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Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Awasthi S, Peto R, Read S, Clark S, Pande V, Bundy D, and the DEVTA (Deworming and Enhanced Vitamin A) team. Vitamin A supplementation every 6 months with retinol in 1 million pre-school children in north India: DEVTA, a cluster-randomised trial. *Lancet* 2013; published online March 14. http://dx.doi.org/10.1016/S0140-6736(12)62125-4.

Protocol for DEVTA, cluster-randomised demonstration & evaluation program among 1 million rural children in 7 districts near Lucknow, Uttar Pradesh, India of routine de-worming and of enhanced vitamin A supplementation [1998]

Background

This short protocol summarises the aims and describes in detail the standard operating procedures (SOPs) of a large 2x2 factorial cluster-randomised trial of six-monthly de-worming with albendazole, six-monthly supplementation with vitamin A (retinol), both, or neither in 72 rural study areas (clusters) in northern India, each within a few hours drive of Lucknow, the state capital of the state of Uttar Pradesh (UP).

The total population at ages 1-6.0 years of these study areas is about one million, the 5-year probability of death at these ages is about 2-3%, and the main aims are to find whether either treatment reduces this mortality.

The study will also be of relevance to another important question: with only a limited amount of outside organisational support, can regular deworming, micronutrient supplementation and other simple health interventions be delivered reliably, sustainably and inexpensively through existing infrastructures to large numbers of preschool children in places such as rural UP?

Among preschool children (which, by definition, excludes infants less than 1 year old), poor growth and high mortality are serious problems in many parts of the world where intestinal worms are prevalent and blood retinol levels are low. Although intestinal worms can in some instances cause death directly, eg, from fatal intestinal obstruction, this is extremely uncommon. There are, however, many other nutritional and infective factors contributing to poor growth and and mortality among such children, and the indirect contribution of chronic worm infestation to the effects of these other factors is not known. Thus, the value of routinely eliminating worms is uncertain.

A recent study of 4000 preschool children in the urban slums of the city of Lucknow in Uttar Pradesh (UP) found that 6-monthly deworming with albendazole for 2 years appeared to cause a weight gain of 1 kg in preschool children, but it is not known either from that study or from studies in other parts of the world whether such routine deworming would also have any measurable effect on preschool child mortality. Likewise, even if vitamin A deficiency is not severe enough to cause blindness, low levels of blood retinol may render preschool children more susceptible to bacterial or viral pathogens such as those that cause diarrhoea, pneumonia or measles, and previous trials have reported that 6-monthly supplementation with retinol can reduce preschool child mortality by about one-quarter in populations where moderate vitamin A deficiency is widespread.

UP is an ideal setting for this study, because government programmes have already been instituted that should be able to supply food supplements and micronutrients to preschool children. The study will use this infrastructure (the anganwady system) to deliver deworming medicine and vitamin A to children in those blocks randomly allocated one and/or other active treatment.

Both albendazole and vitamin A are products with long histories of safe use, and some children in the study areas may already be getting one or both of these interventions (albeit only intermittently, and with low coverage rates). As this study will greatly enhance any such coverage, it is entitled DEVTA (Deworming and Enhanced Vitamin A).

The cost-effectiveness of mass treatments, such as deworming, and micronutrients, such as vitamin A, depends not only on the costs and effectiveness of the drugs themselves, but also on the practicability of delivering them to children reliably and sustainably at low additional cost. Both of these important questions will be addressed.

Objectives

The study will have three major objectives:

- to determine how reliably, efficiently and sustainably the anganwady system can deliver safe, simple health services - in this case, antihelminthics and micronutrient supplements; and
- to determine whether deworming at 6-month intervals can somewhat improve child survival during ages 1-6 (in addition to its expected effects on weight gain in poorly nourished children); and
- to determine whether retinol supplementation at 6-month intervals can somewhat improve child survival during ages 1-6 (in addition to its expected effects on subclinical eye disease in retinol-deficient children).

