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Out-of-sequence DTP and measles vaccinations and child mortality in Guinea-Bissau – a reanalysis

Sanne Marie Thysen[a,b,c], *Amabelia Rodrigues*[a], *Peter Aaby*[a,b], *Ane Bærent Fisker*[a,b,d]

[a] Bandim Health Project, INDEPTH Network, Bissau, Guinea-Bissau

[b] Research Centre for Vitamins and Vaccines, Bandim Health Project, Statens Serum Institut, Copenhagen, Denmark

[c] Center for Global Health (GloHAU), Aarhus University, Aarhus, Denmark

[d] OPEN, Institute of Clinical Research, University of Southern Denmark, Odense, Denmark

Authors:

Sanne Marie Thysen, SAMT@SSI.dk

Amabelia Rodrigues, a.rodrigues@bandim.org

Peter Aaby, p.aaby@bandim.org

Ane Bærent Fisker, ABF@SSI.dk

Corresponding author: Sanne Marie Thysen, Bandim Health Project, INDEPTH Network, Apartado 861, 1004 Bissau Codex, Guinea-Bissau; SAMT@SSI.dk

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Abstract

Objectives To assess whether the sequence of diphtheria-tetanus-pertussis vaccine (DTP) and measles vaccine (MV) was associated with child survival in a dataset previously used to assess non-specific effects of DTP and MV without considering sequence of vaccinations.

Design Prospective cohort study analysed using the landmark approach.

Setting Bandim Health Project's Health and Demographic Surveillance System covering 100 village clusters in rural Guinea-Bissau.

Participants Children aged 9-17 months (recommended age of MV) and 18-35 months (recommended age of booster DTP) with vaccination status assessed between April 1991 and April 1996.

Methods Using Cox-proportional hazards models with age as underlying time, we compared mortality of children vaccinated out-of-sequence with mortality of children vaccinated in the recommended sequence. The analyses were stratified by sex and village cluster.

Main outcome measure Mortality rate ratio (MRR) for out-of-sequence vaccinations compared with in-sequence vaccinations.

Results Among 5937 observations in children aged 9-17 months, included in the main analysis, 1590 observations were classified as in-sequence vaccinations (DTP followed by MV), and 1984 observations were out-of-sequence vaccinations (1491 observations: MV with DTP and 493 observations: MV followed by DTP). Out-of-sequence vaccinations were associated with higher mortality than in-sequence vaccinations (MRR 2.10 (95% CI: 1.07-4.11)); the MRR was 2.30 (1.15-4.58) for MV and DTP administered simultaneously and 1.45 (0.50-4.22) for DTP administered after MV). Associations were similar for boys and girls (p=0.77). After 18 months, the mortality rate increased and the differential effect of out-of-sequence vaccinations disappeared.

Conclusion Out-of-sequence vaccinations may increase child mortality. Hence, sequence of vaccinations should be considered when planning vaccination programmes or introducing new vaccines into the current vaccination schedule.

Strengths and limitations of this study

- Vaccination status of the children were only updated at the inspection of a vaccination card. Hence, this study used the landmark analyses and thus prevented survival bias
- Misclassification of vaccinations due to the landmark approach would yield conservative estimates
- Booster doses of DTP were not registered before 1996, and we were therefore not able to make any firm conclusions of the effect of booster DTP
- Sensitivity analyses were conducted to limit the effect of vaccinations during follow-up

Introduction

Child mortality has declined significantly over the last decades.[1] Part of this decline is due to a reduction in preventable childhood diseases much of which is commonly ascribed to vaccines.[2] Vaccines are designed to protect against specific pathogens.[3] However, vaccines may have broader effects aside from the disease-specific protection with the live vaccines stimulating the immune system and reducing mortality by more than can be explained by preventing the target infection.[4-7] Hence, due to beneficial non-specific effects (NSEs) of live vaccines, vaccines may have played an even larger role in the decline of childhood mortality than usually assumed.

Studies from the introduction of the measles vaccine (MV) in the 1970's and 1980's from Asia and Africa showed larger reductions in mortality than could be ascribed to the prevention of measles infection.[8-10] Both observational studies and randomised trials have later confirmed lower mortality among measles-vaccinated children compared with measles-unvaccinated children.[11-13] In 2014, WHO's Strategic Advisory Group of Experts on immunization (SAGE) reviewed the evidence for NSEs of vaccines, and concluded that the evidence for MV was consistent with beneficial NSEs, especially for girls.[7 14]

The introduction of diphtheria-tetanus-pertussis vaccine (DTP) in the 1980's was associated with higher overall mortality, despite the protection against the specific diseases.[15-17] Other studies comparing mortality of DTP-vaccinated children and DTP-unvaccinated children have later confirmed the negative NSEs, especially for girls.[11 18-21] The WHO review of NSEs stated that beneficial or deleterious NSEs of DTP could not be confirmed nor refuted based on the evidence available.[7 14] However, the WHO review included studies with major survival bias; if the meta-analysis is restricted to studies with documentation of vaccination status and prospective follow-up, DTP-vaccinated children had two-fold higher mortality than DTP-unvaccinated children.[22]

Both observational studies[18 20 23-27] and randomised trials[19 28] suggest that the NSEs depends most strongly on the most recent vaccination and that sequence of vaccinations therefore is important. In a meta-analysis of randomised trials comparing inactivated vaccine after medium- or high-titre MV with standard-titre MV after inactivated vaccine, it was found that receiving an inactivated vaccine after a live MV was associated with a mortality rate-ratio (MRR) of 1.38 (95% CI: 1.05-1.83) compared with receiving live MV after an inactivated vaccine, the negative effect being particularly strong for females.[28]

In the first study that assessed the effect on mortality of MV and DTP, having received MV vs no MV was associated with a MRR of 0.48 (0.27-0.87); in contrast having received DTP vs no DTP was associated with higher mortality (MRR=1.84 (1.10-3.10)).[11] The analysis did not consider sequence of vaccinations, the potential importance of which had not yet been detected. We took advantage of this historical dataset[11] to test if sequence of vaccinations was associated with mortality. The issue is particularly important now because WHO is planning to add several non-live vaccines to the vaccination schedule,[29] including booster DTP and RTS,S malaria vaccine, and some will be given after MV.

Methods

Setting

Data was collected within the Bandim Health Project's Health and Demographic Surveillance System (HDSS) in rural Guinea-Bissau. The HDSS was established in 1990 using the Expanded Programme on Immunizations (EPI) methodology, randomly selecting 20 clusters of 100 women in each of the five largest health regions. Women of fertile age and their children below 5 years of age were followed through biannual visits. Women were registered at 14-16 years of age or when they moved into the village and were followed to death or migration. Newly registered women were interviewed about their past obstetric history, age, ethnicity and whether they had attended school. Children were registered during pregnancy or when they moved into the village. Children were followed until death, migration or 5 years of age.

At all visits, vaccination status, nutritional status and vital status were assessed. Vaccination status was assessed by inspection of a vaccination card. Children with no vaccination card and whose mother stated that the child had never received any vaccine were considered "unvaccinated". Only children with ascertained vaccination status (seen vaccination card, confirmed unvaccinated) were included in the analyses. Nutritional status was assessed by measurement of the child's mid-upper-arm circumference (MUAC).

Vaccination programme and definition of exposure

The vaccination schedule consisted of Bacillus Calmette-Guérin vaccine (BCG) and oral polio vaccine (OPV) at birth, 3 doses of DTP and OPV at 6, 10 and 14 weeks of age, MV at 9 months of age and booster doses of DTP and OPV at 18 months of age. The vaccination schedule did not change during the study period. Vaccinations were provided through the national immunization

programme. Systematic registration of DTP and OPV booster doses were only initiated in 1996, and thus, booster doses were not registered during the study period.

Children were divided into 5 groups according to the most recent vaccination(s) at the time their vaccination card was inspected: One group consisted of children, who were vaccinated in the recommended sequence, having received MV after DTP (DTP<MV). Two groups were vaccinated out-of-sequence: Children who had received DTP and MV simultaneously (DTP=MV), and children who had received DTP after MV (DTP>MV). Two groups had not received MV; children who had received DTP, but had not received MV (DTP, no MV) and children who had not received MV nor DTP (no DTP, no MV).

Study population

Children aged 9 to 35 months when visited between April 9, 1991 and April 24, 1996 were eligible for the study. It will be seen in Figure 1 that the mortality rate decline with age as expected in the beginning, but after 18 months of age the mortality rate started to increase again. The primary analysis is the age group 9-17 months since this is the period after MV is scheduled and before the scheduled age of booster dose of DTP. Children aged 18 to 35 months at the time of visit were included in a secondary analysis since they could have received a booster dose of DTP after their in-sequence or out-of-sequence vaccinations.

Statistical analyses

Baseline characteristics for different vaccination groups were compared using chi^2-test, Kruskal-Wallis rank test and one-way ANOVA comparison. We also compared baseline characteristics of children included in the analyses with children registered in the HDSS, but not included in the analyses using chi^2-test, t-test and Wilcoxon ranksum test. MUAC of children was expressed as a z-score compared with the 2006-WHO growth reference,[30] thus obtaining a standardized measure.

Using a Cox-proportional hazards model with age as underlying timescale, we compared mortality rates of children vaccinated out-of-sequence and children missing MV with the mortality rates of children vaccinated in-sequence. Children entered the analysis at the date of inspection of the vaccination card and remained in the analysis until the subsequent village visit, 6 months after the visit, death or migration, whichever came first. A child could therefore contribute with two non-overlapping periods if the vaccination status was assessed at more than one visit within the relevant age range (9 to 17 months). The booster doses of DTP and OPV administered at 18 months of age

was not registered consistently and we were therefore not able to account for which children had received the booster doses; we therefore censored at 18 months of age in the main analysis. The data was analysed using the landmark approach,[31] in which the child's vaccination status is only updated when the vaccination status is re-assessed at the next home visit.

In a secondary analysis, we assessed the effects of out-of-sequence vaccinations among children who were eligible for the DTP booster dose. In this analysis, we included children aged 18 to 35 months at the time of visit.

Since previous studies have reported sex-differential NSEs, all analyses were stratified by sex and separate estimates by sex are presented. All analyses were stratified by village cluster, thus comparing only children from the same community. Available baseline characteristics (Table 1) were included in the analyses one by one. No variable changed the main estimate by more than 10% and adjusted estimates are therefore not presented.

The original study assessed the effect of MV compared with no MV. To account for sequence of vaccination, we reinterpreted the NSEs of MV comparing children vaccinated in-sequence with MV after DTP with children with no MV (DTP, no MV and no DTP, no MV).

Sensitivity analyses

Since many children were vaccinated during follow up, we conducted two sensitivity analyses to limit the effect of vaccines administered during follow-up. In the first sensitivity analysis, we censored follow-up time at 2 months after entry. In the second sensitivity analysis, we included only children who had completed three DTP vaccinations and were therefore not eligible for further doses during follow-up.

Ethical considerations

The data was derived from the HDSS routine data collection, which has been ongoing since 1990 in collaboration with the Ministry of Health in Guinea-Bissau.[11]

Patient and public involvement

The communities were involved in locating households, when the HDSS was setup and contributed information allowing tracing of internal migrants between villages throughout the study period. No participants were involved in setting the research question or the outcome measure, nor were they involved in developing plans for recruitment, design, or implementation of the study. No participant

was asked to advise on interpretation or writing up the results. The results are disseminated to the national public health institute. There are no plans to disseminate the results of the research to study participants or the community.

Results

Baseline characteristics

Vaccination status was assessed for 4862 children aged 9-17 months contributing with 5956 observations (Figure 1). In addition to the 2536 children not included as their vaccination status was not assessed, we excluded 18 children corresponding to 19 observations from the analyses. These were children with unknown date of MV or DTP (8 children, 9 observations), and children who had received MV, but no DTP (10 children, 10 observations). We compared the distribution of baseline characteristics between children included in and excluded from the study (Supplementary table 1). Children excluded differed from the children included in the analyses with respect to age, region of residence, ethnicity and maternal age, but sex, nutritional status and maternal education did not differ. We also compared the distribution of baseline characteristics for different vaccination groups (Table 1). The age of children differed by vaccination group: children with DTP>MV were older than children who received DTP before or together with MV and children without MV were younger ($p<0.0001$). Mean MUAC z-scores for all groups were around one standard deviation below the reference, but children with DTP>MV and no DTP, no MV tended to deviate more from the WHO reference curve for MUAC compared with the other groups. The distribution of vaccination groups differed by region and ethnicity. More mothers of children vaccinated out-of-sequence or with missing MV had never attended school than mothers of children vaccinated in-sequence. Children vaccinated out-of-sequence had received their most recent vaccine closer to entry in the analysis (Table 1).

Mortality by vaccination group among children aged 9-17 months

Children vaccinated out-of-sequence had higher mortality compared with children vaccinated in-sequence (MRR: 2.10 (95% CI: 1.07-4.11); DTP=MV: 2.30 (1.15-4.58) and DTP>MV: 1.45 (0.50-4.22)). Children who had received DTP, but no MV had higher mortality compared with children vaccinated in-sequence (MRR: 2.57 (1.37-4.83)). Children without DTP and MV had higher mortality than children vaccinated in-sequence (MRR: 3.04 (1.41-6.55)) (Table 2). The associations were similar for boys and girls ($p=0.77$). For boys, out-of-sequence vaccinations were associated

with a MRR of 1.96 (0.80-4.78); for girls, the MRR was 2.25 (0.81-6.30). DTP without MV was associated significantly with higher mortality for boys (MRR: 3.41 (1.50-7.77)); mortality for girls was also higher, but not statistically significant (MRR: 1.67 (0.62-4.50)) (Table 2).

We have previously estimated a MRR of 0.48 (0.27-0.87) for MV versus no MV, without taking sequence of vaccination into consideration[11]. When we examined the NSEs of measles vaccine by comparing children MV-vaccinated in-sequence with children not MV-vaccinated, we found a MRR of 0.40 (0.23-0.69) (data not shown).

Mortality by vaccination group among children aged 18 to 35 months

Initially, mortality declined with age as expected (Figure 2). However, in spite of being older, in-sequence vaccinated children had higher mortality at 18 to 35 months of age (mortality rate (MR): 39.9 per 1000 person years (PYRS)) than children aged 9 to 17 months (MR: 32.6 per 1000 PYRS). Mortality developed differently with age for children vaccinated in-sequence compared with children vaccinated out-of-sequence (Figure 3). Since the in-sequence group had high mortality, there was no real differences in mortality between out-of-sequence and in-sequence vaccinations in the 18-35 months age group (Supplementary table 2). The MRR for out-of-sequence compared with in-sequence vaccinated children differed significantly between the age group 9-17 months (Table 2) and 18-35 months (Supplementary table 2) (test of interactions, p=0.02).

Sensitivity analyses

In the age group 9-17 months at least 20% of children vaccinated out-of-sequence received further doses of DTP during follow-up, but few children vaccinated in-sequence did (Supplementary table 3). To minimise the effect of vaccinations during follow-up, we conducted two sensitivity analyses. First, we censored follow-up 2 months after entry since few additional vaccines would be provided in that time window. This clearly restricted the power, but the trends remained the same: Out-of-sequence vaccinations were associated with a MRR of 2.51 (0.86-7.35) (Table 3). The estimates changed more for girls; out-of-sequence vaccinations being associated with an 8-fold higher mortality for girls (MRR: 7.83 (0.90-67.83)). Second, we restricted the dataset to children who had received DTP3 and therefore were unlikely to receive additional routine DTP vaccinations during follow-up (Supplementary table 4). The MRR of out-of-sequence vaccinations compared with in-sequence vaccinations was 1.85 (0.82-4.16), and the effect was similar for boys and girls (p=0.60) (Supplementary table 4). For girls, both DTP3=MV and DTP3>MV were associated with higher

mortality. For boys, DTP3=MV were associated with higher mortality, whereas DTP3>MV was not (Supplementary table 4).

Discussion

Main findings

Out-of-sequence vaccinations were associated with higher mortality compared with in-sequence vaccinations. After 18 months, the recommended age of booster DTP vaccination, the general mortality rate increased and the differential effect of out-of-sequence vaccinations disappeared.

