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Full Length Research Paper

Effects of DTP on infection with intracellular pathogen *Chlamydia pneumoniae* following booster vaccination

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Increased mortality and morbidity with intracellular pathogens has been reported in females with diphtheria-tetanus-pertussis (DTP) as their most recent vaccination. Within a randomised trial, we investigated the effect of DTP on *Chlamydia pneumoniae* serology. Eighteen-month-old children were randomised to DTP booster and oral polio vaccine (OPV) or OPV only. Blood samples collected from 523 children at baseline and six months later were analysed for *C. pneumoniae* antibodies. The *C. pneumoniae* IgG seroprevalence was high, with 12% positive at 18 months of age. Loss of IgG positivity was common during follow-up (25/65 (38%)), and was associated with lower weight-for-age. DTP did not affect the incidence of IgG seroconversion or IgM positivity. Among normal-weight children, DTP vaccination was associated with an increase in *C. pneumoniae* infection for females, whereas the trend was the opposite among males (p=0.05, test of interaction between sex and DTP). Among DTP-vaccinated children, the male-female risk ratio was 2.23 (95% CI=0.88-5.66) for IgG seroconversion and 0.0 (95% CI=0.0- 0.77) for IgM positivity. The overall *C. pneumoniae* seroprevalence in young children was high even though loss of IgG positivity was common. We found sex differences in IgG seroconversion and in IgM positivity among DTP-vaccinated children.

Key words: Chlamydia pneumoniae, diphtheria-tetanus-pertussis, serology, child, low-income countries, prevalence, non-specific effects, vaccinations.

INTRODUCTION

Child vaccination is one of the most important inventions to improve child survival. However, live vaccines, like the measles vaccination (MV), are associated with a stronger reduction in mortality than can be explained by prevention prevention of the targeted diseases (Aaby et al., 1995).

The opposite has been found for killed vaccines such as the diphtheria-tetanus-pertussis vaccine (DTP). DTP seems to cause higher mortality for females in areas with herd immunity against pertussis (Aaby et al., 2002, 2003, 2004b; Veirum et al., 2005). The immunological background for these effects is not known. Recent studies have found a higher incidence of the intracellular pathogens rotavirus, cryptosporidium (Rodrigues et al., 2006; Valentiner-Branth et al., 2007) and measles (Aaby et al., 2009) in females than in males who had DTP as their most recent vaccination.

The intracellular pathogen *Chlamydia pneumonia* is estimated to cause 10% of community-acquired pneumonias worldwide. The clinical spectrum ranges from mild to severe respiratory illness (Grayston, 2000), but the significance

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Abbreviations: DTP, diphtheria-tetanus-pertussis (vaccination); **OPV,** oral polio vaccine; **MV,** measles vaccination; **HDSS,** health and demographic surveillance system; **cpm,** counts per minute; **EIA,** enzyme immunoassay; **EIU,** enzyme immuno-units; **MIF,** microimmunofluorescence; **RF,** rheumatoid factor; **RR,** relative risk; **IQR,** interquartile range; 95% **CI,** 95% confidence interval.

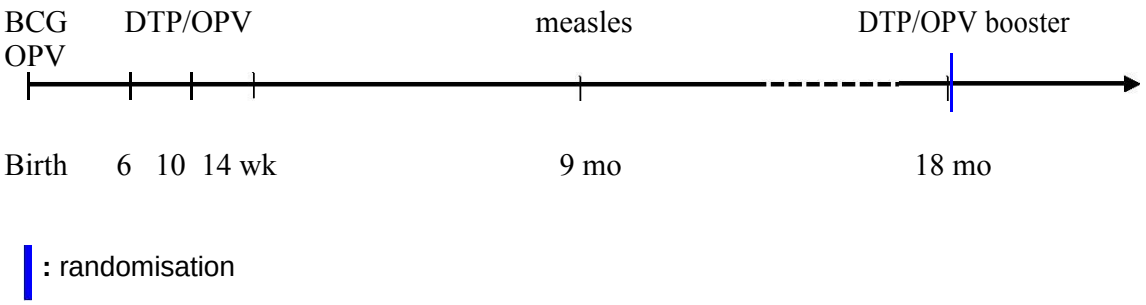

Figure 1. Recommended vaccination schedule in Guinea-Bissau.

of *C. pneumoniae* for child morbidity in low-income countries is ambiguous (Lassmann et al., 2008). *C. pneumoniae* is believed to be less common in childhood than in adult pneumonias, but on the other hand *C. pneumoniae* infection may cause more severe illness in tropical countries (Saikku et al., 1988).

The *C. pneumoniae* seroprevalence worldwide is estimated to be more than 50% in adults, with a sharp increase in children at preschool age, followed by a lifelong increase due to re -infections (Kuo et al., 1995). Studies from high-income countries generally describe low seroprevalence (0 to 5%) in children aged 18 to 24 months (Grayston, 1994; Tuuminen et al., 2000) whereas studies from Taiwan, Korea, Congo and Sudan suggest that the *C. pneumoniae* seroprevalence in low-income countries may be higher (Choi et al., 1998; Kabeya et al., 1999; Mahmoud et al., 1994; Wang and Grayston, 1990). Risk factors for higher *C. pneumoniae* seroprevalence in young children in Africa are not well described.

In Guinea-Bissau DTP booster was provided for 18-months-old children and the coverage was only around 50% before the start of the study. Without affecting herd immunity we used this opportunity to investigate the effects of DTP on mortality in a large randomised trial of the DTP booster vaccination. Children were randomised to DTP booster and OPV or OPV only at 18 months old. Our *a priori* hypothesis was that not receiving DTP booster would be associated with lower mortality. In the present study we aimed to investigate the non-specific effects of DTP on infection with a commonly occurring intracellular pathogen, *C. pneumoniae,* in the six months following vaccination.

METHODS

Setting

In Guinea-Bissau, the Bandim Health Project covers six districts (Belem, Mindera, Bandim I and II and Cuntum I and II) in the capital Bissau. A population of 102,000 is registered by the health and demographic surveillance system (HDSS); approximately 3,500 children are born every year. Basic health indicators and social conditions are registered at birth, including ethnicity, mother's education, number of siblings, type of roof, electricity and toilet facilities. Three health centres provide routine vaccinations for children in the study area. Figure 1 shows the recommended vaccination schedule in Guinea-Bissau.

Study cohort

Eligible for the randomised trial were children who were at least 18 months old and who had received three DTP vaccines and one MV but who had not yet received DTP booster (Figure 1). Children were randomised to the usual DTP booster+OPV or OPV only. The randomised study of non-specific effects of DTP vaccination was initiated in October 2005. We included children in the study of *C. pneumoniae* from 25 September, 2006 to 16 March, 2007. Due to a national measles vaccination campaign in May 2006 and a two-dose measles vaccination trial providing some children with an additional MV, some of the study children had received two MVs before inclusion.

Methods

Lists of children eligible for the randomised trial were drawn from the HDSS database; children were visited at home and the mother or guardian was asked to come to one of the health centres in the afternoon. If the child was not found at home at the first visit, they were visited again every 14 days until they were found at home or had turned 23 months of age. At the health centre a project assistant registered personal data, hospitalisations, cases of tuberculosis (TB) in the home and symptoms (coughing, diarrhoea, vomiting and fever) and medical treatment on the day of inclusion according to the mother. Anthropometric measurements were obtained and a medical examination was performed. Children with an axillary temperature above 37.5^{o}C and children diagnosed at the medical examination with acute febrile illness, were not included in the study. If consenting, the mother was asked to draw a randomisation lot from an envelope which determined whether the child should receive DTP booster+OPV or OPV. The mother was informed about the blood sample sub-study. If the mother accepted, a venous blood sample (denoted 18-month sample) was drawn before vaccination. In case we did not succeed in getting venous blood, a capillary sample was drawn. Six months later, project assistants visited all the children again to ask them to come for a follow-up and a second blood sample. Medical history, symptoms and objective signs of illness were registered and a second sample (denoted 24- month sample) was obtained. In case of a capillary sample at 18 months, the 24-month sample was also performed by capillary procedure. Two venous samples were obtained from 89% of the children. There were no exclusion criteria for the 24-month blood sample. From all blood samples a slide for malaria diagnostics was made and read on the same day. Children with

Table 1. *C. pneumoniae* seroprevalence in children 18 months and 24 months of age, N = 523.

