

Plasmodium falciparum Parasitaemia in Children from Different Breastfeeding Regimens Evaluated by the Deuterium Technique in Southern Benin

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Abstract

Studies on the health benefits of exclusive breastfeeding (EBF) and susceptibility to malaria have shown contradictory results. These studies often failed to account for dose response effects. The deuterium dilution technique was used to evaluate the breastmilk intake, the amount of water from sources other than human milk and the exclusivity of breastfeeding. We determine *Plasmodium falciparum* parasitaemia prevalence in children aged less than six months according to their breastfeeding regimen and maternal milk dose ingested in southern Benin. We conducted a cross sectional study in the Ouidah Kpomassè Tori-Bossito (OKT) health zone in southern Benin from February to December 2014, an intense transmission season. 115 mothers paired with their children aged from 0 to 6 months were enrolled. The daily human milk was measured through saliva of each mother and child pair (MCP) over a period of 14 days by the technique of deuterium oxide "dose to the mother" using the Fourier Transformed Infrared Spectrometer (FTIR). Malaria parasitaemia in children was determined by quantitative Polymerase Chain Reaction (qPCR) in real time at day 14 and day 28. The average age was 2.3 months. The distribution of *Plasmodium falciparum* parasitaemia in children was 0.00%, 5.08% and 3.85% respectively in exclusive, predominant and partial breastfeeding regimens. The mean intake of breast milk was 641.71 mL per day; 256.75 mL of water per day was taken from sources other than breastmilk. Adjusted linear regression analysis revealed a significant association between quantity of breastmilk and parasitaemia prevalence; infected children (qPCR positive) had 164.11 mL of ingested breastmilk per day less than children without parasitaemia ($p=0.00$). Our findings highlighted the association between the low risk of *Plasmodium falciparum* parasitaemia and the ingested breastmilk dose. Study with larger numbers of patients would be necessary to confirm this relationship.

Keywords: Breastfeeding; Deuterium oxide technique; *Plasmodium falciparum* parasitaemia; Children; Breast milk; Benin

Background

Many studies on the health benefits of breastfeeding in developing countries have shown conflicting results. Studies from Uganda and Nigeria suggested that Exclusive Breastfeeding (EBF) had no significant effect on malaria infection risk [1,2]. Some studies from Malawi and Congo, found that maternal milk or EBF protected infants from developing malaria [3,4].

However, in all studies, the classification of EBF were based on questionnaires and is subject to recall bias because it may be affected by bias on mothers self-reported behavior [5,6]. These studies often failed to account for dose-response effects [7]. The dose-to-mother

deuterium dilution technique has made it possible to distinguish between water ingested by the baby via breastfeeding and water from sources other than human milk. This study allows an objective evaluation of whether a mother is exclusively breastfeeding her infant as well as the volume of human milk and water intake. We determined *Plasmodium falciparum* parasitaemia prevalence in breastfed children aged from 0 to 6 months according to breastfeeding regimens and a relation with ingested breastmilk dose evaluated with deuterium dilution technique in southern Benin.

Materials and Methods

Study sites

A cross sectional study was performed between February to December 2014, primarily during the rainy season, in four hospitals,

namely the health centers of Ouidah, Pahou, Kindji and Tokpadomè, all located in the sanitary health zone of Ouidah Kpomassè Tori-Bossito (OKT) a mesoendemic area. Biological analyses were carried out in the Laboratory of Centre de Lutte Intégrée contre le Paludisme (CLIP) located in Cotonou.

Subjects

Sampling and inclusion criteria

The sample size has been calculated by the Schwartz formula using the prevalence of *Plasmodium falciparum* infection (7.8%) obtained in the OKT health region in children under 12 months [7] and has yielded a total of 110 children. We used this sampling size calculation for children from 0 to 6 months in this study.

During the first 2 weeks of the study, all lactating mothers attending the pediatric unit of follow-up and counselling, who agreed to sign the consent form, were included in the study. All Mother-Child Pairs (MCP) and who met the eligibility criteria were enrolled into the study.

Inclusion criteria included:

- Resided for at least 6 months in the OKT area;
- HIV negative serologic status;
- Hemoglobin level greater than 7 g/dl;
- Agreed and signed the consent form.

Training of field workers and data collection

All lactating mothers who consented and met all the inclusion criteria were recruited into the study. Demographic, epidemiological and clinical data were collected by trained nurse, pediatrician, parasitologist and nutritionist.

Each MCP responded to a questionnaire and benefited from a measurement of the anthropometric. Each child received a clinical examination with temperature and a biological check-up (qPCR and quantitative breast milk by oxyde de deuterium).

Variables studied were demographic (age and sex), nutritional (weight, height, body mass index), epidemio-clinics (a fever with a temperature above 37.5°C), taking of at least one dose of Sulfadoxine-Pyrimethamine, and use of the long-lasting insecticidal nets or LLINs.