Strengths and weaknesses

Using the landmark approach, survival bias was prevented since the vaccination status of the children were only updated when vaccination status was re-assessed, thereby preventing that vaccination information was updated for surviving children, but not for dead children. While this approach does not misclassify observation time dependent on the outcome, the misclassification of vaccinations during follow-up would yield conservative estimates.[31]

Data was collected through the rural HDSS in Guinea-Bissau and vaccination status was based on the vaccination card being inspected. Vaccinated children, whose vaccination card was not presented, were not included in the analysis. Mortality as the main outcome is unlikely to be reported wrongly, and with visits every 6 months, the imprecision in date of death is limited. Booster doses of DTP were not registered before 1996 and we could not fully explore the effect of booster DTP in the present cohort. To limit the effect of vaccinations during follow-up, we censored the main analysis at 18 months of age, when the children were eligible for the DTP booster; furthermore, we conducted two sensitivity analyses in which we first restricted follow-up to 2 months after entry and second limited the analysis to children who had received three doses of DTP. The conclusions of the main analysis were robust in these sensitivity analyses. The statistical model used, only compared children within the same village cluster, thus limiting bias from local differences such as epidemics, ethnicity, and access to health care. Comparing children across clusters did not change the conclusions (data not shown).

Comparison with other studies

Similar to our study, previous studies have found that out-of-sequence vaccinations are associated with increased mortality.[24-26 32-34] In the WHO-commissioned review, out-of-sequence vaccinations

with DTP and MV were associated with a relative mortality risk of 2.34 (1.57-3.50) compared with MV after DTP.[7] Hence, the age group 9-17 months in the present study is entirely consistent with previous studies. Out-of-sequence vaccinations may affect not only mortality but also hospital admissions; large population-based cohort studies from Denmark found that out-of-sequence vaccinations of DTP and MV were associated with higher hospitalisation rates.[35 36] To our knowledge, no study without survival bias has found beneficial effects of out-of-sequence vaccinations with DTP and MV.

The original study assessed the effect of MV compared with no MV and found a MRR of 0.48 (0.27-0.87)[11] not accounting for sequence of vaccination. According to our analyses this has underestimated the NSEs of MV. When we considered sequence of vaccination and compared children MV-vaccinated in-sequence with children not MV-vaccinated, we found a MRR of 0.40 (0.23-0.69).

The mortality rate usually declines with age.[37] In our study, among children vaccinated in-sequence, we found higher mortality rate in children aged 18 to 35 months compared with children aged 9 to 17 months (Figure 3). Since mortality did decline with age in the younger age group, we speculate that DTP booster for which children were eligible at 18 months of age may have contributed to this pattern just like DTP out-of-sequence with MV was associated with higher mortality. Unfortunately, our data collection tool in the early 1990 did not systematically assess DTP booster coverage. According to UNICEF figures, the DTP3 coverage was low in 1991-1996 (45-74%),[38] and we would not expect the coverage of booster DTP to be high. In urban Bissau, where the coverage for booster DTP was high, we have previously shown a similar increase in mortality after 18 months of age.[5] Thus, DTP booster doses may partly explain the higher mortality among 18-35 months old children, as observed in Gambia and India.[5 34 39]

Effects were similar for boys and girls, and overall we found no sex-differential effect of out-of-sequence vaccinations. However, other studies have found higher female mortality when DTP was administered after MV[21 39]; for example, high-titre measles vaccine (HTMV) was associated with higher female mortality and had to be withdrawn because most HTMV recipients had received DTP after MV.[28] In the present cohort, few children had received DTP after MV and most out-of-sequence vaccinations were combined administration of DTP and MV. When follow-up was limited to 2 months, estimates for out-of-sequence changed more for girls than for boys even though the difference between boys and girls did not reach statistical significance.

Interpretation and implications

We found that out-of-sequence vaccinations were associated with higher mortality both for children with co-administration of DTP and MV, and children with DTP after MV, compared with children vaccinated in-sequence. It could be speculated that out-of-sequence vaccinated children just had higher mortality because they were frail or their mothers less compliant with health services. In the present study, it speaks against the effect being due to an inherent bias that the difference disappeared completely for 18-35 months old children, possibly due to booster DTP. Furthermore, evidence from RCTs of medium and high-titre MV strongly supported that an inactivated vaccine after MV was associated with higher mortality.[28] Thus, sequence of vaccinations is likely to be important for child survival and should be considered when planning, implementing and evaluating the childhood vaccination programmes.

Current vaccination recommendations are based merely on the disease-specific effects of vaccines, often based on surrogate measures of the ability to prevent targeted infections. However, if vaccines alter the susceptibility to other infections this should be considered. Currently, vaccination programmes are evaluated based on vaccination coverage of DTP and MV at 12 months of age, and timeliness or sequence of vaccination is not taken into account. We found that DTP not succeeded by MV was associated with increased mortality and that out-of-sequence vaccinations were associated with higher mortality compared with children vaccinated in-sequence, thus, the current evaluation criteria emphasising DTP3 coverage may not optimise the impact of the vaccination programme on child health. A stronger emphasis should be put on increasing the MV coverage and getting DTPs and MV in the recommended sequence.

A change of emphasis is urgent: WHO is planning to introduce the second year of life platform with several inactivated vaccines (booster DTP, Meningitis A, RTS,S Malaria vaccine).[29] Hence, in the future children may receive inactivated vaccines after live MV at 9 months of age, not only because they deviate from the recommended schedule, but also if they follow the schedule.

Conclusion

Overall, we found that out-of-sequence vaccinations in children were associated with higher mortality compared with children vaccinated in-sequence. Vaccination programmes should monitor the sequence of vaccinations to optimise the overall effect on child survival.

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Authors' contributions

SMT, ABF and PA designed the study and planned the analyses. SMT extracted, cleaned and analysed the data. PA supervised the data collection and data entry. SMT drafted the paper with assistance from PA and ABF. All authors read and approved the final manuscript.

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Competing interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted

work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Data sharing

Data used for analyses in the present study are available from the corresponding author on reasonable request.

Transparency statement

SMT affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as originally planned have been explained.

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Figure 1: Flowchart of children included and excluded from the analysis

Figure 2 Overall mortality rate among children visited between 9 and 35 months of age.

Note: The figure plots the unadjusted mortality rates for children with a vaccination card seen between 9-35 months of age.

Figure 3 Mortality rate according to sequence of DTP and MV vaccinations among children visited between 9 and 35 months of age.

Note: The figure plots unadjusted mortality rates by vaccination status (in-sequence vs out-of-sequence vaccinations) among children with a vaccination card seen between 9-35 months of age.

Table 1 - Baseline characteristics for observations of children (9-17 months old) included in the analyses by vaccination group

	DTP<MV	DTP=MV	DTP>MV	DTP, no MV	No DTP, no MV	p-value
Numbers (%)	1590 (27)	1491 (25)	493 (8)	1816 (31)	547 (9)	
Sex						.29
Male (%)	834 (52)	729 (49)	255 (52)	931 (51)	290 (53)	
Female (%)	756 (48)	762 (51)	238 (48)	885 (49)	257 (47)	
Median age at start of follow-up (interquartile range)	425 (359 - 484)	420 (361 - 488)	466 (415 - 510)	346 (305 - 411.5)	365 (317 - 446)	<0.0001
MUAC at start of Follow-up	-0.93 (1.09)	-1.09 (1.05)	-1.16 (1.08)	-1.09 (1.13)	-1.13 (1.13)	<0.0001
Region						<0.0001
Oio	303 (19)	337 (23)	87 (18)	413 (23)	167 (31)	
Biombo	405 (25)	283 (19)	108 (22)	386 (21)	147 (27)	
Gabu	158 (10)	484 (32)	184 (37)	437 (24)	87 (16)	
Cacheu	353 (22)	125 (8)	37 (8)	226 (12)	46 (8)	
Bafata	371 (23)	262 (18)	77 (16)	354 (19)	100 (18)	
Ethnicity						<0.0001
Balanta	220 (14)	179 (12)	38 (8)	323 (18)	177 (33)	
Pepel	338 (21)	228 (16)	98 (20)	316 (18)	137 (25)	
Fula/Mandinca	703 (45)	921 (63)	296 (61)	950 (53)	183 (34)	
Manjaco	106 (7)	44 (3)	9 (2)	81 (4)	25 (5)	
Other	208 (13)	96 (7)	45 (9)	134 (7)	18 (3)	
Median maternal age (interquartile range)	25.6 (20.6 - 30.8)	26 (21.2 - 30.6)	25.9 (21.3 - 31)	26.2 (20.8 - 30.9)	26.8 (21.3 - 31.5)	.05
Education of caretaker						<0.0001
0 years	1290 (81)	1302 (87)	426 (86)	1562 (86)	485 (89)	
1-4 years	198 (12)	143 (10)	52 (11)	179 (10)	44 (8)	
>4 years	77 (5)	15 (1)	6 (1)	45 (2)	3 (1)	
Time since MV/ Time since DTP after MV	105 (52 - 169)	85 (38 - 154)	66 (30 - 108)	161 (98 - 238)	N/A	<0.0001

[1] 503 observations with missing MUAC
[2] 64 observations with missing information on ethnicity
[3] 63 observations with missing information on maternal age
[4] 110 observations with missing information on education of caretaker

Table 2 - Main analysis: Mortality of children visited between 9 and 17 months of age according to vaccination group

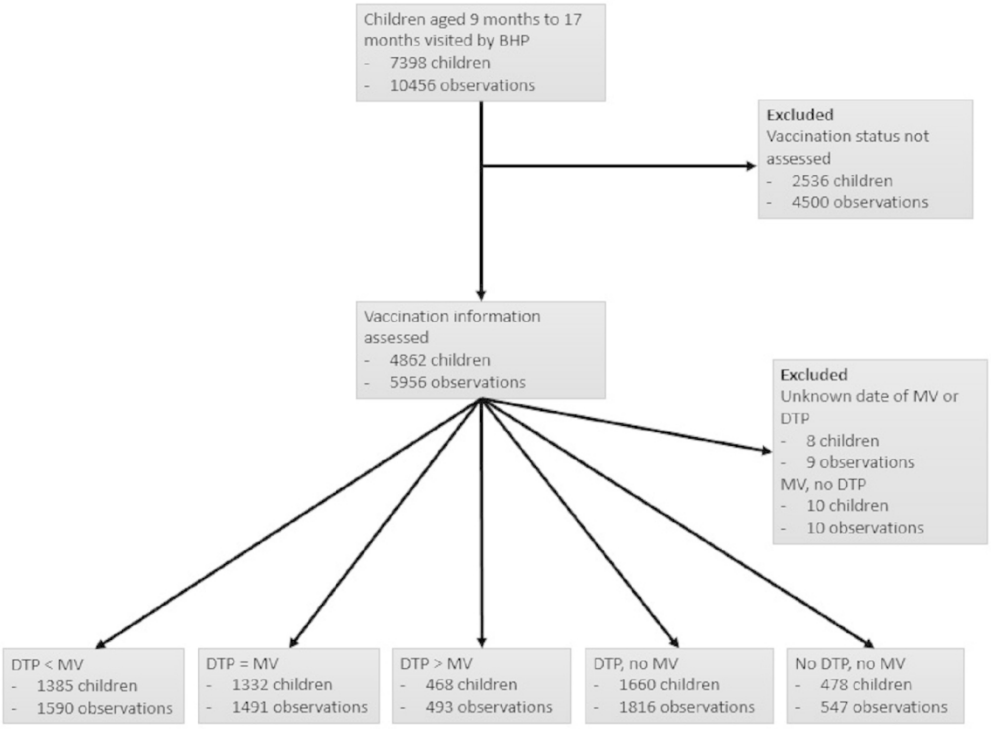
				Boys			Girls		
Vaccination status	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**
DTP < MV	1590	32.6 (16/491)	Ref	834	34.8 (9/258)	Ref	756	30.1 (7/232)	Ref
DTP = MV	1491	63.7 (29/455)	**2.30 (1.15-4.58)**	729	63.6 (14/220)	2.08 (0.83-5.26)	762	63.8 (15/235)	2.50 (0.88-7.12)
DTP > MV	493	43.1 (5/116)	1.45 (0.50-4.22)	255	51.5 (3/58)	1.48 (0.37-5.88)	238	34.6 (2/58)	1.38 (0.25-7.52)
DTP, no MV	1816	78.8 (57/723)	**2.57 (1.37-4.83)**	931	102.3 (38/372)	**3.41 (1.50-7.77)**	885	54.1 (19/351)	1.67 (0.62-4.50)
No DTP, no MV	547	111.3 (22/198)	**3.04 (1.41-6.55)**	290	95.3 (10/105)	2.77 (0.97-7.97)	257	129.5 (12/93)	**3.28 (1.06-10.12)**
Out-of-sequence vaccinations combined									
DTP>=MV	1984	59.5 (34/571)	**2.10 (1.07-4.11)**	984	61.1 (17/278)	1.96 (0.80-4.78)	1000	58.0 (17/293)	2.25 (0.81-6.30)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster.

Table 3 - Mortality of children visited between 9 and 18 months of age according to vaccination group with follow up censored at 2 months after entry into the analysis

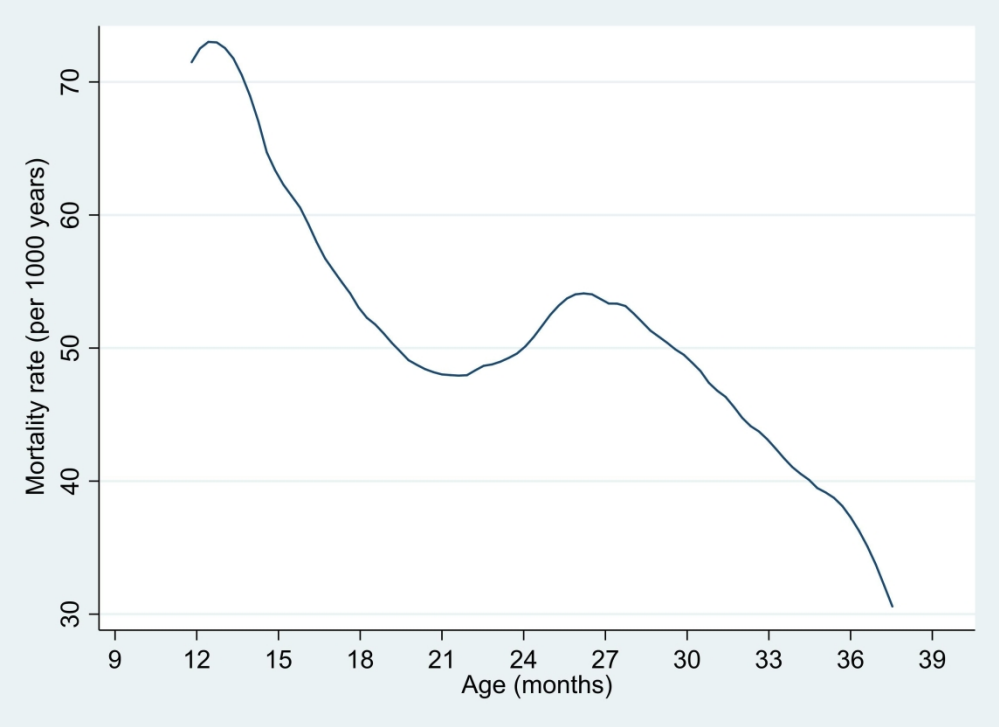
Vaccination status	Boys			Girls		
	Obser-vations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)	Obser-vations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)
DTP < MV	1590	25.8 (6/233)	Ref	834	32.9 (4/122)	Ref	756	18.0 (2/111)	Ref
DTP = MV	1491	60.5 (13/215)	2.34 (0.77-7.12)	729	38.2 (4/105)	1.16 (0.27-5.07)	762	81.8 (9/110)	6.71 (0.77-58.26)
DTP > MV	493	60.4 (4/66)	3.30 (0.77-14.14)	255	58.8 (2/34)	1.61 (0.26-10.18)	238	62.1 (2/32)	**14.61 (1.01-210.68)**
DTP, no MV	1816	93.4 (27/289)	**3.47 (1.24-9.69)**	931	127.8 (19/149)	2.56 (0.80-8.24)	885	57.0 (8/140)	6.34 (0.72-55.92)
No DTP, no MV	547	130.3 (11/84)	3.35 (1.00-11.26)	290	88.6 (4/45)	1.19 (0.24-5.90)	257	178.3 (7/39)	**14.73 (1.55-140.27)**
Out-of-sequence vaccinations combined									
DTP>=MV	1984	60.5 (17/281)	2.51 (0.86-7.35)	984	43.2 (6/139)	1.26 (0.32-4.85)	1000	77.4 (11/142)	7.83 (0.90-67.83)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster.