	Negative, n (%)	Weakly positive, n (%)	Positive, n (%)
	At 18 months[1]	At 18 months[1]	At 18 months[1]
IgG	432 (82)	26 (5)	65 (12)
IgA	454 (86)	27 (5)	42 (8)
IgM	470 (90)	40 (8)	13 (3)
	At 24 months[2]	At 24 months[2]	At 24 months[2]
IgG	403 (77)	34 (7)	86 (16)
IgA	442 (85)	17 (3)	64 (12)
IgM	463 (89)	49 (9)	11 (2)

[1]18 months, 18 - 22 months of age, [2]24 months, 24 - 29 months of age.
Weakly positive, between the negative cut-off and the positive cut-off.

parasitaemia were offered treatment (N = 7). Vaccines received were registered in the sub-study and in the HDSS. In case of additional DTP or MV in the follow-up period, the child was excluded from the analyses of the effect of DTP on *C. pneumoniae*. Children who were not at home at the follow-up visit six months after inclusion were visited weekly until they were found, or until seven-and- a-half months after inclusion. After this, they were considered lost to follow-up.

Laboratory methods

Blood samples were collected in EDTA tubes and kept cold until separation. Plasma was separated by centrifugation at 3500 rpm for 10 min, transferred to new tubes and placed in the freezer for storage, until the tubes could be transported to Denmark. Samples were kept at a temperature of –20°C or lower until analysis. *C. pneumoniae* antibodies were analysed using the commercial *Chlamydia pneumoniae* IgG, IgA and IgM enzyme immunoassay (EIA) kits (Ani-labsystems, Helsinki, Finland) providing measurements in enzyme immuno-units (EIU). Samples from the same child were analysed side-by- side on the same plate. The assay is an indirect solid-phase EIA using *C. pneumoniae* elementary bodies as the antigen and horseradish peroxidase as a marker enzyme. Samples were diluted 1:100 and analyses were performed according to the manufacturer's description.

Capillary sampling was performed when venous sampling was not possible. Capillary samples did not differ in IgG or IgM positivity compared with venous samples (The relative risk (RR) of IgG positivity was 0.81 (95% CI = 0.46 - 1.44) and of IgM positivity was 1.48 (95% CI = 0.45 - 4.85). IgA differed in capillary vs. venous samples (RR was 0.30 (95% CI = 0.10 - 0.93). Since, studies of HSV and measles antibodies show a good correlation in antibody positivity/levels (100 and 99%) between blood sampled by venous and capillary techniques (Laderman et al., 2008; Novello et al., 1996) and since in the present study there was a strong association between capillary sampling and weight -for -age (the z-score being 0.44 (95% CI = 0.10 - 0.78) lower in children with capillary samples), we concluded that differences in antibody-levels were more likely to be due to the health constitution of the child than the sampling method.

Presence of IgM rheumatoid factor (RF) in an IgG-positive sample may cause false-positive IgM results in microimmunofluorescence (MIF) tests of *C. pneumoniae* (Verkooyen et al., 1992; Einarsson et al., 1994). In the first step of the IgM EIA kit, IgG was removed using anti - human IgG . Due to an IgM peak in the months following high IgG prevalence, we examined whether the IgM positivity could still be due to IgG positivity in RF-positive samples. We analysed IgM RF in all IgM-positive samples and in "weakly positive" samples from March 2007. The analyses of IgM-RF were performed at the Department of Clinical Immunology, Aarhus University Hospital, Skejby (in-house method). One sample was very weakly positive. All others were negative (data available on request). Samples at age 18 months collected between October 23 and November 2 were possibly thawed one day due to generator failure. The seroprevalence in these samples did not differ from the other samples (data not shown). Single measurements were performed. Triple-analyses of 23 randomly chosen samples on the same microtiter-plate had a median standard deviation of 1.64 (IQR, 0.98 - 2.29).

Definitions

"Positive" IgG, IgA or IgM was defined as antibody levels above the assay's cut- off for positivity, which was 45 EIU for IgG, 12 EIU for IgA and 1.1 Signal/Cut-Off (S/CO) for IgM respectively. "Negative" IgG, IgA or IgM was defined as antibody levels equal to or below the assay's cut- off for negativity, which was 30 EIU for IgG, 8 EIU for IgA and 0.5 S/CO for IgM respectively. "Weakly positive" IgG, IgA or IgM was defined as the antibody levels between the cut-off for positivity and the cut-off for negativity. Only samples above the positive cut-off were considered positive in the analyses.

C. pneumoniae seroprevalence (of IgG or IgA) is the percentage of positive samples. *C. pneumoniae* seroconversion was the change from negative at 18 months to positive at 24 months (Table 1). Hence, analyses of seroconversion only included children who were negative at 18 months. Overall seroconversion to *C. pneumoniae* infection in the six-month follow-up period was defined as either IgG-seroconversion or IgA-seroconversion or a positive IgM test at 24 months.

In children, IgG antibodies may decline rapidly after primary infections (Volanen et al., 2003). In adults, IgG may not appear until six to eight weeks after the onset of illness and may be detectable after three years, but are usually lost in three to five years unless re-infection occurs (Grayston, 2000; Kuo et al., 1995; Paldanius et al., 2005); levels of IgA fall more rapidly than IgG, while IgM appears about three weeks after onset of the illness and is undetectable after four to six months (Kuo et al., 1995; Saikku et al., 1992; Paldanius et al., 2005). Due to antibody kinetics, we evaluated loss of antibodies. "Loss of positivity" was defined as a decrease in antibody levels from positive at 18 months of age to below the cut-off for positivity at 24 months of age.

Statistical methods

DTP analysis, Relative risks of IgG positivity, "loss of antibodies" and *C. pneumoniae* seroconversion in children who received DTP booster+OPV (DTP group) compared with children who did not receive DTP (OPV group) were estimated by binary regression.

Only a few had an IgM-positive test and these were compared using Fisher's exact test. Since the non-specific effects of DTP are mostly seen in females, all analyses were stratified by sex. Furthermore, we computed male-female risk ratios within each of the two randomisation groups.

Analyses were also done and adjusted for risk factors for being seropositive at 18 months of age. These risk factors were identified using univariate binary regression ($p < 0.05$) and the identification was also checked in stepwise multivariate binary regression to $p < 0.1$. All risk factors in Table 2 were included in the multivariate analyses, except for height and MUAC of the child (which were correlated with weight) and television (correlated with electricity at home). Risk factors did not differ in randomisation groups except for medication. Adjusting DTP analyses for medication or for risk factors affecting antibody positivity (season) did not change the results. All analyses were performed using Stata version 9.

Sample size considerations

In observational studies, mortality was observed to be around two times higher in children who received DTP vaccination after MV compared with children who received MV as the most recent vaccination (Aaby et al., 2004a). Thus DTP-vaccinated children possibly contract twice as many infections as children who do not receive DTP vaccination. In a pilot study of samples from a Varicella study in Guinea- Bissau, 27% (48 of 176) of children aged 18 to 36 months had *C. pneumoniae* IgG seropositive samples. Based on the pilot study and on previous studies of *C. pneumonia*e serology from low-income countries we expected *C. pneumoniae* seroconversion to occur in 10% of children who did not receive DTP during a six-month follow-up period and based on previous studies of non-specific mortality-effects of DTP we expected C. pneumonia seroconversion in 20% of DTP-vaccinated children in the follow- up period. Based on these assumptions we needed 286 children in each group. We analysed all 523 paired plasma samples obtained in the blood sample sub-study in 2006 to 2007.