The SP use for malaria prevention during pregnancy as intermittent preventive treatment in pregnancy (IPTp), child HIV status, and child's birthweight were recorded in the child's health record and in the maternity register of the health centers. Taking the temperature and looking for an abnormality of the physical examination was carried out respectively by nurses and a pediatrician.

Anthropometry

Measurements were made by trained personnel using standard procedures [8,9]. Wearing minimal clothes, the mothers were weighed to the nearest 10 g with an electronic scale (Seca, Hamburg, Germany). Height was measured to the nearest millimeter with gauge of Shorr. The body mass index (BMI) was calculated as weight (kg) over height squared (m²). The children were weighed without clothes using a portable electronic infant scale accurate to 5 g. Length was measured using a standardized infantometer of Shorr.

Assuming the recommended cut-offs for data exclusion, data were excluded if a child's length-for-age z-score (LAZ) was below -6 or above +6, weight-for-age z-score (WAZ) was below -6 or above +5, or weight-for length z-score (WLZ) was below -5 or above +5, as these extreme values were most likely a result of errors in measurement or data entry [10]. All children admitted for pediatric consultation, and who met the inclusion criteria, were tested for *P. falciparum* in a blood specimen that was collected and sent to the CLIP Laboratory at Cotonou.

Laboratory Examinations

Capillary blood sampling

Sample of blood in the heel of the child is extracted: one drops to perform hemoglobin test, and the remaining three drops of blood (50 µL × 3) on Whatman 3 MM filter paper. The dried blood impregnated filter papers were stored at -20°C (under conditions of good quality) in a sealed film with silica after complete air drying until DNA extraction and qPCR in real time was achieved.

Haemoglobin measurement

Finger-picked blood sample was used to measure hemoglobin using a portable spectrophotometer (Hemo-Control, EKF-Diagnostic, Germany) [11]. Individuals found with Plasmodium parasitaemia or hemoglobin level less than 7 g/dL were referred to the pediatrician for evaluation and treatment.

The dose-to-the-mother deuterium-oxide technique

The amount of breast milk consumed by the infant over a period of 14 days was evaluated using the deuterium oxide technique "dose to the mother". This method consists in giving the mother to drink deuterium oxide, then to follow its disappearance through saliva in the mother and its appearance in infants' saliva [12]. In the laboratory, a double reading by the FTIR reading was made for each saliva sample by experienced technicians of the CLIP Laboratory.

The dose-to-mother deuterium dilution technique was used to evaluate the human milk intake, the water from sources other than human milk and the exclusivity of breastfeeding [13-16]. The data about non-breast milk water intake were grouped into three regimens of breastfeeding according to the study reported [17], exclusive breastfeeding (EBF)=non-breast milk water intake <52 mL/day; predominant breastfeeding (preBF)=non-breast milk water intake=52-216 mL/day and partial breastfeeding (parBF)=non-breast milk water intake >216 mL/day).

A baseline sample of 2 mL of saliva from the mother and the child was collected on day 0, after which, the mother received an oral dose (30 g) of deuterium oxide (99.9% purity; Sercon-Cortec Cambridge Isotope Laboratories Inc, Andover, MA, USA). Further, 12 saliva samples were collected from the mother and the infant on days 1, 2, 3, 4, 13 and 14. Saliva samples were stored in ice during transport on the days of collection, brought to the laboratory and stored at -20°C until analysis. Enrichment of saliva samples was measured using a Fourier transform infrared spectrometer (IR-PRESTIGE 21; Shimadzu, Vienna, Austria) with calcium fluoride cell of 0.1 mm path length. Before measuring deuterium in saliva, the calibration procedure involved preparation of D2O calibrator by dilution of D2O with deionized

water. The infrared spectra were measured in the range 2300-2900 cm^{-1} .

Magnitude of the response obtained from the FTIR was deduced from the D2O absorption curve by an algorithm (isotope software) developed by the Medical Research Council (MRC; Human Nutrition Research, Cambridge, UK). Intake of breastmilk and water from non-milk sources was calculated by fitting the deuterium enrichment, anthropometric data, MCP ages to a model for water turnover in the mothers and infants, and the transfer of milk from mother to the infant [13,14] using an algorithm [Excel (2007) spreadsheet developed by the MRC] and clinical information.

Real-time quantitative PCR

DNA extraction was performed using Chelex 100 technique from the filter papers. Real-time duplex qPCR was performed using primers and probes specific to *Plasmodium* spp/*P. falciparum* for the gene encoding the small (18S) subunit of *Plasmodium* rRNA. This technique was used to test the 115 samples of children breastfed, as described [18]. The samples were subjected to 40 cycles of amplification in the real-time PCR system ViiA⁷ (Applied Biosystem) at the Molecular Biology Laboratory of (CLIP) in the Faculté des Sciences de la Santé in Cotonou.

A positive control signal for the human GlycerAldehyde-3-Phosphate De-Hydrogenase (GAPDH) gene was amplified for all samples (control of good performance of DNA extraction from the filter paper). To declare that a sample was *P. falciparum* infection positive, the Ct should be: Plasmoprobe <40 cycles, *Plasmodium falciparum* <37 cycles, Ct GAPDH <40 cycles, the Ct of *P. falciparum* 3D7<23 and the negative control must be undetectable.

