wild poliovirus and the vaccine of choice in polio eradication. However, the mucosal immunity induced by the OPV is still uncertain. Literature has shown that bovine lactoferrin (BLF) is a safe and useful protein found in cow's milk with extraordinary antimicrobial, antiviral, antiinflammatory and immune-modulatory functions that help children's gut to fight against micro-organisms like poliovirus. However, limited data exist regarding the effect of BLF on polio vaccine immune response. The primary objective is to evaluate the effect of BLF in enhancing mucosal and humoral immunity in children following the administration of oral and inactivated polio vaccines.

Methods and analysis This is a two-arm double-blinded randomised controlled trial comparing 462 neonates (231 in both groups) receiving either BLF or placebo with breast milk. The intervention is administered from day 1 till 6 weeks of age to a full-term healthy singleton newborn born at the Aga Khan University Hospitals, Karachi, Pakistan. The primary outcome is the seroconversion, 1 month after the receipt of two doses of OPV (at 10 weeks). For descriptive statistical analysis, Stata will be used, the frequency with percentages will be reported to describe baseline characteristics of the participants. A χ^2 test will be used to compare categorical variables and a simple t test to compare continuous variables. The proportion of seroconversion and shedding will be compared using χ^2 test or Fisher's exact test.

Ethics and dissemination The Ethics approval has been granted by the Ethics Review Committee (ERC) of Aga Khan University for the proposed trial (ID: 2019-1955-5013). Furthermore, the National Bioethics Committee (NBC) of Pakistan has also approved the study for human subject research (ID: 4–87/NBC-443/19/669). Study findings will be disseminated through presentations at scientific conferences and educational practice workshops and will be published in an international peer-reviewed scientific journal.

Trial registration number NCT04432935; ClinicalTrials.gov.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Randomisation will produce comparable groups and eliminate bias in treatment assignment.
⇒ As it is double blinded, the unbiased selection of the participants will be ensured, resulting in reliable and robust data.
⇒ Collection of blood and stool samples at various time points will provide robust data on the effect of Lactoferrin on polio seroconversion.
⇒ Complexity of the trial may result in loss to follow-ups, which has been adjusted in sample size estimation.
⇒ The primary analytic approach will be an intention to treat analysis to avoid the effects of crossover and dropouts.

INTRODUCTION

The global polio eradication initiative was adopted in 1988 and since then the number of cases due to polioviruses has decreased by over 99.9%. In 2019, the WHO reported 143 cases of paralytic poliomyelitis due to wild polioviruses worldwide.[1] In the year 2020, the remaining endemic areas with wild poliovirus circulation were limited to Pakistan, Nigeria and Afghanistan.[2 3] The oral polio vaccine (OPV) has been successful in producing substantial results in eliminating the wild poliovirus and has been the vaccine of choice in polio eradication, especially in developing countries.[4 5] However, its effectiveness studies have shown that the OPV stimulated a lower antibody response in developing countries.[6–11] Factors such as repeated illnesses, malnutrition, gut barrier dysfunction and shorter duration of breast feeding in infancy are associated with diminished OPV response.[12–15]

Malnutrition in Pakistan is a persistent concern. According to the recent National

Nutrition Survey, 40% of under-5 children are stunted with a high prevalence of micronutrient deficiencies.[16] Infectious diseases affecting malnourished children is another public health issue as it is related to high child mortality in under 5 years of children.[17] Studies also suggested that malnourished children have a compromised mucosal and intestinal barrier, which is a risk factor for lower mucosal protection against poliovirus and a major determinant of the humoral response against OPV.[18 19] This phenomenon has been corroborated in studies conducted in Pakistan.[14 20 21]

Breast feeding is a dynamic and highly recommended practice as it is unique in protecting children from infections as a first line of defence and serves as a source of nutrition for their optimal growth and development; being free from environmental contaminants.[22 23] Its anti-infective, anti-inflammatory and immune regulatory compounds include antibodies, glycan, lactoferrin, leukocytes, cytokines, which play a key role in immunity development.[24 25] Exclusive breastfeeding for the first 6 months followed by partial continuation into the second year of life has been staunchly encouraged by international health agencies.[26] However, as per the Pakistan Demographic Health Survey, 20% of children start breast feeding within the first hour of birth, and 48% of children under age 6 months are exclusively breastfed.[27]