Ethical considerations

The study was limited to children who had received at least three doses of DTP to ensure high protection against whooping cough. At four years of age, children randomised to no DTP booster are offered DTP vaccination. The randomised trial and the present sub-study were approved by The Danish National Committee on Biomedical Research Ethics and the Ministry of Health in Guinea-Bissau. The trial was registered at Clinical trials.gov, number NCT00244673.

RESULTS

Study children

A total of 688 children were included in the sub-study. *C. pneumoniae* analyses were performed on samples from the 523 children with paired 18- and 24-month samples (Figure 2). The children were 18 to 22 months old (median 19. 1 , IQR, 18.8 - 19.6 months) when the 18 month samples were obtained and 24 to 29 months old (median 25.2, IQR, 24.8 - 25.9 months) when the 24-month samples were obtained. The median time between the two samples was 6.0 months (IQR, 6.0 - 6.2 months) and it was the same in the two treatment groups. The 165 children without a second sample more often had diarrhoea at inclusion (12% vs. 6%) and more often lived in a house without electricity (74% vs. 66%).

Overall seroprevalence and incidence

The IgG-seroprevalence was 12% at 18 months and increased to 16% at 24 months; the IgA-seroprevalence was 8% at 18 months and 12% at 24 months and the IgM seroprevalence was 3% at 18 months and 2% at 24 months (Table 1). Half (50%) of the samples with IgG-positive tests were also IgA-positive; 71% of IgA-positives were IgG-positive; and no IgM- positives in the 18-month samples were IgM- positive in the 24-month samples. Forty-six per cent of those who had a positive IgM test at 18 months had a positive IgG test six months later.

The IgM-positive samples peaked with 5 - 8% in the dry season in the period February to April 2007; IgG and IgA were already high in the previous months (Figure 3). The *C. pneumoniae* incidence was 23 per 100 person-years based on seroconversion in IgG, IgA or IgM antibodies.

Loss of antibodies

Unexpectedly, loss of antibodies was very common. Among those initially IgG-positive, 38% (25 of 65) had lost their antibodies at 24 months and 31% (13 of 42) had lost IgA positivity. With loss of antibodies it is not surprising that IgM and IgG data did not agree on recent infections; seven of 13 children with a positive IgM sample at 18 months of age did not have a positive IgG sample at 24 months of age.

Risk factors for seropositivity

None of the examined risk factors were related *to IgG-seropositivity* at 18 months except season; there were fewer seropositive children among those tested in the rainy season (Table 2). *IgA-positivity* was not significantly related to any of the examined risk-factors. *IgM positivity* in 18- or 24-month samples was associated with weight and having no television in the home. The z-score was 0.58 (95% CI = 0.09 - 1.07) higher in children with a positive IgM test compared with children with no IgM-positive test and the RR of IgM positivity was 1.70 (95% CI = 1.15 - 2.52) higher in children with "no television in the home" compared with having a "television in the home"; IgA and IgM positivity both tended to be associated with season (data not shown).

Table 2. Risk factors for *C. pneumoniae* IgG seroprevalence at 18 months of age[1], N = 523.

Risk factor	Prevalence 18 mo[1], n (%)	Relative Risk[2] (95% CI)	Relative Risk (adjusted)[2] (95% CI)
All children	65/523 (12)		
Males	35/259 (14)	1.19 (0.75 - 1.88)[3]	1.20 (0.73 - 1.96)
Females	30/264 (11)		
Residence			
Belem/Mindera	6/90 (7)	0.49 (0.22 - 1.10)[4]	0.60 (0.27 - 1.34)
Bandim/Cuntum	59/433 (14)		
Ethnicity			
Pepel	25/163 (15)	1.38 (0.87 - 2.20)	1.22 (0.73 - 2.04)
Other	40/360 (11)		
Mother 4 yrs of schooling [29]	31/219(14)	1.39 (0.86 - 2.25)	1.37 (0.84 - 2.26)
More than two siblings [2]	20/133 (15)	1.30 (0.80 - 2.11)	1.11 (0.64 - 1.93)
Straw roof [2]	5/27 (19)	1.52 (0.67 - 3.49)	1.88 (0.87 - 4.08)
No electricity in house [2]	44/343 (13)	1.09 (0.67 - 1.77)	1.00 (0.60 - 1.69)
No television [2]	42/350 (12)	0.89 (0.55 - 1.43)	0.84 (0.49 - 1.42)
Toilet outside the house [3]	55/452 (12)	0.92 (0.48 - 1.77)	0.70 (0.36 - 1.35)
Not breastfed at 18 mo. [5]	17/161 (11)	0.79 (0.47 - 1.32)	0.80 (0.45 - 1.41)
Previously hospitalised [7]	6/64 (9)	0.72 (0.32 - 1.60)	0.79 (0.33 - 1.89)
TB in the house [11]	4/35 (11)	0.92 (0.36 - 2.40)	0.81 (0.27 - 2.44)
On the day of sample			
Cough	8/81 (10)	0.77 (0.38 - 1.54)	0.82 (0.39 - 1.73)
Diarrhoea	5/29 (17)	1.42 (0.62 - 3.26)	1.32 (0.50 - 3.46)
Fever	8/53 (15)	1.24 (0.63 - 2.47)	1.28 (0.62 - 2.64)
Had medicine [2]	7/39 (18)	1.52 (0.74 - 3.10)	1.18 (0.45 - 3.07)
Respiratory rate >40 cpm	9/46 (20)	1.67 (0.88 - 3.15)	1.41 (0.67 - 2.94)
Temperature 37^{O}C axillary	1/5 (20)	1.62 (0.28 - 9.50)	2.04 (0.42 - 9.88)
Objective signs	7/66 (11)	0.82 (0.39 - 1.73)	0.83 (0.34 - 2.01)
DTP vaccination	31/268 (12)	0.87 (0.55 - 1.37)	0.83 (0.50 - 1.36)
Rainy season	16/206 (8)	0.50 (0.29 - 0.86)	0.40 (0.21 - 0.76)
Capillary sample [1]	5/49 (10)	0.80 (0.34 - 1.91)	1.32 (0.55 - 3.14)
Cried [11]	57/433 (13)	1.25 (0.59 - 2.63)	1.95 (0.81 - 4.67)
	Mean (sd)[5]		
Age (mo)	19.3/19.3 (0.8/0.7)	1.02 (0.75 - 1.38)	1.01 (0.74 - 1.38)
Weight (kg)	10.2/10.1 (1.3/1.3)	1.06 (0.89 - 1.26)	0.98 (0.79 - 1.20)
Height (cm) [5]	80.6/80.5 (3.5/3.3)	1.01 (0.95 - 1.08)	0.99 (0.92 - 1.07)
MUAC (mm)	146/146 (11/12)	1.00 (0.98 - 1.02)	1.00 (0.98 - 1.02)
BCG scar	4.1/4.0 (2.0/1.8)	1.03 (0.90 - 1.19)	1.09 (0.93 - 1.27)

[1]18 months, 18 - 22 months of age [2]Seroprevalence in children with mentioned risk factor compared with children without this risk factor. [3]Males vs. females .[4]Belem/Mindera districts compared with Bandim/Cuntum districts. [5]Mean in children with a positive sample/mean in children without a positive sample. Number of missing values at 18 months. The rainy season in Guinea-Bissau is from June to November. Respiratory rate of at least 40 cpm in a child aged 12 to 59 months is one of the criteria used to diagnose pneumonia (Grant et al., 2009).