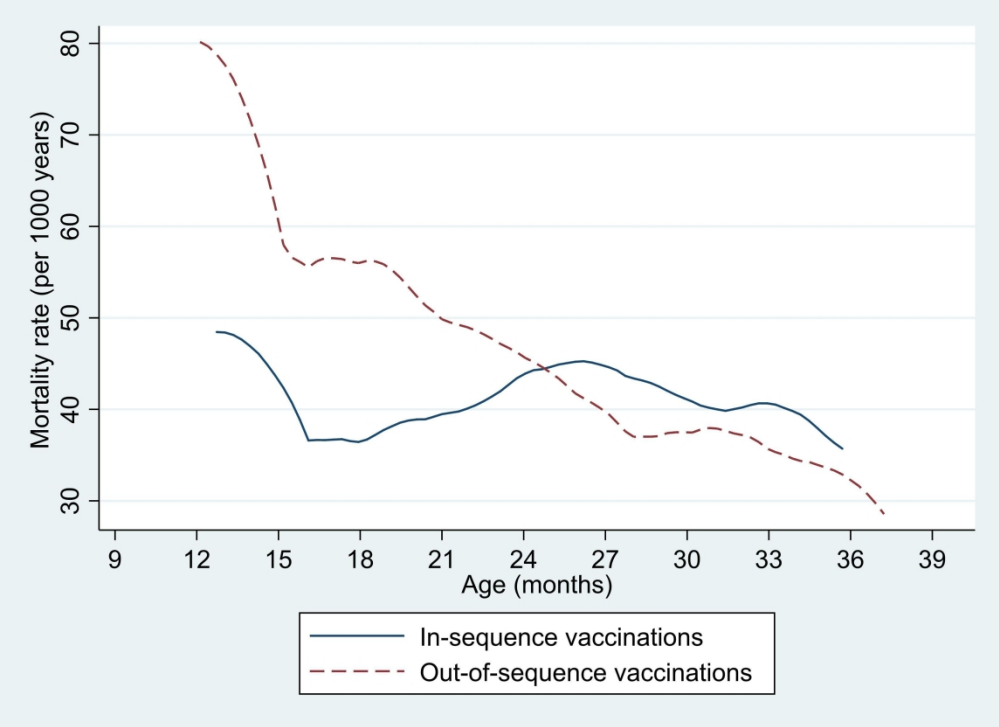
Flowchart of children included and excluded from the analysis

127x93mm (300 x 300 DPI)



Overall mortality rate among children visited between 9 and 35 months of age.

279x203mm (300 x 300 DPI)



Mortality rate according to sequence of DTP and MV vaccinations among children visited between 9 and 35 months of age.

279x203mm (300 x 300 DPI)

Supplementary table 1 - Baseline characteristics among children included and excluded from the analyses

	Included	Excluded	p-value
Numbers (%)	5937 (57)	4519 (43)	
Sex			.11
Male (%)	3039 (51)	2242 (50)	
Female (%)	2898 (49)	2277 (50)	
Median age at start of follow-up (interquartile range)	398 (334 - 471)	407 (338 - 477)	0.0005
MUAC at start of Follow-up[1]	-1.06 (1.1)	-1.09 (1.12)	.42
Region			<0.0001
Oio	1307 (22)	969 (21)	
Biombo	1329 (22)	1380 (31)	
Gabu	1350 (23)	803 (18)	
Cacheu	787 (13)	692 (15)	
Bafata	1164 (20)	675 (15)	
Ethnicity[2]			<0.0001
Balanta	937 (16)	926 (21)	
Pepel	1117 (19)	1172 (26)	
Fula/Mandinca	3053 (52)	1728 (39)	
Manjaco	265 (5)	270 (6)	
Other	501 (9)	369 (8)	
Median maternal age (interquartile range)[3]	26 (20.9 - 30.8)	25.3 (20.4 - 30)	<0.0001
Education of caretaker[4]			0.95
0 years	5065 (85)	3805 (84)	
1-4 years	616 (10)	472 (10)	
>4 years	146 (2)	111 (2)	

[1] 3731 observations with missing MUAC
[2] 118 observations with missing information on ethnicity
[3] 116 observations with missing information on maternal age
[4] 241 observations with missing information on education of caretaker

Supplementary table 2 - Mortality of children visited between 18 and 35 months of age according to vaccination group

Vaccination status	Boys			Girls		
	Observations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)	Observations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)
DTP < MV	1249	45.2 (27/598)	Ref	1166	34.1 (19/557)	Ref
DTP = MV	1031	57.5 (28/487)	1.06 (0.59-1.92)	1034	36.7 (18/491)	0.85 (0.42-1.71)
DTP > MV	954	41.4 (19/458)	0.83 (0.42-1.63)	986	21.3 (10/469)	**0.39 (0.16-0.94)**
DTP, no MV	278	76.2 (10/131)	1.39 (0.63-3.05)	302	56.3 (8/142)	1.37 (0.55-3.42)
No DTP, no MV	238	71.0 (8/113)	1.58 (0.64-3.91)	264	150.8 (18/119)	**2.68 (1.21-5.94)**
Out-of-sequence vaccinations combined						
DTP>=MV	1985	49.7 (47/946)	0.96 (0.56-1.67)	2020	29.2 (28/960)	0.64 (0.33-1.24)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster.

Supplementary table 3 - Registered vaccinations during follow-up (FU) period among those with vaccination status assessed again

9-17 months of age **Vaccination status**	**Observations N**	**Seen card after visit**	**Vaccinated during FU**			
			DTP	**MV**	**Polio**	**BCG**
DTP < MV	1590	1427	31 (2%)	0 (0%)	51 (4%)	3 (0%)
DTP = MV	1491	1324	310 (23%)	0 (0%)	316 (24%)	5 (0%)
DTP > MV	493	433	67 (15%)	0 (0%)	73 (17%)	0 (0%)
DTP, no MV	1816	1561	569 (36%)	866 (55%)	576 (37%)	14 (1%)
No DTP, no MV	547	516	120 (23%)	102 (20%)	118 (23%)	78 (15%)
18-35 months of age **Vaccination status**						
DTP < MV	2415	2053	18 (1%)	1 (0%)	38 (2%)	0 (0%)
DTP = MV	2065	1726	150 (9%)	0 (0%)	156 (9%)	2 (0%)
DTP > MV	1940	1648	75 (5%)	0 (0%)	82 (5%)	1 (0%)
DTP, no MV	580	429	77 (18%)	104 (24%)	79 (18%)	0 (0%)
No DTP, no MV	502	462	24 (5%)	19 (4%)	23 (5%)	13 (3%)

Supplementary table 4 - Mortality of children, who had received 3 doses of DTP, visited between 9 and 17 months of age according to vaccination group

				Boys			Girls		
Vaccination status	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**
DTP3 < MV	1492	30.4 (14/460)	Ref	774	37.7 (9/239)	Ref	718	22.6 (5/221)	Ref
DTP3 = MV	882	49.6 (13/262)	2.07 (0.88-4.86)	422	64.5 (8/124)	1.77 (0.61-5.15)	460	36.3 (5/138)	2.64 (0.65-10.73)
DTP3 > MV	363	35.8 (3/84)	1.27 (0.33-4.81)	191	23.2 (1/43)	0.76 (0.09-6.43)	172	49.1 (2/41)	2.09 (0.34-12.86)
DTP3, no MV	634	78.4 (20/255)	**3.01 (1.42-6.34)**	334	88.1 (12/136)	2.56 (0.97-6.74)	300	67.4 (8/119)	**3.80 (1.17-12.33)**
Out-of-sequence vaccinations combined									
DTP3>=MV	1245	46.3 (16/346)	1.85 (0.82-4.16)	613	53.8 (9/167)	1.53 (0.54-4.29)	632	39.2 (7/179)	2.46 (0.67-9.09)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1	
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3	
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4-5	
Objectives	3	State specific objectives, including any prespecified hypotheses	5	
Methods				
Study design	4	Present key elements of study design early in the paper	5-6	
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5	
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	6	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case		
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*5*	
Bias	9	Describe any efforts to address potential sources of bias	7	
Study size	10	Explain how the study size was arrived at	8	

Continued on next page

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6-7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	7
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8
		(b) Give reasons for non-participation at each stage	Figure 1
		(c) Consider use of a flow diagram	Figure 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8 + table 1
		(b) Indicate number of participants with missing data for each variable of interest	Table 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	Table 2
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	*Table 2*
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8 + Table 2
		(*b*) Report category boundaries when continuous variables were categorized	Table 1
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	

Continued on next page

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9 + Table 3 + Supp tables
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	10
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11-12
Generalisability	21	Discuss the generalisability (external validity) of the study results	10-12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Out-of-sequence DTP and measles vaccinations and child mortality in Guinea-Bissau – a reanalysis

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Keywords:	DTP vaccine, Measles vaccine, Child mortality, Vaccine sequence, Non-specific (heterologous) effects of vaccines

Out-of-sequence DTP and measles vaccinations and child mortality in Guinea-Bissau – a reanalysis

Sanne Marie Thysen[a,b,c], Amabelia Rodrigues[a], Peter Aaby[a,b], Ane Bærent Fisker[a,b,d]

[a] Bandim Health Project, INDEPTH Network, Bissau, Guinea-Bissau

[b] Research Centre for Vitamins and Vaccines, Bandim Health Project, Statens Serum Institut, Copenhagen, Denmark

[c] Center for Global Health (GloHAU), Aarhus University, Aarhus, Denmark

[d] OPEN, Institute of Clinical Research, University of Southern Denmark, Odense, Denmark

Authors:

Sanne Marie Thysen, s.thysen@bandim.org

Amabelia Rodrigues, a.rodrigues@bandim.org

Peter Aaby, p.aaby@bandim.org

Ane Bærent Fisker, a.fisker@bandim.org

Corresponding author: Sanne Marie Thysen, Bandim Health Project, INDEPTH Network, Apartado 861, 1004 Bissau Codex, Guinea-Bissau; s.thysen@bandim.org

Keywords: DTP vaccine, measles vaccine, child mortality, vaccine sequence, non-specific (heterologous) effects of vaccines

Word count: Abstract: 300, Manuscript: 3720

Abstract

Objectives To assess whether the sequence of diphtheria-tetanus-pertussis vaccine (DTP) and measles vaccine (MV) was associated with child survival in a dataset previously used to assess non-specific effects of vaccines with no consideration of vaccination sequence.

Design Prospective cohort study analysed using the landmark approach.

Setting Bandim Health Project's Health and Demographic Surveillance System covering 100 village clusters in rural Guinea-Bissau. The recommended vaccination schedule was BCG and oral polio vaccine (OPV) at birth, DTP and OPV at 6, 10 and 14 weeks, MV at 9 months, and booster-DTP and OPV at 18 months of age.

Participants Children aged 9-17 months (main analysis) and 18-35 months (secondary analysis: age of booster DTP) with vaccination status assessed between April 1991 and April 1996.

Methods Survival during the six months after assessing vaccination status was compared by vaccination sequence in Cox-proportional hazards models with age as underlying time. Analyses were stratified by sex and village cluster.

Main outcome measure Mortality rate ratio (MRR) for out-of-sequence vaccinations compared with in-sequence vaccinations.

Results Among children aged 9-17 months, 60% of observations (3574/5937) were from children who had received both MV and DTP. Among these,1590 observations were classified as in-sequence vaccinations (last DTP before MV), and 1984 observations were out-of-sequence vaccinations (1491: MV with DTP and 493: MV before DTP). Out-of-sequence vaccinations were associated with higher mortality than in-sequence vaccinations (MRR 2.10 (95% CI: 1.07-4.11)); the MRR was 2.30 (1.15-4.58) for MV with DTP and 1.45 (0.50-4.22) for DTP after MV). Associations were similar for boys and girls (p=0.77). Between 18-36 months the mortality rate increased among children vaccinated in-sequence and the differential effect of out-of-sequence vaccinations disappeared.

Conclusion Out-of-sequence vaccinations may increase child mortality. Hence, sequence of vaccinations should be considered when planning vaccination programmes or introducing new vaccines into the current vaccination schedule.

Strengths and limitations of this study

- Vaccination status of the children were only updated at the inspection of a vaccination card. Hence, this study used the landmark analyses and thus prevented survival bias
- Misclassification of vaccinations due to the landmark approach would yield conservative estimates
- Booster doses of DTP were not registered before 1996, and we were therefore not able to make any firm conclusions of the effect of booster DTP
- Sensitivity analyses were conducted to limit the effect of vaccinations during follow-up

Introduction

Child mortality has declined significantly between 2000 and 2015.[1] Part of this decline is due to a reduction in preventable childhood diseases much of which is commonly ascribed to vaccines.[2] Vaccines are designed to protect against specific pathogens.[3] However, vaccines may have broader effects aside from the disease-specific protection with the live vaccines stimulating the immune system and reducing mortality by more than can be explained by preventing the target infection.[4-7] Hence, due to beneficial non-specific effects (NSEs) of live vaccines, vaccines may have played an even larger role in the decline of childhood mortality than usually assumed.

Studies from the introduction of the measles vaccine (MV) in the 1970's and 1980's from Asia and Africa showed larger reductions in mortality than could be ascribed to the prevention of measles infection.[8-10] Both observational studies and randomised trials have later confirmed lower mortality among measles-vaccinated children compared with measles-unvaccinated children.[11-13] Based on accumulating evidence, WHO's Strategic Advisory Group of Experts on immunization (SAGE) recently reviewed the evidence for NSEs of some vaccines, and concluded that the evidence for MV was consistent with beneficial NSEs, especially for girls.[7 14]

The introduction of diphtheria-tetanus-pertussis vaccine (DTP) in the 1980's was associated with higher overall mortality, despite the protection against the specific diseases.[15-17] Other studies comparing mortality of DTP-vaccinated children and DTP-unvaccinated children have later confirmed the negative NSEs, especially for girls.[11 18-21] The WHO review of NSEs stated that beneficial or deleterious NSEs of DTP could not be confirmed nor refuted based on the evidence available.[7 14] However, the WHO review included studies with major survival bias; if the meta-analysis is restricted to studies with documentation of vaccination status and prospective follow-up, DTP-vaccinated children had two-fold higher mortality than DTP-unvaccinated children.[22]

Both observational studies[18 20 23-27] and randomised trials[19 28] suggest that the NSEs depends most strongly on the most recent vaccination and that sequence of vaccinations therefore is important. Randomised trials have compared inactivated vaccine after medium- or high-titre MV with standard-titre MV after inactivated vaccine. A meta-analysis of the trials indicates that receiving an inactivated vaccine after a live MV was associated with a mortality rate-ratio (MRR) of 1.38 (95% CI: 1.05-1.83) compared with receiving live MV after an inactivated vaccine, the negative effect being particularly strong for females.[28]

In the first study that assessed the effect on mortality of MV and DTP, having received MV vs no MV was associated with a MRR of 0.48 (0.27-0.87); in contrast having received DTP vs no DTP was associated with higher mortality (MRR=1.84 (1.10-3.10)).[11] The analysis did not consider sequence of vaccinations, the potential importance of which had not yet been detected. We took advantage of this historical dataset[11] to test if the different sequences of DTP and MV vaccinations were associated with mortality. The issue is particularly important now because WHO is planning to add several non-live vaccines to the vaccination schedule,[29] including booster DTP and RTS,S malaria vaccine, and some will be given after MV.

Methods

Setting

Data was collected within the Bandim Health Project's Health and Demographic Surveillance System (HDSS) in rural Guinea-Bissau. The HDSS was established in 1990 using the Expanded Programme on Immunizations (EPI) methodology, randomly selecting 20 clusters of 100 women in each of the five largest health regions. Women of fertile age and their children below 5 years of age were followed through biannual visits. Women were registered at 14-16 years of age or when they moved into the village and were followed to death or migration. Newly registered women were interviewed about their past obstetric history, age, ethnicity and whether they had attended school. Children were registered during pregnancy or when they moved into the village. Children were followed until death, migration or 5 years of age.