Lactoferrin which is believed to be an immune modulator is found in human colostrum, but its quantity starts decreasing in mature milk.[28] Evidence suggests that lactoferrin enhances a child's immunity against gastrointestinal infections by inhibiting the growth of bacteria by iron deprivation, further it also inhibits the activity of the virus by preventing virus attachment to the intestinal cells and by binding to the viral particle at the postadsorption step. Hence, the children become less susceptible to the virus that replicates in the gut such as rotavirus, poliovirus and adenovirus.[29–32] Several studies have reported the antiviral activity of lactoferrin against different types of viruses that replicate in the gut such as rotaviruses, polioviruses and adenoviruses, and in almost all cases, the effect takes place mainly in the early phase of viral infection by preventing the adsorption of the virus to the target cells.[33 34]

Limited data exist regarding the efficacy of bovine lactoferrin (BLF) on polio immune response. Yet, trials investigating the enhanced efficacy of the Bacille Calmette-Guerin (BCG) vaccine after induction of Lactoferrin have shown promising results.[35 36] Given that lactoferrin has extraordinary antimicrobial, antiviral, anti-inflammatory and immune-modulatory functions, a new window of opportunity can be created if its effects are assessed on the level of seroconversion of polio vaccine in children. Pertaining to the scarcity of data, the present study will add to the existing pool of knowledge regarding the use and effectiveness of lactoferrin on intestinal and humoral immunity produced by the bivalent OPV (bOPV) and trivalent inactivated polio virus vaccine given during the routine immunisation (RI). This trial will be engendered knowledge on the anti-infective properties of lactoferrin that will contribute towards the eradication of this debilitating disease from our country.

HYPOTHESIS

This trial will address the following primary questions:

1. Is there a difference in serum antibody levels against poliovirus types 1 and 3 between children receiving lactoferrin versus those receiving placebo at 10 weeks of age (1 month after second OPV dose)?
2. Is there a difference in serum antibody levels against poliovirus types 1, 2 and 3 between the two groups at 18 weeks of age (1 month after completion of the RI schedule)?
3. Is there a difference between children receiving lactoferrin versus those receiving placebo in excretion of poliovirus types 1 and 3 in stools at 19 weeks of age (1 week after administration of a challenge dose of bOPV)?

SPECIFIC OBJECTIVES

Primary objective

1. To compare humoral immunity represented by seroconversion (presence of detectable antibodies to poliovirus types 1 and 3 in serum) 1 month after receipt of two doses of OPV (at 10 weeks) in both the study arms (lactoferrin vs placebo).

Secondary objectives

1. To compare humoral immunity represented by seroconversion (presence of detectable antibodies to poliovirus types 1, 2 and 3 in serum) after completion of the RI doses/schedule (administered at 6, 10 and 14 weeks of age) at 18 weeks of age (4 weeks after the last RI dose) in both study arms.
2. To compare intestinal immunity represented by shedding of poliovirus types 1 and 3 1 week after a challenge dose of bOPV administered at 18 weeks of age (1 month after completion of the RI doses/schedule at 14 weeks of age) in both the study arms.
3. To compare the duration of shedding among the two study arms (lactoferrin vs placebo) represented by shedding at 1 and 2 weeks, that is, 19 and 20 weeks of age after the challenge dose of bOPV administered at 18 weeks.

METHODOLOGY

Study design

This is a double-blind (participants, healthcare workers and investigators will be blinded) randomised placebo-controlled trial that will compare intestinal and humoral immunity following completion of the RI schedule for poliovirus in children receiving a nutritional supplement (lactoferrin) in the intervention arm vs those receiving placebo in the control arm.

Study setting

The trial will be conducted at the Aga Khan University Hospital and its associated secondary health facility in Kharadar, Karachi and district Dadu of Sindh province in Pakistan. The sites are, providing services in obstetrics, gynaecology, neonatology, paediatrics, child health and family planning/infertility. Services for consulting clinics, diagnostics, immunisation, operating rooms, inpatient wards, laboratory, pharmacy and vaccination as per the Expanded Program on Immunization (EPI) are also offered in these centres.