Risk factors for loss of antibodies

Since loss of *C. pneumoniae* antibodies between 18 and 24 months was very common, we looked at risk factors for losing seropositivity. Low weight-for-age, cough on the day of inclusion and having the 18-month sample collected in the rainy season were associated with a higher risk of losing IgG antibodies (Table 3). Low

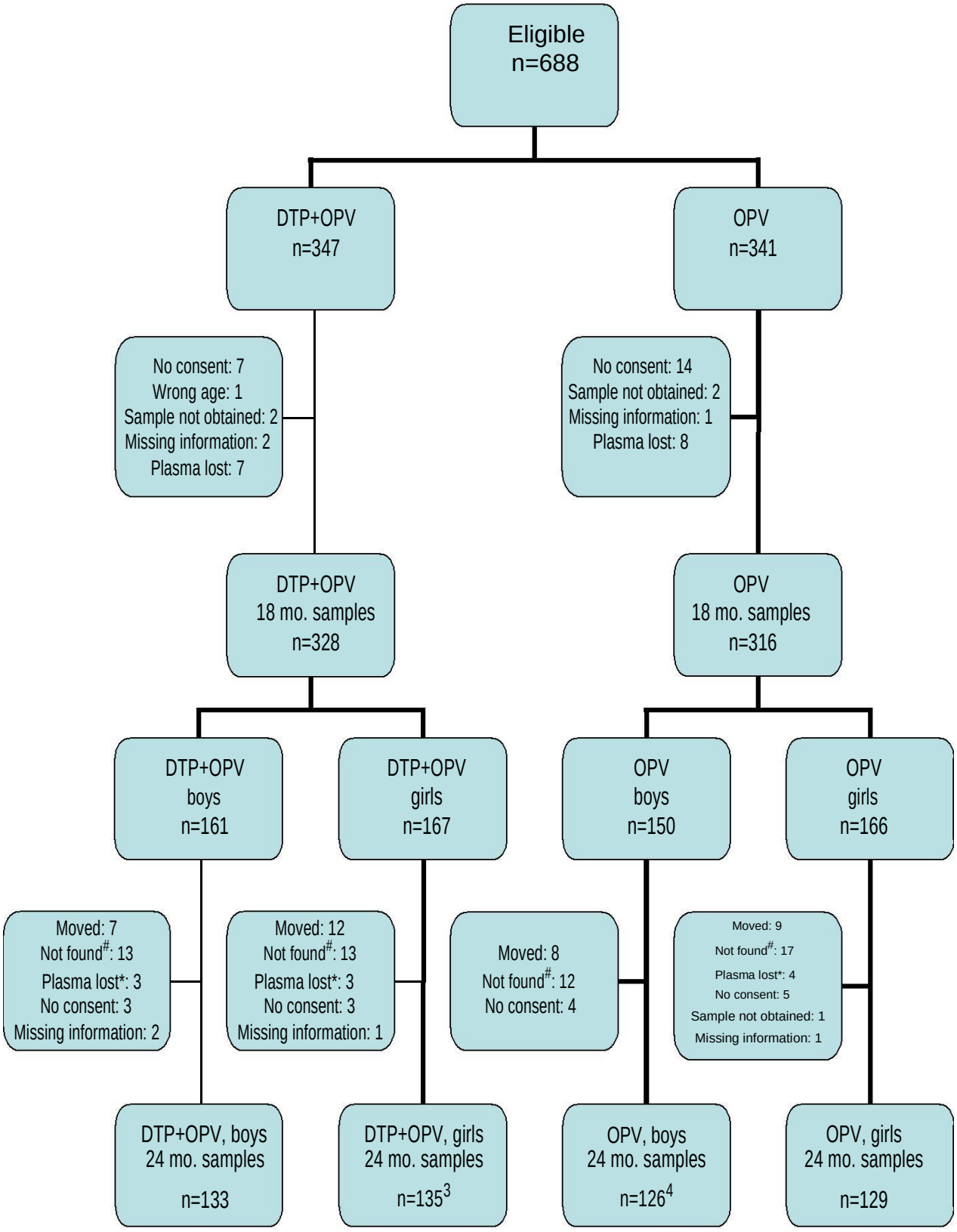

Figure 2. Flow chart. [1]18 months: 18 - 22 months of age, [2]24 months: 24 - 29 months of age. [3] 4 censored and [4]1 censored from DTP analyses due to other type of vaccine received in the 6-month follow-up time period. # Child was not found at home during the interval for 24-month sample (most children "not found" are children travelling with their family). * No electricity at the laboratory, no key to the laboratory or lost during transport.

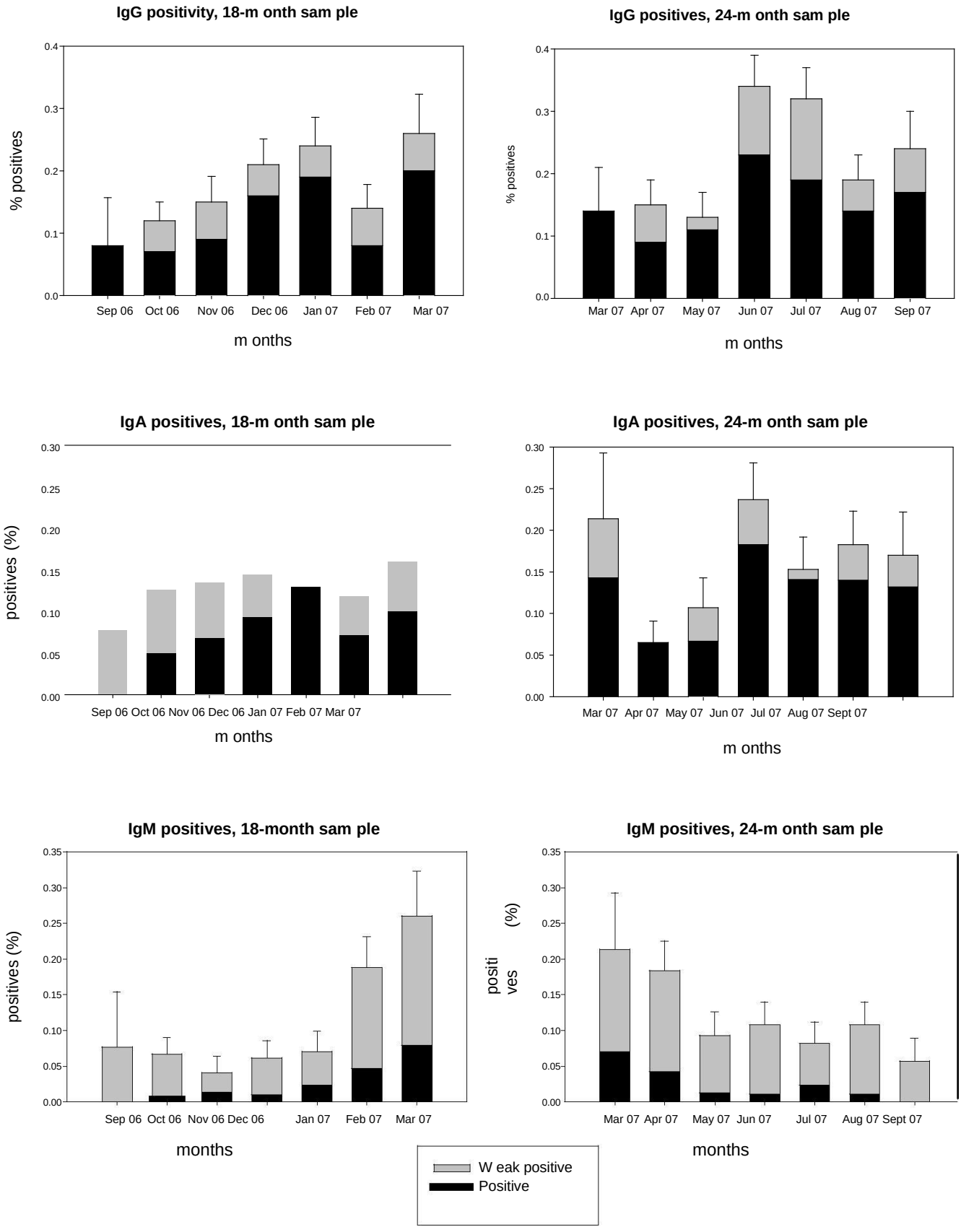

Figure 3. IgG, IgA and IgM seroprevalence by calendar month.