At all visits, vaccination status, nutritional status and vital status were assessed. Vaccination status was assessed by inspection of a vaccination card. Children with no vaccination card and whose mother stated that the child had never received any vaccine were considered "unvaccinated". Only children with ascertained vaccination status (seen vaccination card, confirmed unvaccinated) were included in the analyses. Nutritional status was assessed by measurement of the child's mid-upper-arm circumference (MUAC).

Vaccination programme and definition of exposure

The vaccination schedule consisted of Bacillus Calmette-Guérin vaccine (BCG) and oral polio vaccine (OPV) at birth, 3 doses of DTP and OPV at 6, 10 and 14 weeks of age, MV at 9 months of age and booster doses of DTP and OPV at 18 months of age. The vaccination schedule did not change during the study period. Vaccinations were provided through the national immunization programme. Systematic

registration of DTP and OPV booster doses were only initiated in 1996, and thus, booster doses were not registered during the study period.

Children were divided into 5 groups according to the most recent vaccination(s) at the time their vaccination card was inspected: One group consisted of children, who were vaccinated in the recommended sequence, having received MV after DTP (DTP<MV). Two groups were vaccinated out-of-sequence: Children who had received DTP and MV simultaneously (DTP=MV), and children who had received DTP after MV (DTP>MV). Two groups had not received MV; children who had received DTP, but had not received MV (DTP, no MV) and children who had not received MV nor DTP (no DTP, no MV).

Study population

Children aged 9 to 35 months when visited between April 9, 1991 and April 24, 1996 were eligible for the study. Figure 1 depicts the combined mortality rate of all study children. Mortality declines with age as expected in the beginning, but in the months following 18 months of age the mortality rate increases. The primary analysis is the age group 9-17 months since this is the period after MV is scheduled and before the scheduled age of booster dose of DTP. Children aged 18 to 35 months at the time of visit were included in a secondary analysis since they could have received a booster dose of DTP after their in-sequence or out-of-sequence vaccinations.

Statistical analyses

Baseline characteristics for different vaccination groups were compared using chi^2-test, Kruskal-Wallis rank test and one-way ANOVA comparison. We also compared baseline characteristics of children included in the analyses with children registered in the HDSS, but not included in the analyses using chi^2-test, t-test and Wilcoxon ranksum test. MUAC of children was expressed as a z-score compared with the 2006-WHO growth reference,[30] thus obtaining a standardized measure.

Using a Cox-proportional hazards model with age as underlying timescale, we compared mortality rates of children vaccinated out-of-sequence and children missing MV with the mortality rates of children vaccinated in-sequence. Children entered the analysis at the date of inspection of the vaccination card and remained in the analysis in the same vaccination group until the subsequent village visit, 6 months after the visit, death or migration, whichever came first. A child could therefore contribute with two non-overlapping periods if the vaccination status was assessed at more than one visit within the relevant age range (9 to 17 months). The booster doses of DTP and OPV administered at 18 months of age was not

registered consistently and we were therefore not able to account for which children had received the booster doses; we therefore censored at 18 months of age in the main analysis.

The data was analysed using the landmark approach,[31] in which the child's vaccination status is only updated when the vaccination status is re-assessed at the next home visit. If we had used the actual vaccination dates obtained at subsequent home visits to change the vaccine status, we would have better vaccination information for children who survived and had kept their vaccination cards, whereas the families of children who died between visits were likely to have discarded the vaccination card. As a consequence, the survivors would be given risk-free survival time for their new vaccination status, whereas it would not be known if the dead child had been vaccinated, and the child would therefore be misclassified as less vaccinated or unvaccinated. Such "risk-free" survival time will strongly inflate the estimated benefit of the last vaccination. To avoid such survival bias, we have therefore chosen the landmark approach.[31]

In a secondary analysis, we assessed the effects of out-of-sequence vaccinations among children who were eligible for the DTP booster dose. In this analysis, we included children aged 18 to 35 months at the time of visit.

Since previous studies have reported sex-differential NSEs, all analyses were stratified by sex and separate estimates by sex are presented. All analyses were stratified by village cluster, thus comparing only children from the same community. All available baseline characteristics (Table 1) were included in the analyses one by one. No variable changed the main estimate by more than 10% and adjusted estimates are therefore not presented.

The original study assessed the effect of MV compared with no MV. To account for sequence of vaccination, we reanalysed the NSEs of MV comparing children vaccinated in-sequence with MV after DTP with children with no MV (DTP, no MV and no DTP, no MV).

Sensitivity analyses

Since many children were vaccinated during follow up, i.e. after the inspection of their vaccination card, which allowed their exposure group to be classified, we conducted two sensitivity analyses to limit the effect of vaccines administered during follow-up. In the first sensitivity analysis, we censored observation time at 2 months after entry. In the second sensitivity analysis, we included only children who had completed three DTP vaccinations and were therefore not eligible for further doses during follow-up.

Ethical considerations

The data was derived from the HDSS routine data collection, which has been ongoing since 1990 in collaboration with the Ministry of Health in Guinea-Bissau.[11]

Patient and public involvement

The communities were involved in locating households, when the HDSS was setup and contributed information allowing tracing of internal migrants between villages throughout the study period. No participants were involved in setting the research question or the outcome measure, nor were they involved in developing plans for recruitment, design, or implementation of the study. No participant was asked to advise on interpretation or writing up the results. The results are disseminated to the national public health institute. There are no plans to disseminate the results of the research to study participants or the community.

Results

Baseline characteristics

Vaccination status was assessed for 4862 children aged 9-17 months contributing with 5956 observations (Figure 2). In addition to the 2536 children not included as their vaccination status was not assessed, we excluded 18 children corresponding to 19 observations from the analyses. These were children with unknown date of MV or DTP (8 children, 9 observations), and children who had received MV, but no DTP (10 children, 10 observations). We compared the distribution of baseline characteristics between children included in and excluded from the study (Supplementary table 1). Children excluded differed from the children included in the analyses with respect to age, region of residence, ethnicity and maternal age, but sex, nutritional status and maternal education did not differ. We also compared the distribution of baseline characteristics for different vaccination groups (Table 1). The age of children differed by vaccination group: children with DTP>MV were older than children who received DTP before or together with MV and children without MV were younger ($p<0.0001$). Mean MUAC z-scores for all groups were around one standard deviation below the reference, but children with DTP>MV and no DTP, no MV tended to deviate more from the WHO reference curve for MUAC compared with the other groups. The distribution of vaccination groups differed by region and ethnicity. More mothers of children vaccinated out-of-sequence or with missing MV had never attended school than mothers of children vaccinated in-sequence. Children vaccinated out-of-sequence had received their most recent vaccine closer to entry in the analysis (Table 1).

Mortality by vaccination group among children aged 9-17 months

Children vaccinated out-of-sequence had higher mortality compared with children vaccinated in-sequence (MRR: 2.10 (95% CI: 1.07-4.11); DTP=MV: 2.30 (1.15-4.58) and DTP>MV: 1.45 (0.50-4.22)). Children who had received DTP, but no MV had higher mortality compared with children vaccinated in-sequence (MRR: 2.57 (1.37-4.83)). Children without DTP and MV had higher mortality than children vaccinated in-sequence (MRR: 3.04 (1.41-6.55)) (Table 2). The associations were similar for boys and girls (p=0.77). For boys, out-of-sequence vaccinations were associated with a MRR of 1.96 (0.80-4.78); for girls, the MRR was 2.25 (0.81-6.30). DTP without MV was associated significantly with higher mortality for boys (MRR: 3.41 (1.50-7.77)); mortality for girls was also higher, but not statistically significant (MRR: 1.67 (0.62-4.50)) (Table 2).

We have previously estimated a MRR of 0.48 (0.27-0.87) for MV versus no MV, without taking sequence of vaccination into consideration[11]. When we examined the NSEs of measles vaccine by comparing children MV-vaccinated in-sequence with children not MV-vaccinated, we found a MRR of 0.40 (0.23-0.69) (data not shown).

Mortality by vaccination group among children aged 18 to 35 months

Initially, mortality declined with age as expected (Figure 1). However, in spite of being older, in-sequence vaccinated children had higher mortality at 18 to 35 months of age (mortality rate (MR): 39.9 per 1000 person years (PYRS)) than children aged 9 to 17 months (MR: 32.6 per 1000 PYRS). Mortality developed differently with age for children vaccinated in-sequence compared with children vaccinated out-of-sequence (Figure 3). Since the in-sequence group had high mortality, there was no real differences in mortality between out-of-sequence and in-sequence vaccinations in the 18-35 months age group (Supplementary table 2). The MRR for out-of-sequence compared with in-sequence vaccinated children differed significantly between the age group 9-17 months (Table 2) and 18-35 months (Supplementary table 2) (test of interactions, p=0.02).

Sensitivity analyses

In the age group 9-17 months at least 20% of children vaccinated out-of-sequence received further doses of DTP during follow-up, but few children vaccinated in-sequence did (Supplementary table 3). To minimise the effect of vaccinations during follow-up, we conducted two sensitivity analyses. First, we censored follow-up 2 months after entry since few additional vaccines would be provided in that time window. This clearly restricted the power, but the trends remained the same: Out-of-sequence vaccinations were associated with a MRR of 2.51 (0.86-7.35) (Table 3). The estimates changed more for

girls; out-of-sequence vaccinations being associated with an 8-fold higher mortality for girls (MRR: 7.83 (0.90-67.83)). Second, we restricted the dataset to children who had received DTP3 and therefore were unlikely to receive additional routine DTP vaccinations during follow-up (Supplementary table 4). The MRR of out-of-sequence vaccinations compared with in-sequence vaccinations was 1.85 (0.82-4.16), and the effect was similar for boys and girls (p=0.60) (Supplementary table 4). For girls, both DTP3=MV and DTP3>MV were associated with higher mortality. For boys, DTP3=MV were associated with higher mortality, whereas DTP3>MV was not (Supplementary table 4).

Discussion

Main findings

Out-of-sequence vaccinations were associated with higher mortality compared with in-sequence vaccinations. After 18 months, the recommended age of booster DTP vaccination, the general mortality rate increased and the differential effect of out-of-sequence vaccinations disappeared.

Strengths and weaknesses

Using the landmark approach, survival bias was prevented since the vaccination status of the children was only updated when vaccination status was re-assessed, thereby preventing that vaccination information was updated for surviving children, but not for dead children. While this approach does not misclassify observation time dependent on the outcome, the misclassification of vaccinations during follow-up would yield conservative estimates.[31]

Data was collected through the rural HDSS in Guinea-Bissau and vaccination status was based on the vaccination card being inspected. Vaccinated children, whose vaccination card was not presented, were not included in the analysis. Mortality as the main outcome is unlikely to be reported wrongly, and with visits every 6 months, the imprecision in date of death is limited. Booster doses of DTP were not registered before 1996 and we could not fully explore the effect of booster DTP in the present cohort. To limit the effect of vaccinations during follow-up, we censored the main analysis at 18 months of age, when the children were eligible for the DTP booster; furthermore, we conducted two sensitivity analyses in which we first restricted follow-up to 2 months after entry and second limited the analysis to children who had received three doses of DTP. The conclusions of the main analysis were robust in these sensitivity analyses. The statistical model used, only compared children within the same village cluster, thus limiting bias from local differences such as epidemics, ethnicity, and access to health care. Comparing children across clusters did not change the conclusions (data not shown).

In spite of the careful collection of vaccination information and individual level follow-up, we cannot guarantee that observed mortality differences are caused only by the sequence of vaccinations. To limit confounding, we assessed whether available background factors changed the estimate by more than 10%. As no background factor changed the estimate by more than 10%, we did not present adjusted estimates. However, there may be residual confounding not adjusted for.

To enter the analysis, a child had to survive to have the vaccination card inspected, and a differential mortality pattern before the inspection of the vaccination card would not be captured in our analyses. However, in prior studies of vaccination sequence and mortality, the effects have been similar regardless of whether vaccinations are registered at the time of vaccinations[26 32] or later[24 27], and this is therefore unlikely to explain the pattern.

Comparison with other studies

Similar to our study, previous studies have found that out-of-sequence vaccinations are associated with increased mortality.[24-26 33-35] In the WHO-commissioned review, out-of-sequence vaccinations with DTP and MV were associated with a relative mortality risk of 2.34 (1.57-3.50) compared with MV after DTP.[7] Hence, the age group 9-17 months in the present study is entirely consistent with previous studies. Out-of-sequence vaccinations may affect not only mortality but also hospital admissions; large population-based cohort studies from Denmark found that out-of-sequence vaccinations of DTP and MV were associated with higher hospitalisation rates.[36 37] To our knowledge, no study without survival bias has found beneficial effects of out-of-sequence vaccinations with DTP and MV.

The original study assessed the effect of MV compared with no MV and found a MRR of 0.48 (0.27-0.87)[11] not accounting for sequence of vaccination. According to our analyses this has underestimated the NSEs of MV. When we considered sequence of vaccination and compared children MV-vaccinated in-sequence with children not MV-vaccinated, we found a MRR of 0.40 (0.23-0.69).

The mortality rate usually declines with age.[38] In our study, among children vaccinated in-sequence, we found higher mortality rate in children aged 18 to 35 months compared with children aged 9 to 17 months (Figure 3). Since mortality did decline with age in the younger age group, we speculate that DTP booster for which children were eligible at 18 months of age may have contributed to this pattern just like DTP out-of-sequence with MV was associated with higher mortality. Unfortunately, our data collection tool in the early 1990 did not systematically assess DTP booster coverage. According to UNICEF figures, the DTP3 coverage was low in 1991-1996 (45-74%),[39] and we would not expect the coverage of booster DTP

to be high. In urban Bissau, where the coverage for booster DTP was high, we have previously shown a similar increase in mortality after 18 months of age.[5] Thus, DTP booster doses may partly explain the higher mortality among 18-35 months old children, as observed in Gambia and India.[5 35 40]

Effects were similar for boys and girls, and overall we found no sex-differential effect of out-of-sequence vaccinations. However, other studies have found higher female mortality when DTP was administered after MV[21 40]; for example, high-titre measles vaccine (HTMV) was associated with higher female mortality and had to be withdrawn because most HTMV recipients had received DTP after MV.[28] In the present cohort, few children had received DTP after MV and most out-of-sequence vaccinations were combined administration of DTP and MV. When follow-up was limited to 2 months, estimates for out-of-sequence changed more for girls than for boys even though the difference between boys and girls did not reach statistical significance.

Interpretation and implications

We found that out-of-sequence vaccinations were associated with higher mortality both for children with co-administration of DTP and MV, and children with DTP after MV, compared with children vaccinated in-sequence. It could be speculated that out-of-sequence vaccinated children just had higher mortality because they were frail or their mothers less compliant with health services. In the present study, it speaks against the effect being due to an inherent bias that the difference disappeared completely for 18-35 months old children, possibly due to booster DTP. Furthermore, evidence from RCTs of medium and high-titre MV strongly supported that an inactivated vaccine after MV was associated with higher mortality.[28] Thus, sequence of vaccinations is likely to be important for child survival and should be considered when planning, implementing and evaluating the childhood vaccination programmes.

Current vaccination recommendations are based merely on the disease-specific effects of vaccines, often based on surrogate measures of the ability to prevent targeted infections. However, if vaccines alter the susceptibility to other infections this should be considered. Currently, vaccination programmes are evaluated based on vaccination coverage of DTP and MV at 12 months of age, and timeliness or sequence of vaccination is not taken into account. We found that DTP not succeeded by MV was associated with increased mortality and that out-of-sequence vaccinations were associated with higher mortality compared with children vaccinated in-sequence, thus, the current evaluation criteria emphasising DTP3 coverage may not optimise the impact of the vaccination programme on child health. Our results indicate that a stronger emphasis should be put on increasing the MV coverage and getting DTPs and MV in the recommended sequence.

Currently WHO is planning to introduce the second year of life platform with several inactivated vaccines (booster DTP, Meningitis A, RTS,S Malaria vaccine).[29] Hence, in the future children may receive inactivated vaccines after live MV at 9 months of age, not only because they deviate from the recommended schedule, but also if they follow the schedule. We urge others to test the effect of providing non-live vaccines after MV, preferably prior to the introduction of new vaccines, while RCTs are still possible.