Design

The study will take place in 7 districts in central UP which together include a total of 118 administrative blocks, 72 of which will participate and be randomly allocated in groups of 4 nearby blocks (generally in the same district) to albendazole, vitamin A, both, or neither.

The unit of randomisation will be the *block,* not individual villages or children. Statistically, randomisation by area is just as reliably unbiased as randomisation by individual, but it has important advantages of organisational efficiency and practicability, as all anganwady workers in one block work closely together and meet regularly. Random allocation of the blocks is by the Clinical Trial Service Unit in Oxford.

Within each block, the study area will comprise the catchment areas of all functioning anganwady centres (usually about 100-150 functioning per block, each with a total population at ages 1-6.0 of about 100-150 preschool children). A total of about one million preschool children will therefore be in the study at any one time; as the study will continue for about 5 years, with children entering it as they reach their first birthday and leaving as they reach their sixth, a total of about 2 million preschool children will be in it at one time or another, and a total of about 5 million person-years of experience will accumulate.

The control is open (ie, controls will not get placebos).
The 3 active interventions are:

- 400 mg albendazole every 6 months
- 200,000 IU vitamin A every 6 months
- 400 mg albendazole plus 200,000 IU vitamin A every six months.

As provision already exists within the anganwady system to use deworming treatment whenever there is a clear need for it, the study is not an evaluation of deworming versus nothing, but of routine deworming versus occasional deworming. Likewise, as some non-trial vitamin A supplementation might occur during the study, the comparison should in principle be described as being of routine vitamin A versus occasional vitamin A supplementation.

Albendazole and vitamin A will be distributed through the existing Integrated Child Development Services (ICDS) anganwady system, in which it is intended that all children of age 3-6 years should be cared for each weekday for a few hours by a local anganwady worker.

The coverage achieved by this system is, however, very incomplete; in practice, only about one-third of all the villages in UP have an anganwady centre, and even in villages where there is an anganwady centre most children of age 3-6 years attend it rarely or never, and few attend it regularly.

The younger children (less than 3 years old), although not supposed to be seen daily, are supposed to be enumerated and monitored by the system, partly to ensure that they are developing appropriately. Hence, special efforts are needed to ensure that when study medication is to be given the children do attend the anganwadi centre for this purpose.

Preliminary Measurements in Study Blocks

Within each of the 72 blocks, one anganwady per year will be randomly selected (without replacement) in Oxford through a formula that uses a numbering system for anganwadys within blocks for investigation by a biomedical team. Blood and stool samples will be collected from a total of 30 children (5 of each year of age from 1 to 6) in one anganwady per block. The selected children also will be weighed and measured.

Stool samples will be concentrated and a thick film prepared and read locally. Blood samples (5 ml of venous blood) will be collected according to a standard CTSU protocol (in vacutainers containing EDTA and aprotinin) and spun within 24 hours of collection.

Two aliquots of plasma and one of buffy coat will be placed into bar-coded cryovials. These will be stored in Lucknow in -30°C freezers for a few months, then air freighted to the Oxford CTSU laboratories (packed in styrofoam containers with enough solid CO_2 to provide 100 hours of protection), primarily for careful assay of the retinol levels, many of which may indicate deficiency. Unused portions of the samples will be stored indefinitely at CTSU in liquid nitrogen.

Distribution of Albendazole and Vitamin A

Anganwady workers in blocks allocated albendazole, vitamin A or both will be given supplies appropriate to the treatment assignment of their blocks. Distribution to the anganwady workers will take place twice per year, in April and October, at the monthly meetings that all anganwady workers attend within their blocks.

During each treatment cycle, anganwady workers will attempt to deliver the interventions to all children on a single day. The anganwady workers will record the fact of giving the doses to each child in their regular anganwady registers. Drug supplies that are left over will be collected at the next monthly meeting.