Table 3. Loss of *C. pneumoniae* antibodies from age 18 to 24 months.

	Loss of positivity[#]	Loss of positivity[#]
	IgG relative risk (95% CI), n = 65	IgA relative risk (95% CI), n = 42
Males vs. females	1.28 (0.68 - 2.44)	0.52 (0.20 - 1.33)
Age (months)	1.25 (0.87 - 1.80)	1.46 (0.86 - 2.45)
Belem/Mindera vs. other districts	1.34 (0.56 - 3.21)	1.89 (0.77 - 4.65)
Pepel vs. other ethnicity	0.75 (0.38 - 1.49)	0.54 (0.20 - 1.49)
Mother 4 yrs of schooling	0.99 (0.52 - 1.88)	0.30 (0.10 - 0.94)
More than two siblings	0.88 (0.43 - 1.76)	0.89 (0.33 - 2.42)
Straw roof	0.50 (0.08 - 3.01)	0.79 (0.13 - 4.70)
No electricity in house	0.72 (0.39 - 1.32)	0.38 (0.16 - 0.93)
No television	0.97 (0.51 - 1.85)	1.18 (0.39 - 3.57)
Toilet outside the house	1.20 (0.45 - 3.22)	0.45 (0.18 - 1.11)
Not breastfed at 18 mo.	0.89 (0.42 - 1.87)	0.58 (0.15 - 2.23)
Previously hospitalised	0.41 (0.07 - 2.55)	0.00 (0.00 - 2.37)
TB in the house	1.28 (0.45 - 3.62)	3.70 (2.16 - 6.33)
On the day of 18 mo. sample		
Cough	2.25 (1.30 - 3.89)	1.80 (0.68 - 4.75)
Diarrhoea	0.50 (0.08 - 3.01)	0.00 (0.00 - 87.0)
Fever	0.97 (0.37 - 2.53)	1.09 (0.31 - 3.80)
Had medicine	1.16 (0.46 - 2.94)	1.08 (0.20 - 5.86)
Respiratory rate >40 cpm	1.19 (0.53 - 2.67)	0.42 (0.06 - 2.77)
Weight-for-age-z-score	0.81 (0.64 - 1.01)	0.59 (0.45 - 0.78)
Objective signs	1.58 (0.76 - 3.28)	0.79 (0.13 - 4.70)
DTP-vaccination	1.40 (0.75 - 2.61)	0.89 (0.33 - 2.42)
BCG scar size	0.90 (0.78 - 1.03)	0.87 (0.73 - 1.03)
Rainy vs. dry	1.72 (0.95 - 3.12)	3.29 (1.40 - 7.74)
Capillary sample	1.04 (0.34 - 3.23)	1.67 (0.38 - 7.33)
Cried during procedure	0.86 (0.34 - 2.17)	1.62 (0.26 - 10.17)

"18-month sample positive and 24-month sample not positive" vs. "Two positive samples". The rainy season in Guinea-Bissau is from June to November. Respiratory rate of at least 40 cpm in a child aged 12 to 59 months is one of the criteria used to diagnose pneumonia (Grant et al., 2009).

weight-for-age, rainy season, being exposed to TB at home, having electricity and having a mother with more than four years of schooling were associated with a higher risk of losing IgA antibodies (Table 3).

Conversely, an increase in antibody level in the initially IgG-positive samples tended to be associated with higher weight-for-age, collection of 18- month sample in dry season, larger BCG scar and also venous sampling at 18 months of age (data not shown).

Nutritional status and the risk of infection

Due to the effect of weight-for-age on IgM positivity and loss of antibodies, we stratified the analyses in children with low weight-for-age (weight-for-age z- score below –2) and children with normal weight-for- age (weight-for-age z-score above –2) (Table 4). Children with low weight -for-age had significantly higher IgG seroconversion (RR = 2.08 (95% CI = 1.14 - 3.81)) and higher IgA seroconversion (RR = 2.30 (95% CI = 1.14 - 4.63)) than children with normal weight-for-age. There was no increased risk of IgM positivity among the children with low weight, the RR of IgM positivity at 24 months being 0.99 (95% CI = 0.26 - 3.77).

The effect of DTP

Judged by IgG seroconversion, IgA seroconversion or IgM positivity the overall incidence of *C. pneumoniae* infection was not affected by DTP booster vaccination (Table 4). However, there were indications of sex-differential effects on the incidence among children who received DTP, whereas there were no sex differences among controls. Among children who had a negative IgG test at 18 months of age and received DTP, 19 (9%) had a positive IgG test at 24 months of age, which consisted of 13 males (12%) and 6 females (5%). In the control

Table 4. *C. pneumoniae* seroconversion and IgM positivity in 18- to 24-months-old children vaccinated with DTP+OPV or OPV.

	All			Males			Fe	
	DTP+OPV (%)	**OPV (%)**	**RR (95% CI), DTP+OPV vs. OPV***	**DTP+OPV (%)**	**OPV (%)**	**RR (95% CI) DTP+OPV vs. OPV**	**DTP+ OPV (%)**	**OPV**
All children								
IgG seroconversion	19/219 (9)	19/207 (9)	0.94 (0.51-1.71)	13/108 (12)	10/98 (10)	1.18 (0.54-2.57)	6/111 (5)	9/109
IgA seroconversion	15/239 (6)	14/209 (7)	0.92 (0.45-1.85)	10/121 (8)	7/95 (7)	1.12 (0.44-2.84)	5/118 (4)	7/114
IgM positivity¤	7/264 (3)	4/252 (2)	1.67 (0.50-5.64)	0/133 (0)	2/124 (2)	0.00 (0.00-4.96)	7/131 (5)	2/128
Any seroconversion	30/231 (13)	23/208 (11)	1.17 (0.70-1.95)	16/117 (14)	13/99 (13)	1.04 (0.53-2.06)	14/114 (12)	10/10
Low weight								
IgG seroconversion	8/54 (15)	9/65 (14)	1.00 (0.44-2.31)	7/26 (25)	2/23 (9)	3.09 (0.70-13.6)	1/28 (4)	7/42 (
IgA seroconversion	8/63 (13)	6/66 (9)	1.27 (0.48-3.41)	6/35 (17)	2/23 (9)	1.97 (0.43-8.94)	2/28 (7)	4/43
IgM positivity¤	1/68 (1)	2/77 (3)	0.57 (0.05-6.11)	0/36 (0)	0/31 (0)	NA	1/32 (3)	2/46
Any seroconversion	11/57 (19)	9/65 (14)	1.35 (0.62-2.96)	8/30 (27)	2/24 (8)	3.20 (0.74-13.9)	3/27 (11)	7/41 (
Normal weight								
IgG seroconversion	11/165 (7)	10/142 (7)	0.97 (0.43-2.19)	6/82 (7)	8/75 (11)	0.69 (0.25-1.89)	5/83 (6)	2/67
IgA seroconversion	7/176 (4)	8/143 (6)	0.72 (0.27-1.93)	4/86 (5)	5/72 (7)	0.67 (0.19-2.41)	3/90 (3)	3/71
IgM positivity¤	6/196 (3)	2/175 (1)	2.67 (0.55-13.1)	0/97 (0)	2/93 (2)	0.00 (0.00-5.10)	6/99 (6)	0/82
Any seroconversion	19/174 (11)	14/143 (10)	1.12 (0.59-2.15)	8/87 (9)	11/75 (15)	0.63 (0.27-1.48)	11/87 (13)	3/68

Seroconversion, negative at 18 months and positive at 24 month. ¤ IgM-positive at 24 months, Fisher's exact test used and no adjustment for sex.
() %. * Sex-adjusted. § p-value for interaction between sex and randomisation group.