Conclusion

Overall, we found that out-of-sequence vaccinations in children were associated with higher mortality compared with children vaccinated in-sequence. Vaccination programmes should monitor the sequence of vaccinations to optimise the overall effect on child survival.

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Authors' contributions

SMT, PA and ABF designed the study and planned the analyses. SMT extracted, cleaned and analysed the data. PA supervised the data collection and data entry. SMT drafted the paper with assistance from PA, AR and ABF. All authors read and approved the final manuscript.

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Competing interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Data sharing

Data used for analyses in the present study are available from the corresponding author on reasonable request.

Transparency statement

SMT affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as originally planned have been explained.

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Figure 1 Overall mortality rate among children visited between 9 and 35 months of age.

Note: The figure plots the unadjusted mortality rates for children with a vaccination card seen between 9-35 months of age. The smoothed mortality curve starts shortly before 12 months of age, where the first event occurs. Only few children contribute with observation time between 9 and 12 months.

Figure 2 Flowchart of children included and excluded from the analysis

Figure 3 Mortality rate according to sequence of DTP and MV vaccinations among children visited between 9 and 35 months of age.

Note: The figure plots unadjusted mortality rates by vaccination status (in-sequence vs out-of-sequence vaccinations) among children with a vaccination card seen between 9-35 months of age. The smoothed mortality curve starts shortly before 12 months of age, where the first event occurs. Only few children contribute with observation time between 9 and 12 months.

Table 1 - Baseline characteristics for observations of children (9-17 months old) included in the analyses by vaccination group

	DTP<MV	DTP=MV	DTP>MV	DTP, no MV	No DTP, no MV	p-value
Numbers (%)	1590 (27)	1491 (25)	493 (8)	1816 (31)	547 (9)	
Sex						.29
Male (%)	834 (52)	729 (49)	255 (52)	931 (51)	290 (53)	
Female (%)	756 (48)	762 (51)	238 (48)	885 (49)	257 (47)	
Median age in months at start of follow-up (interquartile range)	14.0 (11.8 – 15.9)	13.8 (11.9 – 16.0)	15.3 (13.6 – 16.8)	11.4 (10.0 - 13.5)	12.0 (10.4 – 14.7)	<0.0001
MUAC z-score at start of Follow-up	-0.93 (1.09)	-1.09 (1.05)	-1.16 (1.08)	-1.09 (1.13)	-1.13 (1.13)	<0.0001
Region						<0.0001
Oio	303 (19)	337 (23)	87 (18)	413 (23)	167 (31)	
Biombo	405 (25)	283 (19)	108 (22)	386 (21)	147 (27)	
Gabu	158 (10)	484 (32)	184 (37)	437 (24)	87 (16)	
Cacheu	353 (22)	125 (8)	37 (8)	226 (12)	46 (8)	
Bafata	371 (23)	262 (18)	77 (16)	354 (19)	100 (18)	
Ethnicity						<0.0001
Balanta	220 (14)	179 (12)	38 (8)	323 (18)	177 (33)	
Pepel	338 (21)	228 (16)	98 (20)	316 (18)	137 (25)	
Fula/Mandinca	703 (45)	921 (63)	296 (61)	950 (53)	183 (34)	
Manjaco	106 (7)	44 (3)	9 (2)	81 (4)	25 (5)	
Other	208 (13)	96 (7)	45 (9)	134 (7)	18 (3)	
Median maternal age in years (interquartile range)	25.6 (20.6 - 30.8)	26 (21.2 - 30.6)	25.9 (21.3 - 31)	26.2 (20.8 - 30.9)	26.8 (21.3 - 31.5)	.05
Education of caretaker						<0.0001
0 years	1290 (81)	1302 (87)	426 (86)	1562 (86)	485 (89)	
1-4 years	198 (12)	143 (10)	52 (11)	179 (10)	44 (8)	
>4 years	77 (5)	15 (1)	6 (1)	45 (2)	3 (1)	
Time since MV/ Time since DTP after MV in days	105 (52 - 169)	85 (38 - 154)	66 (30 - 108)	161 (98 - 238)	N/A	<0.0001

[1] 503 observations with missing MUAC

[2] 64 observations with missing information on ethnicity

[3] 63 observations with missing information on maternal age

[4] 110 observations with missing information on education of caretaker

Table 2 - Main analysis: Mortality of children visited between 9 and 17 months of age according to vaccination group

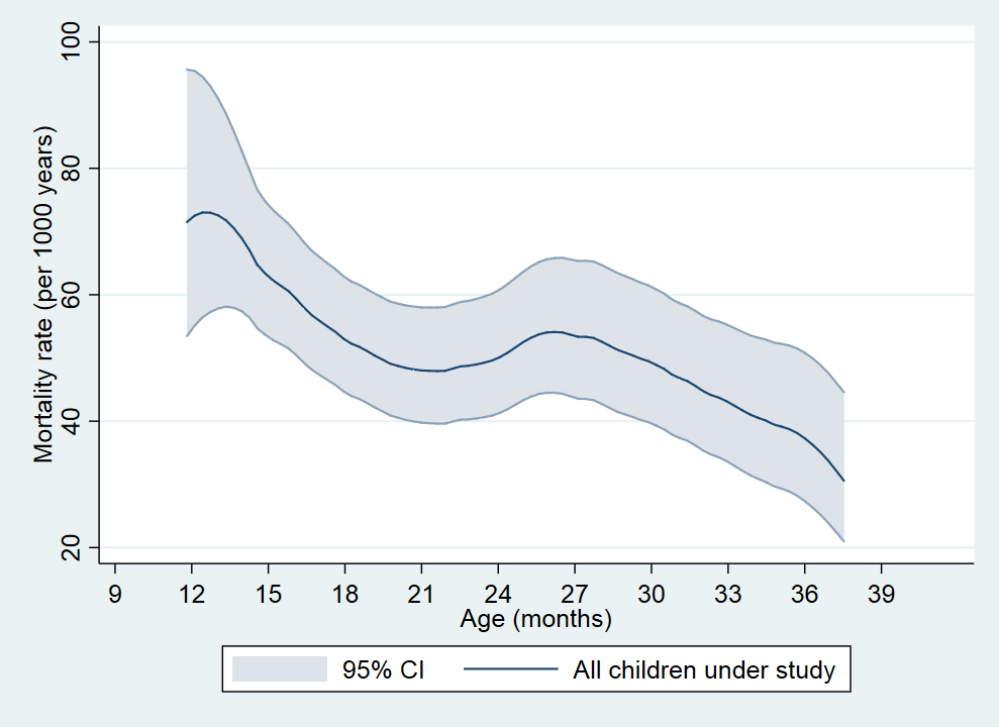
				Boys			Girls		
Vaccination status	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**
DTP < MV	1590	32.6 (16/491)	Ref	834	34.8 (9/258)	Ref	756	30.1 (7/232)	Ref
DTP = MV	1491	63.7 (29/455)	**2.30 (1.15-4.58)**	729	63.6 (14/220)	2.08 (0.83-5.26)	762	63.8 (15/235)	2.50 (0.88-7.12)
DTP > MV	493	43.1 (5/116)	1.45 (0.50-4.22)	255	51.5 (3/58)	1.48 (0.37-5.88)	238	34.6 (2/58)	1.38 (0.25-7.52)
DTP, no MV	1816	78.8 (57/723)	**2.57 (1.37-4.83)**	931	102.3 (38/372)	**3.41 (1.50-7.77)**	885	54.1 (19/351)	1.67 (0.62-4.50)
No DTP, no MV	547	111.3 (22/198)	**3.04 (1.41-6.55)**	290	95.3 (10/105)	2.77 (0.97-7.97)	257	129.5 (12/93)	**3.28 (1.06-10.12)**
Out-of-sequence vaccinations combined									
DTP>=MV	1984	59.5 (34/571)	**2.10 (1.07-4.11)**	984	61.1 (17/278)	1.96 (0.80-4.78)	1000	58.0 (17/293)	2.25 (0.81-6.30)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster.

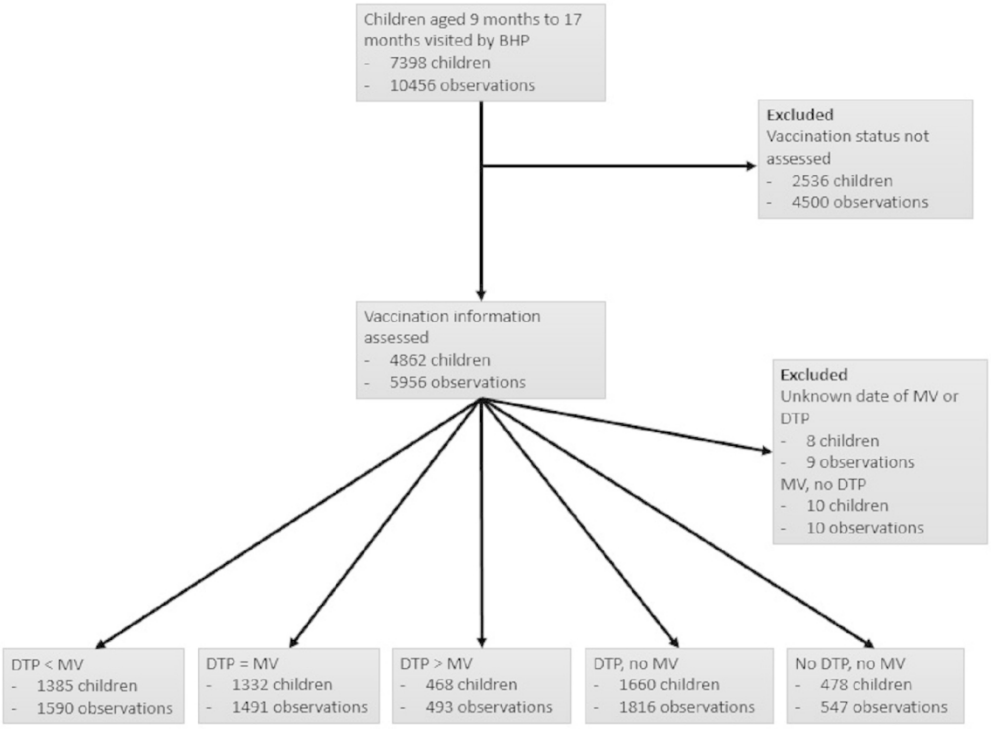
Table 3 - Mortality of children visited between 9 and 18 months of age according to vaccination group with follow up censored at 2 months after entry into the analysis

Vaccination status	Obser-vations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)	Boys			Girls		
				Obser-vations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)	Obser-vations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)
DTP < MV	1590	25.8 (6/233)	Ref	834	32.9 (4/122)	Ref	756	18.0 (2/111)	Ref
DTP = MV	1491	60.5 (13/215)	2.34 (0.77-7.12)	729	38.2 (4/105)	1.16 (0.27-5.07)	762	81.8 (9/110)	6.71 (0.77-58.26)
DTP > MV	493	60.4 (4/66)	3.30 (0.77-14.14)	255	58.8 (2/34)	1.61 (0.26-10.18)	238	62.1 (2/32)	**14.61 (1.01-210.68)**
DTP, no MV	1816	93.4 (27/289)	**3.47 (1.24-9.69)**	931	127.8 (19/149)	2.56 (0.80-8.24)	885	57.0 (8/140)	6.34 (0.72-55.92)
No DTP, no MV	547	130.3 (11/84)	3.35 (1.00-11.26)	290	88.6 (4/45)	1.19 (0.24-5.90)	257	178.3 (7/39)	**14.73 (1.55-140.27)**
Out-of-sequence vaccinations combined									
DTP>=MV	1984	60.5 (17/281)	2.51 (0.86-7.35)	984	43.2 (6/139)	1.26 (0.32-4.85)	1000	77.4 (11/142)	7.83 (0.90-67.83)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster.

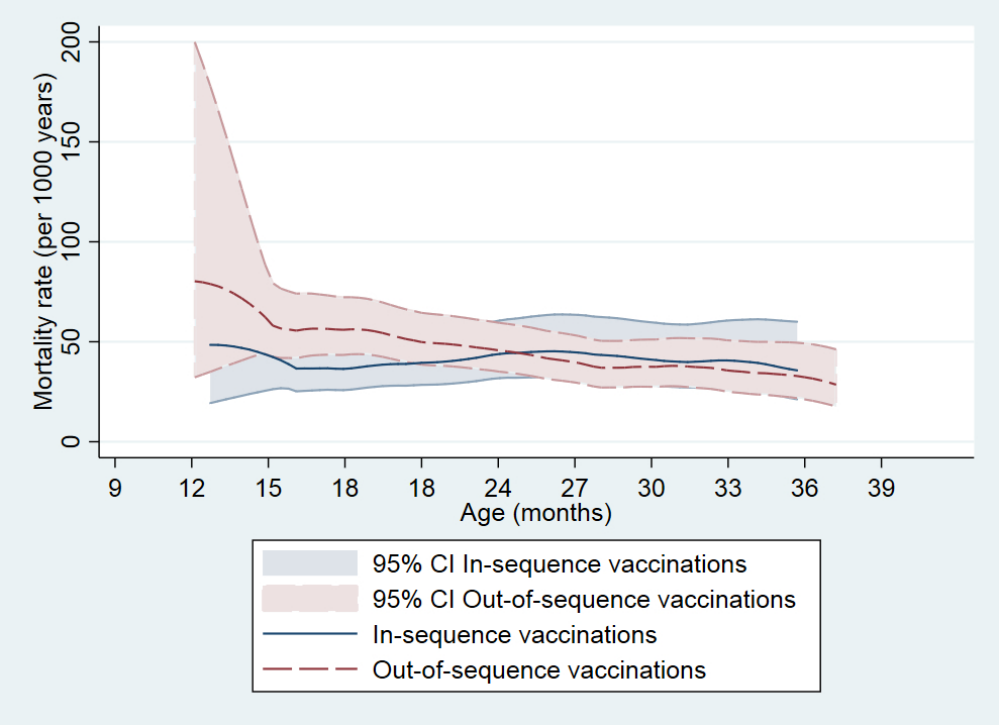


Overall mortality rate among children visited between 9 and 35 months of age.



Flowchart of children included and excluded from the analysis

127x93mm (300 x 300 DPI)



Mortality rate according to sequence of DTP and MV vaccinations among children visited between 9 and 35 months of age.

Supplementary table 1 - Baseline characteristics among children included and excluded from the analyses

	Included	Excluded	p-value
Numbers (%)	5937 (57)	4519 (43)	
Sex			.11
Male (%)	3039 (51)	2242 (50)	
Female (%)	2898 (49)	2277 (50)	
Median age in months at start of follow-up (interquartile range)	13.1 (11.0 – 15.5)	13.4 (11.1 – 15.7)	0.0005
MUAC at start of Follow-up[1]	-1.06 (1.1)	-1.09 (1.12)	.42
Region			<0.0001
Oio	1307 (22)	969 (21)	
Biombo	1329 (22)	1380 (31)	
Gabu	1350 (23)	803 (18)	
Cacheu	787 (13)	692 (15)	
Bafata	1164 (20)	675 (15)	
Ethnicity[2]			<0.0001
Balanta	937 (16)	926 (21)	
Pepel	1117 (19)	1172 (26)	
Fula/Mandinca	3053 (52)	1728 (39)	
Manjaco	265 (5)	270 (6)	
Other	501 (9)	369 (8)	
Median maternal age in years (interquartile range)[3]	26 (20.9 - 30.8)	25.3 (20.4 - 30)	<0.0001
Education of caretaker[4]			0.95
0 years	5065 (85)	3805 (84)	
1-4 years	616 (10)	472 (10)	
>4 years	146 (2)	111 (2)	

[1] 3731 observations with missing MUAC

[2] 118 observations with missing information on ethnicity

[3] 116 observations with missing information on maternal age

[4] 241 observations with missing information on education of caretaker

Supplementary table 2 - Mortality of children visited between 18 and 35 months of age according to vaccination group

				Boys			Girls		
Vaccination status	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**
DTP < MV	2415	39.9 (46/1154)	Ref	1249	45.2 (27/598)	Ref	1166	34.1 (19/557)	Ref
DTP = MV	2065	47.0 (46/978)	0.98 (0.62-1.54)	1031	57.5 (28/487)	1.06 (0.59-1.92)	1034	36.7 (18/491)	0.85 (0.42-1.71)
DTP > MV	1940	31.3 (29/927)	0.62 (0.36-1.06)	954	41.4 (19/458)	0.83 (0.42-1.63)	986	21.3 (10/469)	**0.39 (0.16-0.94)**
DTP, no MV	580	65.9 (18/273)	1.40 (0.77-2.55)	278	76.2 (10/131)	1.39 (0.63-3.05)	302	56.3 (8/142)	1.37 (0.55-3.42)
No DTP, no MV	502	112.0 (26/232)	**2.18 (1.21-3.90)**	238	71.0 (8/113)	1.58 (0.64-3.91)	264	150.8 (18/119)	**2.68 (1.21-5.94)**
Out-of-sequence vaccinations combined									
DTP>=MV	4005	39.4 (75/1,905)	0.82 (0.54-1.24)	1985	49.7 (47/946)	0.96 (0.56-1.67)	2020	29.2 (28/960)	0.64 (0.33-1.24)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster.