Verification of Dosing

Immediately after each round of treatment is due to be given, attempts will be made to verify that the assigned interventions were given. Any problems identified through discussion with anganwady workers or supervisors will be followed up. The exact methods of monitoring and encouraging treatment will remain flexible, and will be adapted in the light of experience. One possibility is to make spot checks in a few randomly selected anganwadys on or within a few days of mass treatment.

Recording of infant and child deaths throughout the study areas

Anganwady workers will be asked to report all deaths of infants and children from birth up to their 6th birthday (ages 0-5, inclusive), as well as stillbirths, to their supervisor during the regular monthly anganwady worker meetings. Each supervisor will, in turn, report deaths by anganwady to the block directors, who will maintain centrally the permanent record in registers designed exclusively for this purpose. Only name, date of birth and date of death will be recorded. It is expected that only a few infants, and even fewer children, would die each year per anganwady.

According to official statistics, nearly all deaths among young children – more than 90% – are from medical causes. The effort in this new system, therefore, will concentrate on getting accurate counts of total mortality, and not on trying to identify precise causes of death.

As, however, the completeness of reporting of deaths by anganwady workers might be biased by whether or not those anganwady workers were routinely giving 6-monthly treatment, direct evidence about any infant and child deaths will be sought by DEVTA moinitors visiting villages every 6 months, not at the times of any study treatment, to search directly for any infant or child deaths.

Timetable

Planning and logistical arrangements for the pilot study began in Fall 1997. It is hoped that all preliminary work will have been completed in time for distribution of albendazole and vitamin A to anganwady workers in a few pilot blocks in mid-1998 (although some delays may be necessary), with follow-up only of compliance and of any organisational problems in achieving it, so that the main study can start in all 72 blocks in Spring 1999. The main study is contingent on a determination during the pilot phase that the interventions can be delivered reliably.

Assuming that the pilot demonstrates this, the main study will get under way as quickly as possible in 1999, and then continue for 5 years (ie, long enough to demonstrate sustainability), unless clear evidence of differences in child mortality emerge earlier.

Ethical approval

The study has been approved on 13 August 1998 by the Ethical Committee of King George's Medical College, Lucknow.

Organisation

The study will be implemented by the INCLEN unit, King George's Medical College, Lucknow. Dr Shally Awasthi, Associate Professor of Pediatrics, is the lead principal investigator.

Professors Donald Bundy and Richard Peto, of the University of Oxford, are co-principal investigators.

Professor Bundy, who also has an appointment with the World Bank, directs the Partnership for Child Development, which is currently responsible for the deworrming of several million school children in various countries around the world.

Professor Peto is (with Professor Rory Collins) co-director of the Clinical Trial Service Unit, which organises some of the world's largest randomised studies of simple medical interventions. Dr. Linda Youngman and Dr. Sarah Clark, director and deputy director of the CTSU laboratory, will supervise all retinol assays carried out in Oxford: details follow below.

Drug donation and study funding

SmithKline Beecham have agreed in principle to donate albendazole, and the Sight and Life Program (an independent aid organisation supported by Hoffmann-LaRoche) has agreed in principle to donate Vitamin A capsules. Albendazole will be supplied as 400 mg chewable tablets (Zentel) in pots of 150; vitamin A will be supplied in gelatin capsules, each containing 200,000 IU of retinol, in bottles of 500 capsules. Repackaging in quantities convenient for dispensing to anganwady workers will take place in Lucknow.

Funding will be sought from USAID and the World Bank for operational expenses in India, augmented if necessary from the Oxford CTSU. The CTSU will also cover any expenses for convening meetings, for travel and for any laboratory assays, computing and administrative expenses in Oxford, using money from the CTSU's core support from the UK Medical Research Council and from a prize recently awarded to Richard Peto and Richard Doll by the Helmut Horten Foundation. The salaries of the principal investigator and co-principal investigators are already covered by their host institutions.