DTP analyses were also performed separately for children with two venous (n = 463) and children with two capillary samples (n = 31). The overall RR of seroconversion, comparing the DTP group with the OPV group, was 1.36 (0.79 - 2.36) in venous and 0.44 (0.03 - 7.38) in capillary samples. In venous samples (like in all samples) the tendencies for seroconversion in the DTP group compared with the OPV group in low-weight children was a higher risk in males and a lower risk in females (p = 0.10 for interaction between sex and DTP) and in normal-weight children was a lower risk in males and a higher risk in females (p = 0.11 for interaction) (data not shown).

DISCUSSION

Main observations

We found higher IgG and IgA seroprevalence at 18 and 24 months of age in Guinea-Bissau than in high-income countries and in previous studies from low-income countries. The IgM results suggested that the *C. pneumoniae* incidence was highest in the dry season, with a peak in March. Surprisingly, there was a very rapid loss of IgG antibodies – 38% within 6 months – which was associated with indicators of poor health status. At the same time, IgG and IgA seroconversion was two times higher among children with a low weight-for -age. DTP vaccination did not affect the overall incidence of *C. pneumoniae* infection. However, whereas there was no sex difference in the control group that had received only OPV, we observed sex differences in the DTP group for both IgG and IgM. Unexpectedly, these results were totally contradictory; whereas the IgM data suggested an increased risk of *C. pneumoniae* infection for females compared with males in the DTP group, the IgG data suggested an increased risk for males compared with females. However, in normal-weight children the effect of DTP in IgG and IgM data consistently indicated higher risk of *C. pneumoniae* infection in girls and a lower risk in boys, whereas the opposite was seen for IgG seroconversion in low-weight children.

Strengths and weaknesses

Strengths

The randomised design provided a unique opportunity to assess the possible non-specific effect of DTP on *C. pneumoniae* serology. However, it should be noted that *C. pneumoniae* infection can be asymptomatic and it is not possible from *C. pneumoniae* serology to evaluate the burden of *C. pneumoniae* infections on childhood morbidity.

Previous studies often used microimmunofluorescence (MIF). The EIA test used in this study was optimised to produce results comparable to a MIF assay (manufacturer's information (Vainas et al., 2003)).

Loss of antibodies

To our surprise, our data suggest that serological data in a low-income-country setting, with many competing infections, are less stable than commonly assumed. Only 46% of those who had a positive IgM test at 18 months had a positive IgG test six months later and 38% of those who were IgG-seropositive at 18 months of age had lost IgG positivity six months later. Hence, IgG may be a poor indicator of past infection. A positive IgG antibody test may measure who is good at maintaining antibody levels rather than who has had *C. pneumoniae* infection. We therefore looked at risk factors for losing antibodies and the analysis suggested that loss of antibodies is related to indicators of poor health, e.g. coughing and low weight-for-age. Conversely, maintaining the antibody level is associated with indicators of good health (e.g. higher weight-for-age and venous sampling). To the extent that this is true, it may cause problems of interpretation in serology studies when assessing the incidence of and risk factors for, *C. pneumoniae* infection in children. For example, in the present study, trends were totally opposite among low-weight and normal-weight children.

Limitations

One of the main limitations in earlier studies of *C. pneumoniae* serology has been problems with cross-reaction to other *Chlamydia* species. In the EIA used in the present study chlamydial LPS was eliminated and the test does not cross-react with *C. tracomatis* antibodies. Due to few data on psittacosis infection in *C pneumoniae* serological negative patients it has not been shown whether cross-reaction occurs to *C. psittaci* (manufacturer's information). No information on the seroprevalence of *Chlamydia psittaci* is available from Guinea-Bissau and it cannot be ruled out that psittacosis infections may cause false positives. False positives may be important for estimation of seroprevalence and would also be important in studies evaluating the importance of *C. pneumoniae* infections for morbidity. However, *C. psittaci* infections might be affected by DTP in the same way as *C. pneumoniae* infections.

Children lost to follow-up were primarily children who moved or travelled with their parents, which in Guinea-Bissau is often due to a lack of stable work. These children would likely live under worse health conditions and in the present study they had more diarrhoea and more often lived in a house without electricity than children from whom two samples were obtained. These children may have had more *C. pneumoniae* infections and could also have been more prone to loss of antibodies. The loss to follow-up was equal in the two

randomisation groups. The loss to follow-up was slightly larger than expected and the sample size was therefore smaller (N = 523) than the estimated 286 children in each group. With the unexpected loss of positivity and the opposite effect of DTP in IgG and IgM data, a larger sample size would probably not show a statistical signify-cant difference in the overall effect of DTP on seroconversion.

We did find differences in IgA positivity, but not in IgG or IgM positivity, in capillary vs. venous samples. However, low weight was significantly associated with the capillary sampling technique and the difference in capillary samples and venous samples may reflect the loss of antibodies in low-weight children rather than be caused by the sampling method.

Consistency with previous studies

Decline and maintenance of antibody levels

In children IgG levels can decline within one year follow-ing a primary infection; Volanen found 13 of 36 children (36%) who lost positivity (Volanen et al., 2003). In our study, 38% lost IgG positivity within a six -month time period and loss of antibodies was more frequent in children with poor health conditions (low weight, cough at inclusion). Repeated infections and the concomitant low protein intake can cause general loss of antibodies, but loss of *C. pneumoniae* antibodies due to infections and nutritional status has to our knowledge not been described previously.

Overall seroprevalence and incidence

Most studies from high-income countries report the IgG and IgA seroprevalence in children between 18 and 24 months of age to be less than 5% (Aldous et al., 1992; Grayston, 1994; Podsiadly et al., 2005; Tuuminen et al., 2000; Volanen et al., 2003). In low-income countries, a study from Congo observed an IgG seroprevalence of 6% in children aged one to six years (Kabeya et al., 1999); in Taiwan the seroprevalence was 23% in 6-month to 10-year-olds (n = 26) (Wang et al., 1993) and 8% in children under 10 years of age (n = 77) (Lin et al., 2004); in Korea the seroprevalence was 11% (n = 28) in two to five-year-olds (Choi et al., 1998) and in a study of tracoma infection from Sudan the seroprevalence was 13% in children aged six years or younger (n = 336) (Mahmoud et al., 1994). IgA-seroprevalence has not been described in children in low-income countries. The general trend is that due to maternal antibodies the IgG seroprevalence is high before one year of age (Choi et al., 1998; Grayston, 2000; Volanen et al., 2003) followed by a nadir at two to four years of age (Grayston, 2000; Volanen et al., 2003) and a sharp increase at preschool age (four to six years of age) (Aldous et al., 1992; Grayston, 1994; Tuuminen et al., 2000; Volanen et al., 2003; Wang and Grayston, 1990). From this perspective, the seroprevalence at one-and-a-half to two years of age was very high in the present study. There may be no nadir at two years and the increase in *C. pneumoniae* seroprevalence starts ear-lier than preschool age in Guinea-Bissau.

An even higher seroprevalence could possibly be found in Bissau. Children who refused to participatewere likely to be sicker and epidemics may occur with variable strength (Grayston et al., 1989; Karvonen et al., 1993). In the pilot study, 27% were IgG positive. When also taking the rapid decline in antibody levels into account, it is apparent that *C. pneumoniae* infection may be very common amongst children in Guinea-Bissau.

Risk factors for C. pneumoniae infection

Only season of sampling was associated with the IgG seroprevalence at 18 months, IgG positivity being higher in samples collected in the dry season. We controlled for season in the analysis of the effect of DTP. Studies from countries with a tropical climate do not report seasonal variations and the seasonal variations in our study may simply reflect that an epidemic seemed to have occurred during the dry season. Previous studies report the prevalence of *C. pneumoniae* infection to be associated with living close together (in population, institutions or families) (Csango et al., 1997; Mordhorst et al., 1992; Pether et al., 1989) but some studies found no effect (Dal et al., 2005; Grayston, 1994). We did not observe an association with number of siblings. However, it should be noted that virtually everyone in Bissau lives under crowded conditions.