Study population

The trial population will be healthy term newborn babies born. For this purpose, pregnant women of 18–45 years, in 28–38 weeks of gestation age will be identified and screened in antenatal clinics. Infants born to these women will be enrolled in the trial.

Eligibility Criteria

Inclusion Criteria

Healthy singleton full-term births weighing at least 1200 g, and with an APGAR (A-Apperance, P-pulse, G-grimace, A-activity, R-respiration) score of 7 or higher at 5 min after delivery, tolerating oral feed, with no respiratory distress, absence of danger signs and congenital anomaly will be included in the trial after obtaining consent from the mother/family.

Exclusion criteria

Preterm and newborns less than 1200 g, sick newborns according to predefined criteria (does not tolerate oral feeds, severe respiratory distress including respiratory rate less than 20 bpm, grunting, central cyanosis, severe chest in-drawing, convulsions, unconsciousness and hypothermia of less than 32°C, apnea, congenital malformation) will be excluded from the trial.

Discontinuation criteria

The discontinuation criteria will be withdrawal of consent for participation, identification of immunodeficiency disorder, blood disorder or other medical condition for which continued participation would pose risk to participants, receipt of immunosuppressive medications and premature termination of the trial.

Sample size

We calculated an overall sample size of 231 neonates in each group (462 neonates in both groups). We estimated the sample size assuming one-sided test with a probability of error (alpha) of 0.05, a seroprevalence of 70% after two doses of OPV at 10 weeks, a power of 80%, an absolute increase of 10% in the seroconversion in the intervention group, a loss to follow-up of 10% and a 95% level of confidence. This sample will be adequate for measuring the outcome for all the objectives mentioned above.

Randomisation

After birth, screening of the participant will be conducted by the trial physician. All eligibility criteria will be checked using a checklist before randomisation. With the confirmation of the eligibility, the participant will be randomly assigned to either control or intervention group. A unique randomisation ID or code will be assigned to the participant in either of these arms. Assignment of treatment will be conducted as a 1:1 ratio per computer-generated randomisation sequence, with random block sizes of 4 or 6. This will be done using a dedicated study computer. A predefined list of randomisation IDs will be given to the study personnel and the study staff will take the next sequentially numbered sachet from the packet. Allocation concealment will be achieved by having identical sachets, that is, lactoferrin and placebo both will look identical and will be in identical packaging.

Participants, field staff interacting with participants, and lab personnel will be blinded to randomisation assignment. To ensure appropriate concealment, both the lactoferrin and placebo are made identical with the same colour, consistency and quantity and using identical packaging in identical ways. Only Data Safety and Monitoring Board (DSMB) will have the authority to unblind in the case of adverse events (AE) or serious adverse events (SAE).

Sampling process

Eligible participants will be enrolled at the time of birth. Cord blood for maternal antibodies will be collected along with the first bOPV (OPV0=zero-dose OPV) administration. The intervention arm will receive BLF and the control arm will receive a placebo with breast milk on daily basis from day 1 to 6 weeks of life. The total follow-up period per participant will be 20 weeks of life. A detailed methodology explanation is demonstrated in table 1.

For both intervention and control arms, a blood sample will be collected during the clinic visit before administration of RI of bOPV, at 6 weeks and 10 weeks. The challenge dose of bOPV will be administered at 18 weeks of age after 4 weeks of the last RI dose given at 14 weeks of age, and a blood sample will also be collected. The serum will be assessed for the presence of poliovirus types 1, 2 and 3 antibodies. Stool samples will be collected at 6, 7, 8, 14, 18, 19 and 20 weeks of age and assessed for the presence of poliovirus types 1 and 3 through virus isolation techniques. The container for stool collection will be provided to the parent beforehand by the field staff.