Lucknow, 1998

Webappendix (S Clark): Plasma assay methods valid at low retinol levels

Sample preparation in Oxford

Prior to analysis, the frozen samples were left to stand at room temperature to thaw, then inverted several times to mix. The concentration of retinol was measured by high performance liquid chromatography (HPLC) in extracts from plasma prepared as follows: 200 μl plasma was added to 250 μl distilled water and 50 μl internal standard (retinyl acetate in ethanol). 400 μL ethanol solution (containing 0.5 g/L butylated hydroxytoluene and 10 mmol/L lauryl sulphate) was added and the contents vortex-mixed for 5 minutes. 1 mL hexane was then added, the mixture centrifuged (4,500 rpm for 10 minutes at 4°C) and the top layer transferred to another tube containing 200 μL methanol. A further 1 mL hexane was added to the original tube for a second extraction step; the mixture was vortex-mixed and centrifuged, with the top layer removed and combined with the previous hexane layer from the first extraction step. his was then vacuum-dried and the residue re-dissolved in 50 μL dichloromethane and 150 μL mobile phase (0.05% tri-ethylamine in 45% acetonitrile, 45% methanol, 10% chloroform) for injection on the HPLC system. From commencement of analyses in 1998 until January 2002, standard samples were prepared by adding known concentrations of retinol to a solvent mixture (75% 50:50 acetonitrile:methanol with 0.05% triethylamine and 25% dichloromethane) with addition of 50μL of internal standard.

After January 2002, to improve long-term stability of standards and assay reproducibility, standard samples were prepared by adding known concentrations of retinol in solvent to a plasma pool. Following this change from solvent-based to plasma-based standards, the extraction procedure needed a slight modification to 175 μL plasma added to 220 μL distilled water. All other details remained unchanged. A study was done to compare sample results obtained using the solvent-based standards and the plasma-based standards and showed that retinol results obtained using plasma-based standards were 0.5% higher, on average, than those obtained using solvent-based standards (n = 42) with a correlation coefficient (r) of 0.997. For samples containing less than 175 μL plasma, a reduced-volume extraction method was performed in which the volumes of plasma and all solvents were half of those given above (i.e. 100 μL plasma). A comparison of results from the full volume and reduced volume assays on the same sample demonstrated excellent correlation (r = 0.976, regression:

[reduced volume result] = 1.07 x [full volume result] 0.04 mol/L, n = 143).

Chromatography

Chromatographic separation was performed by reverse phase HPLC using a Waters system fitted with a photodiode array detector (Waters Ltd, Watford, UK). When sample analyses commenced in 1998 the separation column was a C18 Ultramex PEEK 3μm 100 mm x 4.6 mm column (Phenomenex, Macclesfield, UK) with flow rate set at 0.85 mL/min. This was subsequently discontinued by the manufacturer and from September 2003 the column was changed to a C18 Luna steel 3μm 75 mm x 4.6 mm column (Phenomenex) with flow rate set at 1.0 mL/min. This change in column also required the mobile phase to be changed to 0.05% tri-ethylamine in 90% acetonitrile and 10% chloroform, but all other extraction method details remained unchanged. A comparison of results obtained from analysis of samples using the Ultramex and Luna columns demonstrated excellent correlation (r = 0.998, regression: Luna = 0.9455 x Ultramex + 0.0319, n = 304 samples). Chromatograms were extracted at 325 nm and quantification of retinol was performed using Waters Millennium software.

Assay performance (Webtables 1 and 2, Webfigures 1 and 2)

The laboratory participated in an external quality assurance scheme for carotene and vitamins A & E that was organised by Department of Chemical Pathology and Metabolism, St Helier Hospital, Carshalton, Surrey, UK. Based on all samples received between June 2001 and February 2005, our results on average were 83.8% (SD 4.3%), and within 2SD, of the mean result obtained from all participating laboratories performing HPLC assays. The average number of laboratories returning results was 31 and the average betweenlaboratory coefficient of variation (CV) associated with the mean result from all laboratories was 11.4%: Webfigure 1. Retinol levels in the samples ranged from 1.36 to 6.35 mol/L (based on the mean result from all laboratories).