Supplementary table 3 - Registered vaccinations during follow-up (FU) period among those with vaccination status assessed again

9-17 months of age **Vaccination status**	**Observations N**	**Seen card after visit**	**Vaccinated during FU**			
			DTP	**MV**	**Polio**	**BCG**
DTP < MV	1590	1427	31 (2%)	0 (0%)	51 (4%)	3 (0%)
DTP = MV	1491	1324	310 (23%)	0 (0%)	316 (24%)	5 (0%)
DTP > MV	493	433	67 (15%)	0 (0%)	73 (17%)	0 (0%)
DTP, no MV	1816	1561	569 (36%)	866 (55%)	576 (37%)	14 (1%)
No DTP, no MV	547	516	120 (23%)	102 (20%)	118 (23%)	78 (15%)
18-35 months of age **Vaccination status**						
DTP < MV	2415	2053	18 (1%)	1 (0%)	38 (2%)	0 (0%)
DTP = MV	2065	1726	150 (9%)	0 (0%)	156 (9%)	2 (0%)
DTP > MV	1940	1648	75 (5%)	0 (0%)	82 (5%)	1 (0%)
DTP, no MV	580	429	77 (18%)	104 (24%)	79 (18%)	0 (0%)
No DTP, no MV	502	462	24 (5%)	19 (4%)	23 (5%)	13 (3%)

Supplementary table 4 - Mortality of children, who had received 3 doses of DTP, visited between 9 and 17 months of age according to vaccination group

				Boys			Girls		
Vaccination status	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**
DTP3 < MV	1492	30.4 (14/460)	Ref	774	37.7 (9/239)	Ref	718	22.6 (5/221)	Ref
DTP3 = MV	882	49.6 (13/262)	2.07 (0.88-4.86)	422	64.5 (8/124)	1.77 (0.61-5.15)	460	36.3 (5/138)	2.64 (0.65-10.73)
DTP3 > MV	363	35.8 (3/84)	1.27 (0.33-4.81)	191	23.2 (1/43)	0.76 (0.09-6.43)	172	49.1 (2/41)	2.09 (0.34-12.86)
DTP3, no MV	634	78.4 (20/255)	**3.01 (1.42-6.34)**	334	88.1 (12/136)	2.56 (0.97-6.74)	300	67.4 (8/119)	**3.80 (1.17-12.33)**
Out-of-sequence vaccinations combined									
DTP3>=MV	1245	46.3 (16/346)	1.85 (0.82-4.16)	613	53.8 (9/167)	1.53 (0.54-4.29)	632	39.2 (7/179)	2.46 (0.67-9.09)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1	
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3	
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4-5	
Objectives	3	State specific objectives, including any prespecified hypotheses	5	
Methods				
Study design	4	Present key elements of study design early in the paper	5-6	
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5	
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	6	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case		
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*5*	
Bias	9	Describe any efforts to address potential sources of bias	7	
Study size	10	Explain how the study size was arrived at	8	

Continued on next page

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6-7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	7
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8
		(b) Give reasons for non-participation at each stage	Figure 1
		(c) Consider use of a flow diagram	Figure 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8 + table 1
		(b) Indicate number of participants with missing data for each variable of interest	Table 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	Table 2
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	*Table 2*
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8 + Table 2
		(*b*) Report category boundaries when continuous variables were categorized	Table 1
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	

Continued on next page

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9 + Table 3 + Supp tables
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	10
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11-12
Generalisability	21	Discuss the generalisability (external validity) of the study results	10-12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Out-of-sequence DTP and measles vaccinations and child mortality in Guinea-Bissau – a reanalysis

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Secondary Subject Heading:	Epidemiology, Health policy, Infectious diseases, Paediatrics, Public health
Keywords:	DTP vaccine, Measles vaccine, Child mortality, Vaccine sequence, Non-specific (heterologous) effects of vaccines

Out-of-sequence DTP and measles vaccinations and child mortality in Guinea-Bissau – a reanalysis

Sanne Marie Thysen[a,b,c,d], *Amabelia Rodrigues*[b], *Peter Aaby*[b,c], *Ane Bærent Fisker*[a,b,c]

[a] OPEN, Institute of Clinical Research, University of Southern Denmark, Odense, Denmark

[b] Bandim Health Project, INDEPTH Network, Bissau, Guinea-Bissau

[c] Research Centre for Vitamins and Vaccines, Bandim Health Project, Statens Serum Institut, Copenhagen, Denmark

[d] Center for Global Health (GloHAU), Aarhus University, Aarhus, Denmark

Authors:

Sanne Marie Thysen, s.thysen@bandim.org

Amabelia Rodrigues, a.rodrigues@bandim.org

Peter Aaby, p.aaby@bandim.org

Ane Bærent Fisker, a.fisker@bandim.org

Corresponding author: Sanne Marie Thysen, Bandim Health Project, INDEPTH Network, Apartado 861, 1004 Bissau Codex, Guinea-Bissau; s.thysen@bandim.org

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Abstract

Objectives To assess whether the sequence of diphtheria-tetanus-pertussis vaccine (DTP) and measles vaccine (MV) was associated with child survival in a dataset previously used to assess non-specific effects of vaccines with no consideration of vaccination sequence.

Design Prospective cohort study analysed using the landmark approach.

Setting Bandim Health Project's Health and Demographic Surveillance System covering 100 village clusters in rural Guinea-Bissau. The recommended vaccination schedule was BCG and oral polio vaccine (OPV) at birth, DTP and OPV at 6, 10 and 14 weeks, MV at 9 months, and booster-DTP and OPV at 18 months of age.

Participants Children aged 9-17 months (main analysis) and 18-35 months (secondary analysis: age of booster DTP) with vaccination status assessed between April 1991 and April 1996.

Methods Survival during the six months after assessing vaccination status was compared by vaccination sequence in Cox-proportional hazards models with age as underlying time. Analyses were stratified by sex and village cluster.

Main outcome measure Mortality rate ratio (MRR) for out-of-sequence vaccinations compared with in-sequence vaccinations.

Results Among children aged 9-17 months, 60% of observations (3574/5937) were from children who had received both MV and DTP. Among these,1590 observations were classified as in-sequence vaccinations (last DTP before MV), and 1984 observations were out-of-sequence vaccinations (1491: MV with DTP and 493: MV before DTP). Out-of-sequence vaccinations were associated with higher mortality than in-sequence vaccinations (MRR 2.10 (95% CI: 1.07-4.11)); the MRR was 2.30 (1.15-4.58) for MV with DTP and 1.45 (0.50-4.22) for DTP after MV). Associations were similar for boys and girls (p=0.77). Between 18-35 months the mortality rate increased among children vaccinated in-sequence and the differential effect of out-of-sequence vaccinations disappeared.

Conclusion Out-of-sequence vaccinations may increase child mortality. Hence, sequence of vaccinations should be considered when planning vaccination programmes or introducing new vaccines into the current vaccination schedule.

Strengths and limitations of this study

- Vaccination status of the children were only updated at the inspection of a vaccination card. Hence, this study used the landmark analyses and thus prevented survival bias
- Misclassification of vaccinations due to the landmark approach would yield conservative estimates
- Booster doses of DTP were not registered before 1996, and we were therefore not able to make any firm conclusions of the effect of booster DTP
- Sensitivity analyses were conducted to limit the effect of vaccinations during follow-up

Introduction

Child mortality has declined significantly between 2000 and 2015.[1] Part of this decline is due to a reduction in preventable childhood diseases much of which is commonly ascribed to vaccines.[2] Vaccines are designed to protect against specific pathogens.[3] However, vaccines may have broader effects aside from the disease-specific protection with the live vaccines stimulating the immune system and reducing mortality by more than can be explained by preventing the target infection.[4-7] Hence, due to beneficial non-specific effects (NSEs) of live vaccines, vaccines may have played an even larger role in the decline of childhood mortality than usually assumed.

Studies from the introduction of the measles vaccine (MV) in the 1970's and 1980's from Asia and Africa showed larger reductions in mortality than could be ascribed to the prevention of measles infection.[8-10] Both observational studies and randomised trials have later confirmed lower mortality among measles-vaccinated children compared with measles-unvaccinated children.[11-13] Based on accumulating evidence, WHO's Strategic Advisory Group of Experts on immunization (SAGE) recently reviewed the evidence for NSEs of some vaccines, and concluded that the evidence for MV was consistent with beneficial NSEs, especially for girls.[7 14]

The introduction of diphtheria-tetanus-pertussis vaccine (DTP) in the 1980's was associated with higher overall mortality, despite the protection against the specific diseases.[15-17] Other studies comparing mortality of DTP-vaccinated children and DTP-unvaccinated children have later confirmed the negative NSEs, especially for girls.[11 18-21] The WHO review of NSEs stated that beneficial or deleterious NSEs of DTP could not be confirmed nor refuted based on the evidence available.[7 14] However, the WHO review included studies with major survival bias; if the meta-analysis is restricted to studies with documentation of vaccination status and prospective follow-up, DTP-vaccinated children had two-fold higher mortality than DTP-unvaccinated children.[22]

Both observational studies[18 20 23-27] and randomised trials[19 28] suggest that the NSEs depends most strongly on the most recent vaccination and that sequence of vaccinations therefore is important. Randomised trials have compared inactivated vaccine after medium- or high-titre MV with standard-titre MV after inactivated vaccine. A meta-analysis of the trials indicates that receiving an inactivated vaccine after a live MV was associated with a mortality rate-ratio (MRR) of 1.38 (95% CI: 1.05-1.83) compared with receiving live MV after an inactivated vaccine, the negative effect being particularly strong for females.[28]

In the first study that assessed the effect on mortality of MV and DTP, having received MV vs no MV was associated with a MRR of 0.48 (0.27-0.87); in contrast having received DTP vs no DTP was associated with higher mortality (MRR=1.84 (1.10-3.10)).[11] The analysis did not consider sequence of vaccinations, the potential importance of which had not yet been detected. We took advantage of this historical dataset[11] to test if the different sequences of DTP and MV vaccinations were associated with mortality. The issue is particularly important now because WHO is planning to add several non-live vaccines to the vaccination schedule,[29] including booster DTP and RTS,S malaria vaccine, and some will be given after MV.

Methods

Setting

Data was collected within the Bandim Health Project's Health and Demographic Surveillance System (HDSS) in rural Guinea-Bissau. The HDSS was established in 1990 using the Expanded Programme on Immunizations (EPI) methodology, randomly selecting 20 clusters of 100 women in each of the five largest health regions. Women of fertile age and their children below 5 years of age were followed through biannual visits. Women were registered at 14-16 years of age or when they moved into the village and were followed to death or migration. Newly registered women were interviewed about their past obstetric history, age, ethnicity and whether they had attended school. Children were registered during pregnancy or when they moved into the village. Children were followed until death, migration or 5 years of age.

At all visits, vaccination status, nutritional status and vital status were assessed. Vaccination status was assessed by inspection of a vaccination card. Children with no vaccination card and whose mother stated that the child had never received any vaccine were considered "unvaccinated". Only children with ascertained vaccination status (seen vaccination card, confirmed unvaccinated) were included in the analyses. Nutritional status was assessed by measurement of the child's mid-upper-arm circumference (MUAC).

Vaccination programme and definition of exposure

The vaccination schedule consisted of Bacillus Calmette-Guérin vaccine (BCG) and oral polio vaccine (OPV) at birth, 3 doses of DTP and OPV at 6, 10 and 14 weeks of age, MV at 9 months of age and booster doses of DTP and OPV at 18 months of age. The vaccination schedule did not change during the study period. Vaccinations were provided through the national immunization programme. Systematic

registration of DTP and OPV booster doses were only initiated in 1996, and thus, booster doses were not registered during the study period.

Children were divided into 5 groups according to the most recent vaccination(s) at the time their vaccination card was inspected: One group consisted of children, who were vaccinated in the recommended sequence, having received MV after DTP (DTP<MV). Two groups were vaccinated out-of-sequence: Children who had received DTP and MV simultaneously (DTP=MV), and children who had received DTP after MV (DTP>MV). Two groups had not received MV; children who had received DTP, but had not received MV (DTP, no MV) and children who had not received MV nor DTP (no DTP, no MV).

Study population

Children aged 9 to 35 months when visited between April 9, 1991 and April 24, 1996 were eligible for the study. Figure 1 depicts the combined mortality rate of all study children. Mortality declines with age as expected in the beginning, but around 21 months of age the mortality rate increases. The primary analysis is the age group 9-17 months since this is the period after MV is scheduled and before the scheduled age of booster dose of DTP. Children aged 18 to 35 months at the time of visit were included in a secondary analysis since they could have received a booster dose of DTP after their in-sequence or out-of-sequence vaccinations.

Statistical analyses

Baseline characteristics for different vaccination groups were compared using chi^2-test, Kruskal-Wallis rank test and one-way ANOVA comparison. We also compared baseline characteristics of children included in the analyses with children registered in the HDSS, but not included in the analyses using chi^2-test, t-test and Wilcoxon ranksum test. MUAC of children was expressed as a z-score compared with the 2006-WHO growth reference,[30] thus obtaining a standardized measure.

Using a Cox-proportional hazards model with age as underlying timescale, we compared mortality rates of children vaccinated out-of-sequence and children missing MV with the mortality rates of children vaccinated in-sequence. Children entered the analysis at the date of inspection of the vaccination card and remained in the analysis in the same vaccination group until the subsequent village visit, 6 months after the visit, death or migration, whichever came first. A child could therefore contribute with two non-overlapping periods if the vaccination status was assessed at more than one visit within the relevant age range (9 to 17 months). The booster doses of DTP and OPV administered at 18 months of age was not

registered consistently and we were therefore not able to account for which children had received the booster doses; we therefore censored at 18 months of age in the main analysis.

The data was analysed using the landmark approach,[31] in which the child's vaccination status is only updated when the vaccination status is re-assessed at the next home visit. If we had used the actual vaccination dates obtained at subsequent home visits to change the vaccine status, we would have better vaccination information for children who survived and had kept their vaccination cards, whereas the families of children who died between visits were likely to have discarded the vaccination card. As a consequence, the survivors would be given risk-free survival time for their new vaccination status, whereas it would not be known if the dead child had been vaccinated, and the child would therefore be misclassified as less vaccinated or unvaccinated. Such "risk-free" survival time will strongly inflate the estimated benefit of the last vaccination. To avoid such survival bias, we have therefore chosen the landmark approach.[31]

In a secondary analysis, we assessed the effects of out-of-sequence vaccinations among children who were eligible for the DTP booster dose. In this analysis, we included children aged 18 to 35 months at the time of visit.

Since previous studies have reported sex-differential NSEs, all analyses were stratified by sex and separate estimates by sex are presented. All analyses were stratified by village cluster, thus comparing only children from the same community. All available baseline characteristics (Table 1) were included in the analyses one by one. No variable changed the main estimate by more than 10% and adjusted estimates are therefore not presented.

The original study assessed the effect of MV compared with no MV. To account for sequence of vaccination, we reanalysed the NSEs of MV comparing children vaccinated in-sequence with MV after DTP with children with no MV (DTP, no MV and no DTP, no MV).

Sensitivity analyses

Since many children were vaccinated during follow up, i.e. after the inspection of their vaccination card, which allowed their exposure group to be classified, we conducted two sensitivity analyses to limit the effect of vaccines administered during follow-up. In the first sensitivity analysis, we censored observation time at 2 months after entry. In the second sensitivity analysis, we included only children who had completed three DTP vaccinations and were therefore not eligible for further doses during follow-up.