More important in this instance may be the observation that both seroconversion and loss of antibodies depended on nutritional status. IgG and IgA seroconversion was two times higher among children with low weight-for -age and loss of antibodies was more frequent by lower weight- for-age. Hence, it is clear we may underestimate the prevalence of *C. pneumoniae* infection in populations with a high proportion of children with low weight-for-age.

The effect of DTP

We investigated whether *C. pneumoniae* infection, meas-ured as an increase in *C. pneumoniae* seroconversion or as IgM positivity, in the six months follow-up period was more frequent in DTP-vaccinated than in OPV-only-vaccinated children. The immunological mechanisms underlying the non-specific effects of DTP (Aaby et al., 2002, 2003, 2004a, 2004b; Kristensen et al., 2000; Veirum et al., 2005) are not known. A possible increase in susceptibility to intracellular pathogens is supported by the earlier studies showing that DTP increased the

relative incidence of rotavirus, cryptosporidium and measles infection in females over males (Rodrigues et al., 2006; Valentiner-Branth et al., 2007; Aaby et al., 2009). These studies did not have a randomised control group but showed that the female-male morbidity ratio changed depending on which type of vaccine was most recently received. The IgM data in the present study suggested a similar pattern, *C. pneumoniae* infection being significantly more common in females than in males in the DTP group, whereas there was no sex difference among controls. Surprisingly, the IgG data suggested exactly the opposite conclusion. *C. pneumoniae* infection tended to be more common in males than in females in the DTP group, whereas there was no sex difference among controls who had not received DTP booster. In the light of the association of loss of antibodies with low weight-for-age it is noteworthy that the sex-differential effect of DTP on IgG seroconversion was opposite than expected only among children with low weight-for- age. Among children with normal weight there was a significant sex-differential effect of DTP on overall risk of *C. pneumoniae* infection with a higher risk in females and lower in males.

The contrasting effects of DTP on IgG and IgM could be due to problems with the test. We did exclude false-positive IgM tests due to IgM rheumatoid factor, but there might be other problems of cross-reactivity in an environment with many infections. Still, it is surprising that the IgM data indicate a sex-differential effect consistent with our previous studies in the DTP group, but absolutely no sex difference in the control group or in the IgM tests at 18 months of age. On the other hand, the IgG seroconversion data may have problems if interpreted as reflecting the true picture of *C. pneumoniae* infection. Most of those who were IgM positive at 18 months did not present IgG seroconversion and many of those who were IgG positive lost their antibody level. Hence, many may have had *C. pneumoniae* infection in the interval but did not maintain sufficiently high antibody levels to be classified as having seroconverted. This is particularly critical because poor health was associated with poor maintenance of antibody level and low weight was a risk factor for *C. pneumoniae* infection. From the data presented in Table 4, it may appear that maintenance of IgG antibodies has been par-ticularly bad among low-weight females in the DTP group.

The non-specific effects of DTP and MV were previously observed in the six-months time period in which the vaccine was the most recent vaccination (Aaby et al., 2002, 2004a) . Children in the present study did have three DTP and one or two MV vaccinations prior to randomisation. MV was associated with a decrease in mortality (Aaby et al., 1995) and provided after DTP with a decline in female-male incidence of cryptosporidia infection (Valentiner-Branth et al., 2007) and a decrease in hospital mortality – especially pneumonia deaths (Veirum et al., 2005). Thus, MV may affect infections but were, in the present study, equally distributed in the two randomisation groups. Furthermore, both randomisation groups had OPV vaccination at randomisation. The damaging effect of the combined vaccination with DTP and OPV seems to be due to DTP and not OPV (Aaby et al., 2004a). OPV on the contrary seems to be beneficial (Aaby et al., 2004c), but may be harmful to males when provided at birth (Benn et al., 2008). However, the sex differences in IgG and IgM data were only present in the DTP group, which suggested the effect was due to DTP and not to an opposite effect of OPV only.

The follow-up of mortality in the main study was not yet completed. However, mortality has declined dramatically in the study. The pathogen load has changed in the study area (Rodrigues et al., 2008) and the non-specific effects of DTP in the study population might now primarily be found for specific infections and morbidity measures. Thus, in an adverse- events sub- group study we found a sex-differential effect of DTP on symptoms and signs of morbidity, with tendencies for fewer symptoms in males and more symptoms in females comparing the DTP group with the OPV group (unpublished data). This was in agreement with the sex-differential effect of DTP on overall IgM positivity and on *C. pneumoniae* seroconversion in normal-weight children.

Implications and conclusion

The present sub-study within a randomised clinical trial suggests that *C. pneumoniae* infection is very prevalent among young children in low- income countries. The study both supported and negated the hypothesised increased risk of *C. pneumoniae* infection among females following DTP vaccination. Only future studies can resolve the enigma created by the present study. In future studies evaluating the burden of *C. pneumoniae* infection in children in low-income countries, it should be considered that serology may be hampered by an inability to maintain IgG antibody levels above the diagnostic cut-off in an environment with many competing infections and conditions contributing to poor health.

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REFERENCES

Aaby P, Martins C, Bale C, Garly MLRA, Biai S, Lisse I, Whittle H, Benn CS. Sex differences in the effect of vaccines on the risk of hospitalisation due to measles in Guinea-Bissau (2009). PIDJ (in revision).

Aaby P, Jensen H, Garly ML, Bale C, Martins C, Lisse I (2002). Routine vaccinations and child survival in a war situation with high mortality, effect of gender. Vaccine 21: 15-20.

Aaby P, Jensen H, Gomes J, Fernandes M, Lisse IM (2004a). The introduction of diphtheria-tetanus-pertussis vaccine and child mortality in rural Guinea-Bissau, an observational study. Int. J. Epidemiol. 33: 374-380.

Aaby P, Jensen H, Rodrigues A, Garly ML, Benn CS, Lisse IM, Simondon F (2004b). Divergent female-male mortality ratios associated with different routine vaccinations among female-male twin pairs. Int. J. Epidemiol. 33: 367-373.

Aaby P, Jensen H, Samb B, Cisse B, Sodemann M, Jakobsen M, Poulsen A, Rodrigues A, Lisse IM, Simondon F, Whittle H (2003) Differences in female-male mortality after high-titre measles vaccine and association with subsequent vaccination with diphtheria-tetanus-pertussis and inactivated poliovirus, reanalysis of West African studies. Lancet. 361: 2183-2188.

Aaby P, Rodrigues A, Biai S, Martins C, Veirum JE, Benn CS, Jensen H (2004c). Oral polio vaccination and low case fatality at the paediatric ward in Bissau, Guinea-Bissau. Vaccine 22: 3014-3017.

Aaby P, Samb B, Simondon F, Seck AM, Knudsen K, Whittle H (1995). Non-specific beneficial effect of measles immunisation, analysis of mortality studies from developing countries. BMJ 311: 481-485.

Aldous MB, Grayston JT, Wang SP, Foy HM (1992). Seroepidemiology of Chlamydia pneumoniae TWAR infection in Seattle families, 1966-1979. J. Infect. Dis. 166: 646-649.

Benn CS, Fisker AB, Rodrigues A, Ravn H, Sartono E, Whittle H, Yazdanbakhsh M, Aaby P (2008). Sex-differential effect on infant mortality of oral polio vaccine administered with BCG at birth in Guinea-Bissau. A natural experiment. PLoS One 3, e4056.

Choi TY, Kim DA, Kim SK, Kang JO, Park SS, Jung SR (1998). Prevalence of specific antibodies to Chlamydia pneumoniae in Korea. J. Clin. Microbiol. 36: 3426-3428.

Csango PA, Haraldstad S, Pedersen JE, Jagars G, Foreland I (1997). Respiratory tract infection due to Chlamydia pneumoniae in military personnel. Scand. J. Infect. Dis. Suppl. 104: 26-29.