Considering only the samples with blood retinol < 2 mol/ L, our results were on average 81.3% (SD 3.7%) of the mean result obtained from all participating laboratories. In the light of this average, our raw retinol results were multiplied by 1.2 to avoid bias.

Repeat measurement of retinol, using a second stored sample taken at the same time as the first, was performed on a subset of 349 individuals and resulted in an excellent correlation (r = 0.952, regression equation: [Tube B result] = 0.952 x [Tube A result] + 0.003). For the 295 individuals within this group who had a Tube A retinol result < 0.7 mol/L, the correlation between the first and the repeat measurement was again excellent: Webfigure 2 (r = 0.920, regression equation: [Tube B result] = 0.962 x [Tube A result] + 0.001).

The inter-assay coefficient of variation (CV) for retinol, derived from analysis of human plasma pools (low and medium levels) and manufactured control material (high level) within each batch, was 8% at a level of 0.29 mol/L (n = 217), 4% at a level of 0.49 mol/L (n = 216) and 3% at a level of 1.08 mol/L (n = 172), see Webtable 1.

Reliability at low retinol levels was further assessed by measurement of serial dilutions of a sample of known retinol concentration. The sample with neat concentration of 2.22 mol/Lwas diluted 1 in 2, 1 in 4, 1 in 8 and 1 in 16 (using 0.9% saline) to provide samples with expected concentrations of 1.11, 0.56, 0.28 and 0.14 mol/Lrespectively. The observed results for these samples were within 6% of the expected results: see Webtable 2.

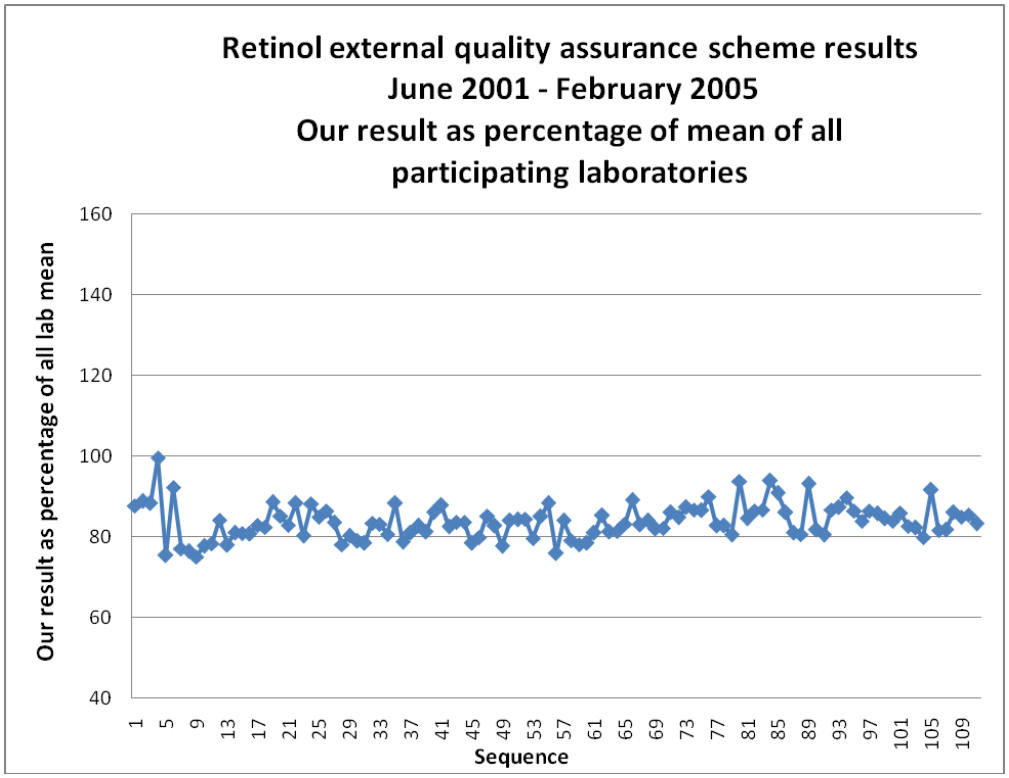
Webtable 1: Retinol internal QC performance