Ethical considerations

The data was derived from the HDSS routine data collection, which has been ongoing since 1990 in collaboration with the Ministry of Health in Guinea-Bissau.[11]

Patient and public involvement

The communities were involved in locating households, when the HDSS was setup and contributed information allowing tracing of internal migrants between villages throughout the study period. No participants were involved in setting the research question or the outcome measure, nor were they involved in developing plans for recruitment, design, or implementation of the study. No participant was asked to advise on interpretation or writing up the results. The results are disseminated to the national public health institute. There are no plans to disseminate the results of the research to study participants or the community.

Results

Baseline characteristics

Vaccination status was assessed for 4862 children aged 9-17 months contributing with 5956 observations (Figure 2). In addition to the 2536 children not included as their vaccination status was not assessed, we excluded 18 children corresponding to 19 observations from the analyses. These were children with unknown date of MV or DTP (8 children, 9 observations), and children who had received MV, but no DTP (10 children, 10 observations). We compared the distribution of baseline characteristics between children included in and excluded from the study (Supplementary table 1). Children excluded differed from the children included in the analyses with respect to age, region of residence, ethnicity and maternal age, but sex, nutritional status and maternal education did not differ. We also compared the distribution of baseline characteristics for different vaccination groups (Table 1). The age of children differed by vaccination group: children with DTP>MV were older than children who received DTP before or together with MV and children without MV were younger ($p<0.0001$). Mean MUAC z-scores for all groups were around one standard deviation below the reference, but children with DTP>MV and no DTP, no MV tended to deviate more from the WHO reference curve for MUAC compared with the other groups. The distribution of vaccination groups differed by region and ethnicity. More mothers of children vaccinated out-of-sequence or with missing MV had never attended school than mothers of children vaccinated in-sequence. Children vaccinated out-of-sequence had received their most recent vaccine closer to entry in the analysis (Table 1).

Mortality by vaccination group among children aged 9-17 months

Children vaccinated out-of-sequence had higher mortality compared with children vaccinated in-sequence (MRR: 2.10 (95% CI: 1.07-4.11); DTP=MV: 2.30 (1.15-4.58) and DTP>MV: 1.45 (0.50-4.22)). Children who had received DTP, but no MV had higher mortality compared with children vaccinated in-sequence (MRR: 2.57 (1.37-4.83)). Children without DTP and MV had higher mortality than children vaccinated in-sequence (MRR: 3.04 (1.41-6.55)) (Table 2). The associations were similar for boys and girls (p=0.77). For boys, out-of-sequence vaccinations were associated with a MRR of 1.96 (0.80-4.78); for girls, the MRR was 2.25 (0.81-6.30). DTP without MV was associated with significantly higher mortality for boys (MRR: 3.41 (1.50-7.77)); mortality for girls was also higher, but not statistically significant (MRR: 1.67 (0.62-4.50)) (Table 2).

We have previously estimated a MRR of 0.48 (0.27-0.87) for MV versus no MV, without taking sequence of vaccination into consideration[11]. When we examined the NSEs of measles vaccine by comparing children MV-vaccinated in-sequence with children not MV-vaccinated, we found a MRR of 0.40 (0.23-0.69) (data not shown).

Mortality by vaccination group among children aged 18 to 35 months

Initially, mortality declined with age as expected (Figure 1). However, in spite of being older, in-sequence vaccinated children had higher mortality at 18 to 35 months of age (mortality rate (MR): 39.9 per 1000 person years (PYRS)) than children aged 9 to 17 months (MR: 32.6 per 1000 PYRS). Mortality developed differently with age for children vaccinated in-sequence compared with children vaccinated out-of-sequence (Figure 3). Since the in-sequence group had high mortality, there was no real differences in mortality between out-of-sequence and in-sequence vaccinations in the 18-35 months age group (Supplementary table 2). The MRR for out-of-sequence compared with in-sequence vaccinated children differed significantly between the age group 9-17 months (Table 2) and 18-35 months (Supplementary table 2) (test of interactions, p=0.02).

Sensitivity analyses

In the age group 9-17 months at least 20% of children vaccinated out-of-sequence received further doses of DTP during follow-up, but few children vaccinated in-sequence did (Supplementary table 3). To minimise the effect of vaccinations during follow-up, we conducted two sensitivity analyses. First, we censored follow-up 2 months after entry since few additional vaccines would be provided in that time window. This clearly restricted the power, but the trends remained the same: Out-of-sequence vaccinations were associated with a MRR of 2.51 (0.86-7.35) (Table 3). The estimates changed more for

girls; out-of-sequence vaccinations being associated with an 8-fold higher mortality for girls (MRR: 7.83 (0.90-67.83)). Second, we restricted the dataset to children who had received DTP3 and therefore were unlikely to receive additional routine DTP vaccinations during follow-up (Supplementary table 4). The MRR of out-of-sequence vaccinations compared with in-sequence vaccinations was 1.85 (0.82-4.16), and the effect was similar for boys and girls (p=0.60) (Supplementary table 4). For girls, both DTP3=MV and DTP3>MV were associated with higher mortality. For boys, DTP3=MV were associated with higher mortality, whereas DTP3>MV was not (Supplementary table 4).

Discussion

Main findings

Out-of-sequence vaccinations were associated with higher mortality compared with in-sequence vaccinations. After 18 months, the recommended age of booster DTP vaccination, the general mortality rate increased and the differential effect of out-of-sequence vaccinations disappeared.

Strengths and weaknesses

Using the landmark approach, survival bias was prevented since the vaccination status of the children was only updated when vaccination status was re-assessed, thereby preventing that vaccination information was updated for surviving children, but not for dead children. While this approach does not misclassify observation time dependent on the outcome, the misclassification of vaccinations during follow-up would yield conservative estimates.[31]

Data was collected through the rural HDSS in Guinea-Bissau and vaccination status was based on the vaccination card being inspected. Vaccinated children, whose vaccination card was not presented, were not included in the analysis. Mortality as the main outcome is unlikely to be reported wrongly, and with visits every 6 months, the imprecision in date of death is limited. Booster doses of DTP were not registered before 1996 and we could not fully explore the effect of booster DTP in the present cohort. To limit the effect of vaccinations during follow-up, we censored the main analysis at 18 months of age, when the children were eligible for the DTP booster; furthermore, we conducted two sensitivity analyses in which we first restricted follow-up to 2 months after entry and second limited the analysis to children who had received three doses of DTP. The conclusions of the main analysis were robust in these sensitivity analyses. The statistical model used, only compared children within the same village cluster, thus limiting bias from local differences such as epidemics, ethnicity, and access to health care. Comparing children across clusters did not change the conclusions (data not shown).

In spite of the careful collection of vaccination information and individual level follow-up, we cannot guarantee that observed mortality differences are caused only by the sequence of vaccinations. To limit confounding, we assessed whether available background factors changed the estimate by more than 10%. As no background factor changed the estimate by more than 10%, we did not present adjusted estimates. However, there may be residual confounding not adjusted for.

To enter the analysis, a child had to survive to have the vaccination card inspected, and a differential mortality pattern before the inspection of the vaccination card would not be captured in our analyses. However, in prior studies of vaccination sequence and mortality, the effects have been similar regardless of whether vaccinations are registered at the time of vaccinations[26 32] or later[24 27], and this is therefore unlikely to explain the pattern.

Comparison with other studies

Similar to our study, previous studies have found that out-of-sequence vaccinations are associated with increased mortality.[24-26 33-35] In the WHO-commissioned review, out-of-sequence vaccinations with DTP and MV were associated with a relative mortality risk of 2.34 (1.57-3.50) compared with MV after DTP.[7] Hence, the age group 9-17 months in the present study is entirely consistent with previous studies. Out-of-sequence vaccinations may affect not only mortality but also hospital admissions; large population-based cohort studies from Denmark found that out-of-sequence vaccinations of DTP and MV were associated with higher hospitalisation rates.[36 37] To our knowledge, no study without survival bias has found beneficial effects of out-of-sequence vaccinations with DTP and MV.

The original study assessed the effect of MV compared with no MV and found a MRR of 0.48 (0.27-0.87)[11] not accounting for sequence of vaccination. According to our analyses this has underestimated the NSEs of MV. When we considered sequence of vaccination and compared children MV-vaccinated in-sequence with children not MV-vaccinated, we found a MRR of 0.40 (0.23-0.69).

The mortality rate usually declines with age.[38] In our study, among children vaccinated in-sequence, we found higher mortality rate in children aged 18 to 35 months compared with children aged 9 to 17 months (Figure 3). Since mortality did decline with age in the younger age group, we speculate that DTP booster for which children were eligible at 18 months of age may have contributed to this pattern just like DTP out-of-sequence with MV was associated with higher mortality. Unfortunately, our data collection tool in the early 1990 did not systematically assess DTP booster coverage. According to UNICEF figures, the DTP3 coverage was low in 1991-1996 (45-74%),[39] and we would not expect the coverage of booster DTP

to be high. In urban Bissau, where the coverage for booster DTP was high, we have previously shown a similar increase in mortality after 18 months of age.[5] Thus, DTP booster doses may partly explain the higher mortality among 18-35 months old children, as observed in Gambia and India.[5 35 40]

Effects were similar for boys and girls, and overall we found no sex-differential effect of out-of-sequence vaccinations. However, other studies have found higher female mortality when DTP was administered after MV[21 40]; for example, high-titre measles vaccine (HTMV) was associated with higher female mortality and had to be withdrawn because most HTMV recipients had received DTP after MV.[28] In the present cohort, few children had received DTP after MV and most out-of-sequence vaccinations were combined administration of DTP and MV. When follow-up was limited to 2 months, estimates for out-of-sequence changed more for girls than for boys even though the difference between boys and girls did not reach statistical significance.

Interpretation and implications

We found that out-of-sequence vaccinations were associated with higher mortality both for children with co-administration of DTP and MV, and children with DTP after MV, compared with children vaccinated in-sequence. It could be speculated that out-of-sequence vaccinated children just had higher mortality because they were frail or their mothers less compliant with health services. In the present study, it speaks against the effect being due to an inherent bias that the difference disappeared completely for 18-35 months old children, possibly due to booster DTP. Furthermore, evidence from RCTs of medium and high-titre MV strongly supported that an inactivated vaccine after MV was associated with higher mortality.[28] Thus, sequence of vaccinations is likely to be important for child survival and should be considered when planning, implementing and evaluating the childhood vaccination programmes.

Current vaccination recommendations are based merely on the disease-specific effects of vaccines, often based on surrogate measures of the ability to prevent targeted infections. However, if vaccines alter the susceptibility to other infections this should be considered. Currently, vaccination programmes are evaluated based on vaccination coverage of DTP and MV at 12 months of age, and timeliness or sequence of vaccination is not taken into account. We found that DTP not succeeded by MV was associated with increased mortality and that out-of-sequence vaccinations were associated with higher mortality compared with children vaccinated in-sequence, thus, the current evaluation criteria emphasising DTP3 coverage may not optimise the impact of the vaccination programme on child health. Our results indicate that a stronger emphasis should be put on increasing the MV coverage and getting DTPs and MV in the recommended sequence.

Currently WHO is planning to introduce the second year of life platform with several inactivated vaccines (booster DTP, Meningitis A, RTS,S Malaria vaccine).[29] Hence, in the future children may receive inactivated vaccines after live MV at 9 months of age, not only because they deviate from the recommended schedule, but also if they follow the schedule. We urge others to test the effect of providing non-live vaccines after MV, preferably prior to the introduction of new vaccines, while RCTs are still possible.

Conclusion

Overall, we found that out-of-sequence vaccinations in children were associated with higher mortality compared with children vaccinated in-sequence. Vaccination programmes should monitor the sequence of vaccinations to optimise the overall effect on child survival.

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Authors' contributions

SMT, PA and ABF designed the study and planned the analyses. SMT extracted, cleaned and analysed the data. PA supervised the data collection and data entry. SMT drafted the paper with assistance from PA, AR and ABF. All authors read and approved the final manuscript.

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Competing interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Data sharing

Data used for analyses in the present study are available from the corresponding author on reasonable request.

Transparency statement

SMT affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as originally planned have been explained.

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Figure 1 Overall mortality rate among children visited between 9 and 35 months of age.

Note: The figure plots the unadjusted mortality rates for children with a vaccination card seen between 9-35 months of age. The smoothed mortality curve starts shortly before 12 months of age, where the first event occurs. Only few children contribute with observation time between 9 and 12 months.

Figure 2 Flowchart of children included and excluded from the analysis

Figure 3 Mortality rate according to sequence of DTP and MV vaccinations among children visited between 9 and 35 months of age.

Note: The figure plots unadjusted mortality rates by vaccination status (in-sequence vs out-of-sequence vaccinations) among children with a vaccination card seen between 9-35 months of age. The smoothed mortality curve starts shortly before 12 months of age, where the first event occurs. Only few children contribute with observation time between 9 and 12 months.

Table 1 - Baseline characteristics for observations of children (9-17 months old) included in the analyses by vaccination group

	DTP<MV	DTP=MV	DTP>MV	DTP, no MV	No DTP, no MV	p-value
Numbers (%)	1590 (27)	1491 (25)	493 (8)	1816 (31)	547 (9)	
Sex						.29
Male (%)	834 (52)	729 (49)	255 (52)	931 (51)	290 (53)	
Female (%)	756 (48)	762 (51)	238 (48)	885 (49)	257 (47)	
Median age in months at start of follow-up (interquartile range)	14.0 (11.8 – 15.9)	13.8 (11.9 – 16.0)	15.3 (13.6 – 16.8)	11.4 (10.0 - 13.5)	12.0 (10.4 – 14.7)	<0.0001
MUAC z-score at start of Follow-up	-0.93 (1.09)	-1.09 (1.05)	-1.16 (1.08)	-1.09 (1.13)	-1.13 (1.13)	<0.0001
Region						<0.0001
Oio	303 (19)	337 (23)	87 (18)	413 (23)	167 (31)	
Biombo	405 (25)	283 (19)	108 (22)	386 (21)	147 (27)	
Gabu	158 (10)	484 (32)	184 (37)	437 (24)	87 (16)	
Cacheu	353 (22)	125 (8)	37 (8)	226 (12)	46 (8)	
Bafata	371 (23)	262 (18)	77 (16)	354 (19)	100 (18)	
Ethnicity						<0.0001
Balanta	220 (14)	179 (12)	38 (8)	323 (18)	177 (33)	
Pepel	338 (21)	228 (16)	98 (20)	316 (18)	137 (25)	
Fula/Mandinca	703 (45)	921 (63)	296 (61)	950 (53)	183 (34)	
Manjaco	106 (7)	44 (3)	9 (2)	81 (4)	25 (5)	
Other	208 (13)	96 (7)	45 (9)	134 (7)	18 (3)	
Median maternal age in years (interquartile range)	25.6 (20.6 - 30.8)	26 (21.2 - 30.6)	25.9 (21.3 - 31)	26.2 (20.8 - 30.9)	26.8 (21.3 - 31.5)	.05
Education of caretaker						<0.0001
0 years	1290 (81)	1302 (87)	426 (86)	1562 (86)	485 (89)	
1-4 years	198 (12)	143 (10)	52 (11)	179 (10)	44 (8)	
>4 years	77 (5)	15 (1)	6 (1)	45 (2)	3 (1)	
Time since MV/ Time since DTP after MV in days	105 (52 - 169)	85 (38 - 154)	66 (30 - 108)	161 (98 - 238)	N/A	<0.0001

[1] 503 observations with missing MUAC

[2] 64 observations with missing information on ethnicity

[3] 63 observations with missing information on maternal age

[4] 110 observations with missing information on education of caretaker

Table 2 - Main analysis: Mortality of children visited between 9 and 17 months of age according to vaccination group