Trial team and training

The trial team will be comprised of a research manager, research medical officers, research assistants and a phlebotomist. The manager will supervise the overall activity of the trial, whereas the medical officers will screen, recruit and counsel mothers on how to use lactoferrin. The research assistants will collect data on compliance and trial outcomes, whereas the lab assistants will be responsible for blood and stool sample collection and processing. The trial teams will undergo intensive 3–5 days of training in trial procedures and good clinical practice. This will be conducted by AKU faculty members who

Table 1 Summary of trial procedures

Arm	Age of participant									
	Birth	Day 1 to 6 weeks	6 weeks	7 weeks	8 weeks	10 weeks	14 weeks	18 weeks	19 weeks	20 weeks
Lactoferrin	Blood* bOPV	Lactoferrin	Blood Stool bOPV	Stool	Stool	bOPV Blood	bOPV +IPV Stool	Blood Stool bOPV	Stool	Stool
Placebo	Blood* bOPV	Placebo	Blood Stool bOPV	Stool	Stool	bOPV Blood	bOPV +IPV Stool	Blood Stool bOPV	Stool	Stool

*Cord blood=maternal antibodies.
IPV, injectable polio vaccine ; OPV, oral polio vaccine.

will provide essential information on standard operating procedures and data collection instruments. The training will be conducted at the field site, for which multimedia, demonstrative videos and flip charts will be used with relevance to the trial. Furthermore, lecture rooms, as well as operational fields for real-life field scenarios, will be used. Similarly, the teams will also be trained on effective communication for delivering information and counselling the family at the household level as per the study requirements. Educational materials such as information, pictorial booklet and flip charts in the local language will be provided to trial staff. The sample collection team will be trained as per the WHO guidelines[37] by trained lab staff.

Interventions

First dose of BLF

The first dose of BLF or placebo will be dispensed by a trained trial physician in front of the mother/caregiver at the hospital. The BLF or placebo will begin on the first day of life of the newborn (day 1) with a single dose dissolved in milk (preferably breast milk, otherwise formula milk); preferably before the first dose of BCG, and OPV is given to the baby. Daily follow-up of the newborns in both arms will be organised during the entire course of the hospital stay to assess vital information along with administration of BLF and placebo daily single dose.

Follow-up for compliance and to assess the well-being of participants

At discharge, the mother of the enrolled newborn dyad will be provided with 1 week supply of BLF or placebo along with instructions on how and when to administer it to the infant. Lactoferrin or placebo will be administered daily until 6 weeks of life. Newborns from both arms will be followed weekly until 6 weeks of life for general well-being and compliance. Compliance will be assessed by counting the used sachets of lactoferrin or placebo as mothers will be instructed to keep the empty sachets in a dedicated container issued by trial staff. Mothers will also be asked about intake of the supplement by the infant (ie, did the infant spit it out, vomited, etc). If the infant had vomited or spit out the lactoferrin/placebo, then the dose would not be repeated for the day.

Routine Immunisation

The parents of the enrolled infant will be strictly advised to administer all vaccinations according to the EPI schedule (table 2). They will be asked to come to the trial hospitals for vaccinations and follow-up. Compliance with vaccination will be facilitated by reminder calls before the date of vaccination and if the participant does not come to the hospital for 2 days after the due date of vaccination, the vaccine will be administered at home by trial staff.

Measures to ensure compliance and minimise loss to follow-up and missing data

- During recruitment: only women who have lived at their current residence for the previous year and

Table 2 Routine immunisation schedule in Pakistan

Age	Vaccines
At birth	BCG and bOPV
6 weeks	Penta-I, Pneumo-I, bOPV-I and Rota-I
10 weeks	Penta-II, Pneumo-II, bOPV-II and Rota-II
14 Weeks	Penta-III, Pneumo-III, bOPV-III and IPV
9 Months	Measles-I
15 Months	Measles-II

BCG, Bacille Calmette-Guerin; OPV, oral polio vaccine.

confirm that they will not move away from their home for 5 months following delivery will be recruited. Moreover, after the delivery of the child, plans for moving will be ascertained (before randomisation of the child). If the mother's plans have changed, the child will not be recruited.

- After randomisation, families will be instructed to inform the study team if they plan to move from their residence and to provide the new address for follow-up. Even if a family is not within the catchment area of the health facility, they will be followed by the study team for the administration of immunisation and supplement/placebo.
- To ensure compliance with the RI schedule, mothers will be contacted 1 week before the child is due to receive their vaccine dose to remind them. If the mother does not bring the child for up to 2 days after the vaccination due date, study staff will administer the vaccine at home.
- To facilitate the assessment of compliance with administration of the supplement/placebo, mothers will be instructed to keep the empty sachets of supplement/placebo in a dedicated container. Study staff will visit the families weekly to check on the child, count the empty and full sachets and record basic information regarding the child's acceptance of the supplement/placebo.
- Blood will be collected before administration of the vaccine, the participant will be reminded ahead of the appointment date and if they do not show up, the next day study staff will do a home visit to collect blood or stool specimen.
- In case of withdrawal of consent/refusal to continue the study, data will be collected on the reasons for withdrawal to determine if it is related to the vaccine, supplement or other reasons. This will facilitate a sensitivity analysis to determine the possible effects of missing data on the results of this trial.