	Level 1	Level 2	Level 3
n	217	216	172
SD	0.02	0.02	0.03
%CV	7.6	3.8	2.8

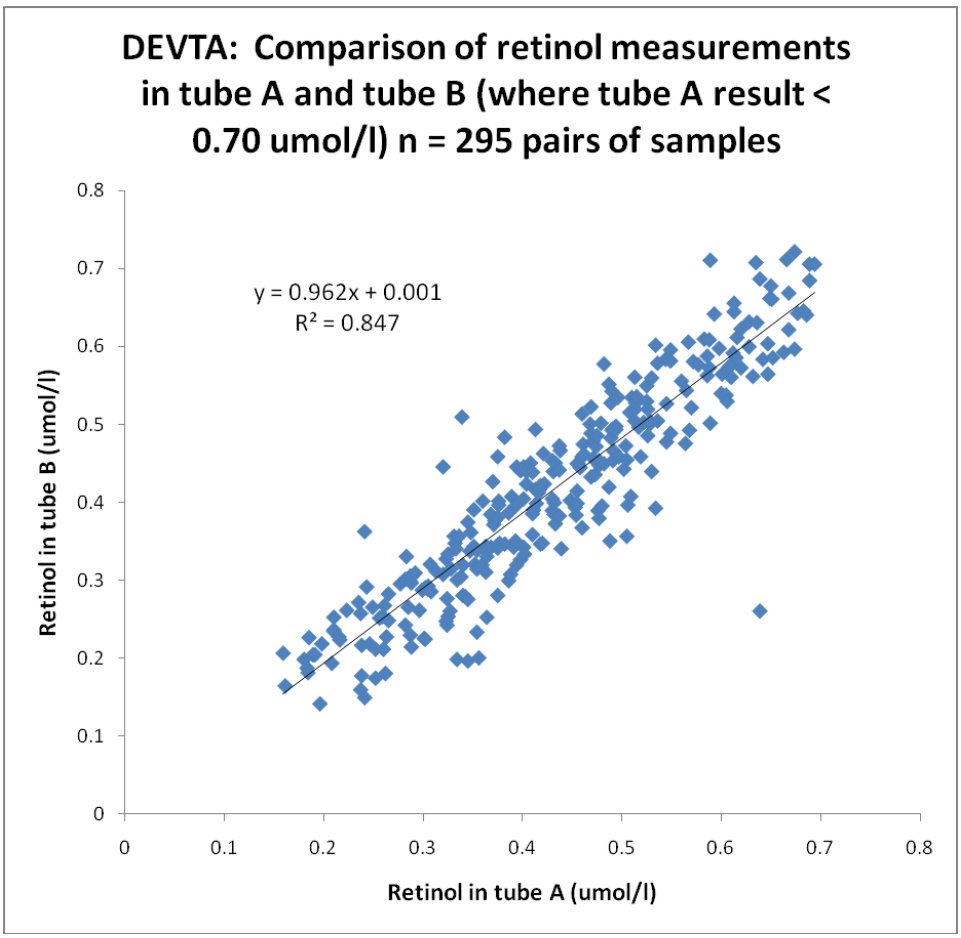
Webtable 2: Retinol reliability at low retinol levels