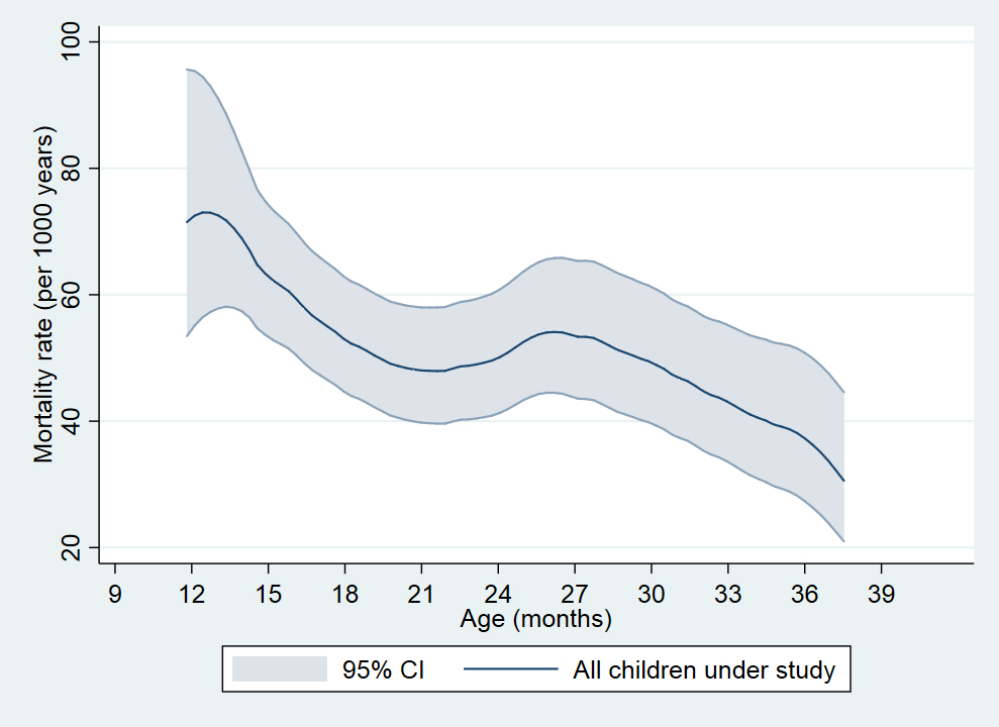
				Boys			Girls		
Vaccination status	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**
DTP < MV	1590	32.6 (16/491)	Ref	834	34.8 (9/258)	Ref	756	30.1 (7/232)	Ref
DTP = MV	1491	63.7 (29/455)	**2.30 (1.15-4.58)**	729	63.6 (14/220)	2.08 (0.83-5.26)	762	63.8 (15/235)	2.50 (0.88-7.12)
DTP > MV	493	43.1 (5/116)	1.45 (0.50-4.22)	255	51.5 (3/58)	1.48 (0.37-5.88)	238	34.6 (2/58)	1.38 (0.25-7.52)
DTP, no MV	1816	78.8 (57/723)	**2.57 (1.37-4.83)**	931	102.3 (38/372)	**3.41 (1.50-7.77)**	885	54.1 (19/351)	1.67 (0.62-4.50)
No DTP, no MV	547	111.3 (22/198)	**3.04 (1.41-6.55)**	290	95.3 (10/105)	2.77 (0.97-7.97)	257	129.5 (12/93)	**3.28 (1.06-10.12)**
Out-of-sequence vaccinations combined									
DTP>=MV	1984	59.5 (34/571)	**2.10 (1.07-4.11)**	984	61.1 (17/278)	1.96 (0.80-4.78)	1000	58.0 (17/293)	2.25 (0.81-6.30)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster.

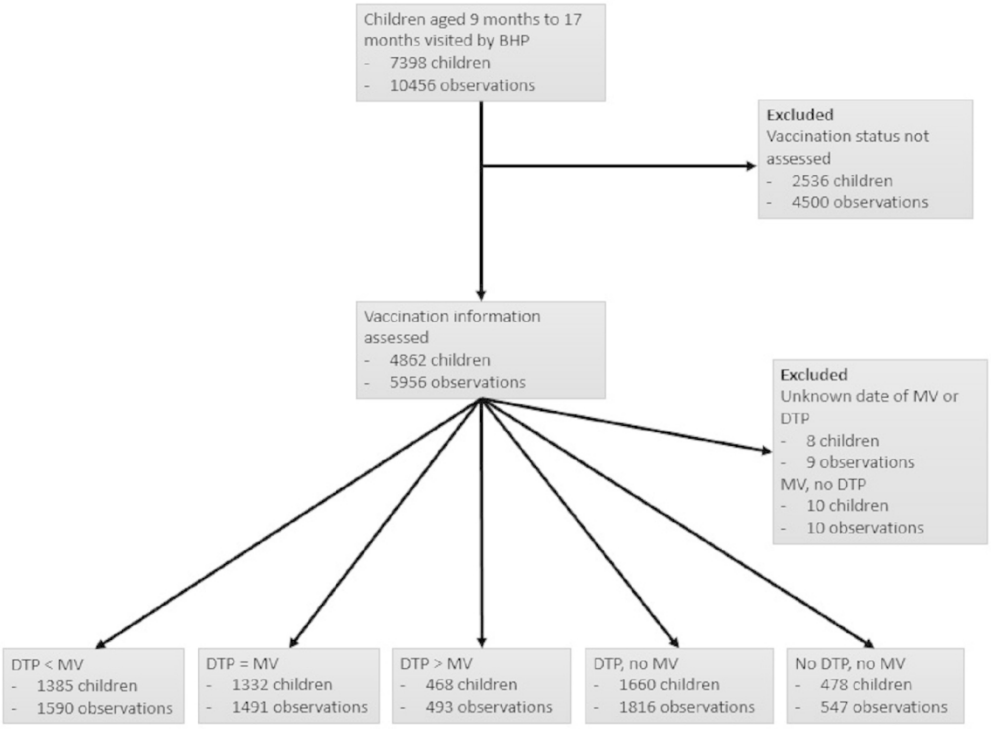
Table 3 - Mortality of children visited between 9 and 18 months of age according to vaccination group with follow up censored at 2 months after entry into the analysis

Vaccination status	Obser-vations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)	Boys			Girls		
				Obser-vations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)	Obser-vations N	MR per 1000 PYRS (deaths/PYRS)	MRR (95% CI)
DTP < MV	1590	25.8 (6/233)	Ref	834	32.9 (4/122)	Ref	756	18.0 (2/111)	Ref
DTP = MV	1491	60.5 (13/215)	2.34 (0.77-7.12)	729	38.2 (4/105)	1.16 (0.27-5.07)	762	81.8 (9/110)	6.71 (0.77-58.26)
DTP > MV	493	60.4 (4/66)	3.30 (0.77-14.14)	255	58.8 (2/34)	1.61 (0.26-10.18)	238	62.1 (2/32)	**14.61 (1.01-210.68)**
DTP, no MV	1816	93.4 (27/289)	**3.47 (1.24-9.69)**	931	127.8 (19/149)	2.56 (0.80-8.24)	885	57.0 (8/140)	6.34 (0.72-55.92)
No DTP, no MV	547	130.3 (11/84)	3.35 (1.00-11.26)	290	88.6 (4/45)	1.19 (0.24-5.90)	257	178.3 (7/39)	**14.73 (1.55-140.27)**
Out-of-sequence vaccinations combined									
DTP>=MV	1984	60.5 (17/281)	2.51 (0.86-7.35)	984	43.2 (6/139)	1.26 (0.32-4.85)	1000	77.4 (11/142)	7.83 (0.90-67.83)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster.

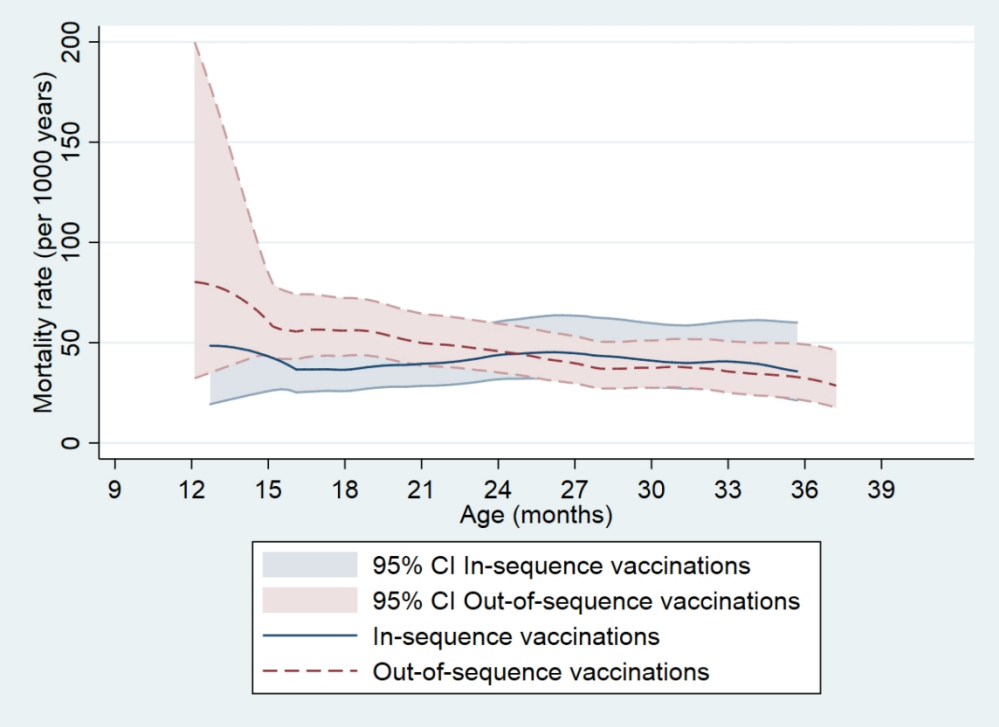


Overall mortality rate among children visited between 9 and 35 months of age.



Flowchart of children included and excluded from the analysis

127x93mm (300 x 300 DPI)



Mortality rate according to sequence of DTP and MV vaccinations among children visited between 9 and 35 months of age.

428x312mm (72 x 72 DPI)

Supplementary table 1 - Baseline characteristics among children included and excluded from the analyses

	Included	Excluded	p-value
Numbers (%)	5937 (57)	4519 (43)	
Sex			.11
Male (%)	3039 (51)	2242 (50)	
Female (%)	2898 (49)	2277 (50)	
Median age in months at start of follow-up (interquartile range)	13.1 (11.0 – 15.5)	13.4 (11.1 – 15.7)	0.0005
MUAC at start of Follow-up[1]	-1.06 (1.1)	-1.09 (1.12)	.42
Region			<0.0001
Oio	1307 (22)	969 (21)	
Biombo	1329 (22)	1380 (31)	
Gabu	1350 (23)	803 (18)	
Cacheu	787 (13)	692 (15)	
Bafata	1164 (20)	675 (15)	
Ethnicity[2]			<0.0001
Balanta	937 (16)	926 (21)	
Pepel	1117 (19)	1172 (26)	
Fula/Mandinca	3053 (52)	1728 (39)	
Manjaco	265 (5)	270 (6)	
Other	501 (9)	369 (8)	
Median maternal age in years (interquartile range)[3]	26 (20.9 - 30.8)	25.3 (20.4 - 30)	<0.0001
Education of caretaker[4]			0.95
0 years	5065 (85)	3805 (84)	
1-4 years	616 (10)	472 (10)	
>4 years	146 (2)	111 (2)	

[1] 3731 observations with missing MUAC

[2] 118 observations with missing information on ethnicity

[3] 116 observations with missing information on maternal age

[4] 241 observations with missing information on education of caretaker

Supplementary table 2 - Mortality of children visited between 18 and 35 months of age according to vaccination group

				Boys			Girls		
Vaccination status	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**
DTP < MV	2415	39.9 (46/1154)	Ref	1249	45.2 (27/598)	Ref	1166	34.1 (19/557)	Ref
DTP = MV	2065	47.0 (46/978)	0.98 (0.62-1.54)	1031	57.5 (28/487)	1.06 (0.59-1.92)	1034	36.7 (18/491)	0.85 (0.42-1.71)
DTP > MV	1940	31.3 (29/927)	0.62 (0.36-1.06)	954	41.4 (19/458)	0.83 (0.42-1.63)	986	21.3 (10/469)	**0.39 (0.16-0.94)**
DTP, no MV	580	65.9 (18/273)	1.40 (0.77-2.55)	278	76.2 (10/131)	1.39 (0.63-3.05)	302	56.3 (8/142)	1.37 (0.55-3.42)
No DTP, no MV	502	112.0 (26/232)	**2.18 (1.21-3.90)**	238	71.0 (8/113)	1.58 (0.64-3.91)	264	150.8 (18/119)	**2.68 (1.21-5.94)**
Out-of-sequence vaccinations combined									
DTP>=MV	4005	39.4 (75/1,905)	0.82 (0.54-1.24)	1985	49.7 (47/946)	0.96 (0.56-1.67)	2020	29.2 (28/960)	0.64 (0.33-1.24)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster.

Supplementary table 3 - Registered vaccinations during follow-up (FU) period among those with vaccination status assessed again

9-17 months of age **Vaccination status**	**Observations N**	**Seen card after visit**	**Vaccinated during FU**			
			DTP	**MV**	**Polio**	**BCG**
DTP < MV	1590	1427	31 (2%)	0 (0%)	51 (4%)	3 (0%)
DTP = MV	1491	1324	310 (23%)	0 (0%)	316 (24%)	5 (0%)
DTP > MV	493	433	67 (15%)	0 (0%)	73 (17%)	0 (0%)
DTP, no MV	1816	1561	569 (36%)	866 (55%)	576 (37%)	14 (1%)
No DTP, no MV	547	516	120 (23%)	102 (20%)	118 (23%)	78 (15%)
18-35 months of age **Vaccination status**						
DTP < MV	2415	2053	18 (1%)	1 (0%)	38 (2%)	0 (0%)
DTP = MV	2065	1726	150 (9%)	0 (0%)	156 (9%)	2 (0%)
DTP > MV	1940	1648	75 (5%)	0 (0%)	82 (5%)	1 (0%)
DTP, no MV	580	429	77 (18%)	104 (24%)	79 (18%)	0 (0%)
No DTP, no MV	502	462	24 (5%)	19 (4%)	23 (5%)	13 (3%)

Supplementary table 4 - Mortality of children, who had received 3 doses of DTP, visited between 9 and 17 months of age according to vaccination group

				Boys			Girls		
Vaccination status	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**	**Obser-vations N**	**MR per 1000 PYRS (deaths/PYRS)**	**MRR (95% CI)**
DTP3 < MV	1492	30.4 (14/460)	Ref	774	37.7 (9/239)	Ref	718	22.6 (5/221)	Ref
DTP3 = MV	882	49.6 (13/262)	2.07 (0.88-4.86)	422	64.5 (8/124)	1.77 (0.61-5.15)	460	36.3 (5/138)	2.64 (0.65-10.73)
DTP3 > MV	363	35.8 (3/84)	1.27 (0.33-4.81)	191	23.2 (1/43)	0.76 (0.09-6.43)	172	49.1 (2/41)	2.09 (0.34-12.86)
DTP3, no MV	634	78.4 (20/255)	**3.01 (1.42-6.34)**	334	88.1 (12/136)	2.56 (0.97-6.74)	300	67.4 (8/119)	**3.80 (1.17-12.33)**
Out-of-sequence vaccinations combined									
DTP3>=MV	1245	46.3 (16/346)	1.85 (0.82-4.16)	613	53.8 (9/167)	1.53 (0.54-4.29)	632	39.2 (7/179)	2.46 (0.67-9.09)

Note: Mortality rate ratios were calculated using Cox proportional hazards models with age as underlying time, stratified by sex and village cluster

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1	
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3	
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4-5	
Objectives	3	State specific objectives, including any prespecified hypotheses	5	
Methods				
Study design	4	Present key elements of study design early in the paper	5-6	
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5	
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	6	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case		
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*5*	
Bias	9	Describe any efforts to address potential sources of bias	7	
Study size	10	Explain how the study size was arrived at	8	

Continued on next page

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6-7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	7
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8
		(b) Give reasons for non-participation at each stage	Figure 1
		(c) Consider use of a flow diagram	Figure 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8 + table 1
		(b) Indicate number of participants with missing data for each variable of interest	Table 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	Table 2
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	*Table 2*
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8 + Table 2
		(*b*) Report category boundaries when continuous variables were categorized	Table 1
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	

Continued on next page

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9 + Table 3 + Supp tables
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	10
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11-12
Generalisability	21	Discuss the generalisability (external validity) of the study results	10-12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.