Dal MG, Longo B, Not T, Poli A, Campello C (2005). A population based seroepidemiological survey of Chlamydia pneumoniae infections in schoolchildren. J. Clin. Pathol. 58: 617-620.

Einarsson S, Sigurdsson HK, Magnusdottir SD, Erlendsdottir H, Briem H,Gudmundsson S (1994). Age specific prevalence of antibodies against Chlamydia pneumoniae in Iceland. Scand. J. Infect. Dis. 26: 393-397.

Grant GB, Campbell H, Dowell SF, Graham SM, Klugman KP Mulholland EK, Steinhoff M, Weber MW, Qazi S (2009). Recommendations for treatment of childhood non-severe pneumonia. Lancet. Infect. Dis. 9: 185 -196.

Grayston JT (2000). Background and current knowledge of Chlamydia pneumoniae and atherosclerosis. J. Infect. Dis. 181 Suppl. 3, S402-S410.

Grayston JT (1994). Chlamydia pneumoniae (TWAR) infections in children. Pediatr. Infect. Dis. J. 13: 675-684.

Grayston JT, Mordhorst C, Bruu AL, Vene S, Wang SP (1989). Countrywide epidemics of Chlamydia pneumoniae, strain TWAR, in Scandinavia. 1981-1983. J. Infect. Dis. 159: 1111-1114.

Kabeya BK, Eb F, Ngwanza I, Corbel C, Biendo M, Orfila J (1999). Prevalence of anti-Chlamydia pneumoniae antibodies in preadolescent children in Congo. Bull. Soc. Pathol. Exot. 92: 6-8.

Karvonen M, Tuomilehto J, Pitkaniemi J, Saikku P (1993). The epidemic cycle of Chlamydia pneumoniae infection in eastern Finland.1972-1987. Epidemiol. Infect. 110: 349-360.

Kristensen I, Aaby P, Jensen H (2000). Routine vaccinations and child survival, follow up study in Guinea-Bissau, West Africa. BMJ. 321: 1435-1438.

Kuo CC, Jackson LA, Campbell LA, Grayston JT (1995). Chlamydia pneumoniae (TWAR). Clin. Microbiol. Rev. 8: 451-461.

Laderman EI, Whitworth E, Dumaual E, Jones M, Hudak A, Hogrefe W, Carney J, Groen J (2008). Rapid, sensitive, and specific lateral-flow immunochromatographic point-of-care device for detection of herpes simplex virus type 2-specific immunoglobulin G antibodies in serum and whole blood. Clin. Vaccine Immunol. 15: 159-163.

Lassmann B, Poetschke M, Ninteretse B, Issifou S, Winkler S, Kremsner PG, Graninger W, Apfalter P (2008). Community-acquired pneumonia in children in Lambarene, Gabon. Am. J. Trop. Med. Hyg. 79: 109 -114.

Lin TM, Kuo CC, Chen WJ, Lin FJ, Eng HL (2004). Seroprevalence of Chlamydia pneumoniae infection in Taiwan. J. Infect. 48: 91-95.

Mahmoud E, Elshibly S, Mardh PA (1994). Seroepidemiologic study of Chlamydia pneumoniae and other chlamydial species in a hyperendemic area for trachoma in the Sudan. Am. J. Trop. Med. Hyg. 51: 489-494.

Mordhorst CH, Wang SP, Grayston JT (1992). Outbreak of Chlamydia pneumoniae infection in four farm families. Eur. J. Clin. Microbiol. Infect. Dis. 11: 617-620.

Novello F, Ridolfi B, Fiore L, Buttinelli G, Medda E, Favero A, Marchetti D, Gaglioppa F (1996). Comparison of capillary blood versus venous blood samples in the assessment of immunity to measles. J. Virol. Methods 61: 73-77.

Paldanius M, Bloigu A, Alho M, Leinonen M, Saikku P (2005). Prevalence and persistence of Chlamydia pneumoniae antibodies in healthy laboratory personnel in Finland. Clin. Diagn. Lab. Immunol. 12: 654-659.

Pether JV, Wang SP, Grayston JT (1989). Chlamydia pneumoniae, strain TWAR, as the cause of an outbreak in a boys' school previously called psittacosis. Epidemiol. Infect. 103: 395-400.

Podsiadly E, Fracka B, Szmigielska A, Tylewska-Wierzbanowska S (2005). Seroepidemiological studies of *Chlamydia pneumoniae* infections in 1-36 months old children with respiratory tract infections and other diseases in Poland. Pol. J. Microbiol. 54: 215-219.

Rodrigues A, Fischer TK, Valentiner-Branth P, Nielsen J, Steinsland H, Perch M, Garly ML, Molbak K, Aaby P (2006). Community cohort study of rotavirus and other enteropathogens, are routine vaccinations associated with sex-differential incidence rates? Vaccine 24: 4737-4746.

Rodrigues A, Schellenberg JA, Kofoed PE, Aaby P and Greenwood B (2008). Changing pattern of malaria in Bissau, Guinea Bissau. Trop. Med. Int. Health. 13: 410-417.

Saikku P, Leinonen M, Tenkanen L, Linnanmaki E, Ekman MR, Manninen V, Manttari M, Frick MH, Huttunen JK (1992). Chronic *Chlamydia pneumoniae* infection as a risk factor for coronary heart disease in the Helsinki Heart Study. Ann. Intern. Med. 116: 273-278.

Saikku P, Ruutu P, Leinonen M, Panelius J, Tupasi TE, Grayston JT (1988). Acute lower-respiratory-tract infection associated with chlamydial TWAR antibody in Filipino children. J. Infect. Dis. 158: 1095-1097.

Tuuminen T, Varjo S, Ingman H, Weber T, Oksi J, Viljanen M (2000). Prevalence of *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* immunoglobulin G and A antibodies in a healthy Finnish population as analyzed by quantitative enzyme immunoassays. Clin. Diagn. Lab. Immunol. 7: 734-738.

Vainas T, De GR, Stassen FR, Kurvers HA, Grauls GE, Kitslaar PJ, Bruggeman CA (2003). Chlamydia pneumoniae serology, comparing a commercial enzyme immunoassay and microimmunofluorescence test in patients with cardiovascular disease. APMIS 111: 363- 69.

Valentiner-Branth P, Perch M, Nielsen J, Steinsland H, Garly ML, FischerTK, Sommerfelt H, Molbak K, Aaby P (2007). Community cohort study of Cryptosporidium parvum infections, sex-differential incidences associated with BCG and diphtheria – tetanu-pertussis

vaccinations. Vaccine 25: 2733-2741.
Veirum JE, Sodemann M, Biai S, Jakobsen M, Garly ML, Hedegaard K, Jensen H and Aaby P (2005). Routine vaccinations associated with divergent effects on female and male mortality at the paediatric ward in Bissau, Guinea-Bissau. Vaccine 23: 1197-1204.
Verkooyen RP, Hazenberg MA, Van Haaren GH, Van Den Bosch JM, Snijder RJ, Van Helden HP, Verbrugh HA (1992). Age-related interference with *Chlamydia pneumoniae* microimmunofluorescence serology due to circulating rheumatoid factor. J. Clin. Microbiol. 30: 1287-1290.
Volanen I, Vainionpaa R, Ilonen J, Markula P, Kallio K, Kaitosaari T, Helenius H, Simell O (2003). A prospective study of Chlamydia pneumoniae antibodies in children between 7 months and 8 years of age. Scand. J. Infect. Dis. 35: 471-477.
Wang JH, Liu YC, Cheng DL, Yeng MY, Chen YS, Chen BC (1993). Seroprevalence of Chlamydia pneumoniae in Taiwan. Scand. J. Infect. Dis. 25: 565-568.
Wang SP, Grayston JT (1990). Population prevalence of Chlamydia pneumonia, strain TWAR, in Clamydial infections (Bowie WR, Caldwell HD, Jones RP and et all eds).. Cambridge University Press pp. 402-405.