Dilution	Expected (µmol/L)	Observed (µmol/L)	Recovery (%)
1 in 2	1.112	1.076	96.8
1 in 4	0.556	0.549	98.7
1 in 8	0.278	0.264	95.0
1 in 16	0.139	0.131	94.2

Webfigure 1: Retinol external quality assurance scheme



Webfigure 2: Repeat on 2 aliquots from the same child



Webtable 3: Results of 8 large community-based trials of regular vitamin A supplementation & child mortality, DEVTA results, and details of calculation of weighted averages of results from these 8 trials & from all 9 trials

Calculation of weighted average of log RR in different trials does not assume the real mortality rate ratios in the trials are the same. Equivalent numbers of deaths are approximately additive when calculating weighted averages, but are not used in calculations.

Year trial published, name, country and reference	Vit. A vs control mortality rate ratio, RR, & 95% CI (L, U)*	Log RR	Weight, w † 1/var(log RR)	Product, w.log RR	Approximately equivalent numbers of deaths, ‡ Vitamin A vs Control
1986, Sommer, Indonesia [1]	0.66 (0.44, 0.97)	-0.416	24.6	-10.2	41 vs 62
1990, Vijayaraghavan, India [2]	1.00 (0.65, 1.55)	0.000	20.3	0.0	40 vs 40
1990, Rahmathullah, India [3]	0.46 (0.30, 0.71)	-0.777	20.7	-16.1	30 vs 66
1990, West, Nepal [4]	0.70 (0.56, 0.88)	-0.357	75.2	-26.8	128 vs 183
1992, Daulaire, Nepal [5]	0.74 (0.55, 0.99)	-0.301	44.5	-13.4	77 vs 105
1992, Herrera, Sudan [6]	1.06 (0.82, 1.37)	0.058	58.3	3.4	120 vs 113
1992, Arthur, Ghana [7,8]	0.30 (0.12, 0.75)	-1.204	4.6	-5.5	6 vs 20
1993, VAST, Ghana [8]	0.81 (0.68, 0.98)	-0.211	115.0	-24.2	208 vs 257
Weighted average, 8 trials †	0.775 (0.699, 0.858)	-0.256	Subtotal= 363.2	Subtotal= -92.8	Subtotal= 650 vs 846
2013, DEVTA, India	0.956 (0.890, 1.026)	-0.045	752.7	-34.1	1472 vs 1540
Overall weighted average, DEVTA + 8 other trials †	**0.893 (0.842, 0.946) 2-sided p=0.00015**	**-0.114**	**Total= 1115.9**	**Total= -126.9**	**Total= 2122 vs 2386**

* For previous trials, RR and its 95% CI (both to 2 dp; DEVTA calculations use 5dp) derive from the trial publication, either as calculated by the authors[1,3-6,8] (allowing for any cluster randomisation or covariate adjustment) or using[18] the estimated RR & numbers of deaths in each group.[2,7]

† To minimise the effects of chance on averaged results, the weight w is $(3.92 / \log [U/L])^2$, the inverse of the estimated variance of (log RR). Heterogeneity between 8 previous trials $\chi^2_7 = 18.5$ (p=0.010); heterogeneity between DEVTA and 8 previous trials $\chi^2_1 = 10.8$ (p=0.0010).

‡ For illustrative purposes, this table provides for each separate trial the equivalent numbers of deaths that would, in a large, individually randomised (50:50) trial, yield the same RR and CI. (For a trial with weight w, these numbers are w+w.RR and w+w/RR). Such numbers are approximately additive when a weighted average of several trial results is calculated (so the given subtotals and totals of these numbers correspond approximately to the weighted averages). Although DEVTA recorded 25,000 child deaths, its clusters were large. So, its statistical power is that of an individually randomised trial with only about 3000 child deaths (double the equivalent number in all other trials combined). Trials were excluded if they had a total of <20 equivalent numbers of deaths, recruited patients with disease, or gave single-dose treatment.