Post vaccination procedures

For both intervention and control groups, a blood sample will be collected in the hospital at 18 weeks of age (4 weeks after the last RI dose given at 14 weeks of age). One day before 18 weeks of visit, the parents will collect stool from the participant/infant and bring it the next day to the hospital. During the 18-week visit, after the blood draw and collection of the stool specimen, the participant will be given a challenge dose of OPV. Thereafter, at 19 and 20 weeks of age, stool will be collected from the participants and field workers will collect the specimens from the participants' homes. The serum will be assessed for the presence of poliovirus type 1; 2 and 3 antibodies and stools will be assessed for the presence of poliovirus types 1 and 3 through virus isolation techniques.

Sample collection and processing

For immunity assessment, 3 mL of whole blood will be collected by a trained phlebotomist from each subject. After collection, the blood samples will be allowed to clot, centrifuged to separate serum and transported to the Nutrition Research Laboratory (NRL) at the AKU, Karachi under cold chain conditions, where they will be stored at −20 C until shipment to the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. Neutralising antibodies will be determined by the method recommended by the WHO and CDC.[38 39]

For stool collection, containers will be provided to the families, which will be collected by the study team for processing. The stool samples will be transported with ice packs to the NRL at the AKU, Karachi under cold chain conditions. At NRL, three aliquots of stool samples will be prepared, two of them will be transported to the Polio Reference Laboratory at NIH Pakistan under strict cold chain maintenance, whereas one aliquot will be achieved at NRL as a backup. Stool specimens will be examined for the presence of poliovirus as per the standard procedures and guidelines.[38 39]

Data collection

Source document file for each participant will have a trial-assigned separator or tag, which will help the hospital staff in identifying the enrolled participants. Data will be collected from the participants of the trial at the hospital and will be managed at the time of screening, enrollment and follow-up visits and during identifying AE/SAE. This data will be collected on hard copies and in electronic format using tablets wherever possible and will be entered in a predesigned screen made by the Data Management Unit, AKU trial staff daily. Screening/eligibility forms will be used for the participants who meet the eligibility criteria. Participants who will be determined to be eligible after screening will be enrolled after the informed consent by the parents (online supplemental file 1). Enrolment form mainly comprises the basic information, which will include study ID, randomisation ID, supplement or placebo details in both the cases whether given or reason, if it is not given. Assigned trial staff will conduct the weekly follow-up visits within the hospital, home visits, inpatient (readmission in the study site hospital or any other hospital) or via phone calls. These weekly follow-ups are designed to get maximum information from the parents about the enrolled study participant, which mainly includes questions about feeding practices,

history in case of in-patient admission or illness, supplement administration and history of supplement consumption and outcomes of the visits. Furthermore, AE/SAE will be examined and assessed separately through AE/SAE form and will be initially examined by the trial physician and will be reported to the DSMB and the trial investigators. For participants who are non-compliant or lost to follow-up, data on the reason for dropping out will be collected including whether they did not complete lactoferrin/placebo doses or immunisation per RI schedule (number of doses and timing of vaccination).

Process of informed consent

The consent form (online supplemental file 1) briefly states the project objectives, procedures, process of confidentiality and gives participants the right to withdraw at any time. Trained research staff preferably nurses will introduce the trial to potential participants. All women will receive information about the trial in their language of choice either English or Urdu. The language used will be non-technical and easily understood. Participants will be given time to reflect on the information and given an opportunity to ask questions. If willing to participate, the informed consent form (online supplemental file 1) will be signed by the participant and research assistant.

If the mother is illiterate, Independent Literate Witness will sign the consent (online supplemental file 1) and the mother will affix a thumb impression on the consent form (online supplemental file 1). Participants will be free to withdraw from the trial at any stage without loss of benefits. The telephone numbers of investigators will be made available to participants in the event that they require further information or assistance.

Data management and analysis

The primary outcomes will be measured for both the arms, that is, control and intervention. Two approaches will be followed to assess the impact of the intervention on the outcome. Following the descriptive statistical analysis for assessment within and between the control and intervention arms, while adjusting for the confounding variables, and then will do the same at the end of the trial. Stata will be used for descriptive statistical analysis, the frequency with percentages will be reported to describe baseline characteristics of the trial population.

A χ^2 test will be used to compare categorical variables and a simple t test will be used to compare continuous variables. Seropositivity is defined as reciprocal titers of poliovirus neutralising antibodies ≥8; seroconversion is defined as the change from seronegative to seropositive (from reciprocal titre of <8 to ≥8) or participants who demonstrate a titre fourfold higher than the expected fall in maternally derived antibodies, assuming a half-life of 28 days. Shedding of poliovirus is defined as isolation of poliovirus in a stool sample. The primary analytic approach will be the intention to treat analysis. For participants who are non-compliant or lost to follow-up data on the reason for dropping out will be collected including whether they did not complete lactoferrin/placebo doses or immunisation per RI schedule (number of doses and timing of vaccination). The proportion of seroconversion and shedding in the trial arms will be compared using the χ^2 test or Fisher's exact test.

DATA AND SAFETY MONITORING BOARD

A DSMB has been constituted to review the data of AE/SAE cases to monitor the progress of the trial and assess the safety of the intervention and the participants. The members include an infectious disease specialist, a paediatrics gastroenterologist, a neonatologist and a biostatistician. The DSMB will examine all the AE/SAE cases, rate of enrollment and compliance to the intervention throughout the study. An interim analysis will be conducted when approximately 50% of the infants are enrolled. This analysis will be conducted in a blinded manner (intervention groups X and Y). The randomisation code will only be unblinded by following the recommendation of the DSMB if there is any clear evidence of an AE/SAE or mortality between the two groups. The DSMB will advise the trial investigators on continuation, modification or termination based on pre-established stopping rules.

ETHICAL CONSIDERATION AND CONFIDENTIALITY

All personally identifiable information of the trial participants will be kept confidential. It may only be accessed by the DSMB. Written informed consent (online supplemental file 1) will be obtained from all participants' parents before the recruitment in the trial, data collection and specimen collection. The Ethics Review Committee (ERC) of Aga Khan University has approved the proposed trial (ID: 2019-1955-5013). Furthermore, the National Bioethics Committee (NBC) of Pakistan has also approved the study for human subject research (ID: 4–87/NBC-443/19/669). The study contains all items required by the WHO Trial Registration Data Set. The study does not plan to share individual deidentified participant data, including data dictionaries. The results will be shared as conference presentations and publications in peer-reviewed journals. However, the data sets used for the article and the study will be available from the corresponding author on request.

DISCUSSION

The potential use of the findings of this study at the community level in Pakistan will serve as a new hope for the eradication of polio from the country. The ultimate goal of polio vaccination is to protect against the disease, encompassing the ability to limit transmission between infected individuals and those non-infected that are at risk from contact. Supporting evidence from this study will provide a unique opportunity to assess the effects of lactoferrin on the level of seroconversion following

poliovirus vaccination in children as an effective adjuvant to enhance efficacy of the polio vaccines. Being one of the last reservoirs of poliomyelitis, Pakistan needs innovative and safe approaches like lactoferrin that can interfere with the endemic occurrence of polio, along with addressing various factors like malnutrition, breast feeding, administration of RI, etc, which decrease the immunity levels among the children, making them more prone to the infection.

PATIENT AND PUBLIC INVOLVEMENT

The public was not involved in the design of the research tools, but they will be a part of the trial and their feedback will be sought regarding intervention acceptability. The key findings will be shared with their representatives as part of the dissemination plan at the local level.

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Contributors The study was conceptualised by SS. AH drafted the first version. Field instruments and data collection process were developed by SN, TJ and AH. Subsequent drafts were reviewed and edited by SN, TS, SuN, SeA, MU, LS, IH, SA and SS. All authors have read the final version and approved it.

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Competing interests None declared.

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