The parasites were quantified using a standard range of DNA made from an *in vitro* culture suspension of a *P. falciparum* 3D7 line offered by IRD/NIH: (*P. falciparum* 3D7 GL, MRA-1001) filed by Megan Dowler, (Walter Reed Army Institute of Research). Children testing

positive were traced and treated according to national guidelines or referred to a local health facility.

Data interpretation and analysis

P. falciparum infection was defined as positivity of Qpcr (Ct<37). For an episode of malaria, there is more a fever. The use of LLIN is verified by the nurse who had the contact of each CMPs. The use of SP is verified in the register of hospital. The HIV status of each child was sought in the registry. Data were handled and analyzed using Excel software and STATA version 12 (Stata Corp, College Station, TX, USA).

Descriptive analyses were done by determining means, and standard deviations. Variables studied were demographic (age and sex), nutritional (weight, height, body mass index), epidemio-clinics (a fever with a temperature above 37.5°C, taking at least one dose of Sulphadoxine-Pyrimethamine or SP, the use of the long-lasting insecticidal nets or LLIN, positivity of qPCR). For quantitative variables, averages and standard deviations were calculated. $p < 0.05$ is considered statistically significant.

Ethics statement

The Ethical Committee of the Faculté des Sciences de la Santé gave the ethical approval N°0103/2014/CE/FSS/UAC du03/03/2014 for the study. The study was approved by the health district of OKT authorities. Written informed consent was obtained from the mother of all the children enrolled in the study.

Results

A total of 115 pairs of mothers and child were recruited. The average age of the mothers was 26.9 years, with values going from 18 to 40. Their average weight and height were respectively 61.5 kg and 161 cm.

Mothers Characteristics	Mean \pm SD or n (%)
Age (years)	26.9 \pm 5.4
Weight (kg)	61.3 \pm 13.6
Height (cm)	160.1 \pm 6.3
BMI (kg/m ²)	23.9 \pm 4.9
Education level	
Never attended	32 (27.8)
Elementary school	44 (38.3)
Middle school or more	39 (33.9)
IPTp-SP one dose use (%)	113 (98.3)
Infants Characteristics	
Age (months)	2.3 \pm 1.6
Weight (kg)	5.0 \pm 1.4
Height (cm)	57.1 \pm 5.4

Gender ratio (Male/Female)	59/56
LLINS use (%)	114 (99.1)
n: number, %: percentage, sd: standard deviation	

Table 1: Characteristics of mothers and children.

The average Body Mass Index (BMI) was 23.9 kg/m² with measurements between normal values 18.5 and 25. 66.09% of the women had at most the elementary school education. 99.1% used the long lasting insecticide treated nets (LLINs) and 98.3% benefited from Intermittent Preventive Treatment in pregnancy (IPTp) by using at least one dose of sulfadoxine-pyrimethamine (SP). The children had an average age of 2.3 months. The gender ratio was balanced (59 boys and 56 girls), and their weight was 5.0 kg in average as shown in Table 1.

Plasmodium falciparum parasitaemia prevalences

The distribution of *Plasmodium falciparum* parasitaemia according the breastfeeding practices is presented in Table 2.

Breastfeeding regimen	N	n	Prévalence (%)
Exclusive	4	0	0
Prédominant	59	3	5.08
Partial	52	2	3.85
Total	115	5	4.35

N: number examined, n: number of parasitaemia positive, %: prevalence of parasitaemia

Table 2: *Plasmodium falciparum* parasitaemia in different breastfeeding regimens at OKT health area.

Of the 115 children examined, 5 were infected with *Plasmodium falciparum* identified by qPCR, giving a global prevalence of 4.35% with 0% in EBF regimen, 5.08% (3/59) in PreEBF regimen, and 3.85% (2/52) in ParEBF regimen.

Breast Milk and Non-Breast Milk Water

Age (month)	N	Maternal milk (mL /day)	Non maternal water (mL /day)
0-3	71	587.68	223
3-6	44	729.05	308.70

N: number of children; CI: Confidence Interval

Table 3: Quantities of maternal milk and non-breast water ingested by children according to age range.

Table 3 shows the average quantity of breast milk and water absorbed by the child. The average amount of breastmilk and water ingested by children from 0 to 3 month were respectively 587.68 and 729.05 ml per day.

Relation between the quantity of milk taken and *Plasmodium falciparum*

Table 4 presents the relation between the quantity of milk taken and *Plasmodium falciparum* parasitaemia adjusted to age. There is a statistically significant difference between the average quantity of breastmilk taken by the infected (*Plasmodium falciparum* parasitaemia with qPCR positive) children and children without parasitaemia.

The infected children have taken about 164.11 mL of breastmilk less than the children who were not infected. Children of age of 3 months or less have taken an average 140.9 mL of breastmilk less than the children who are older than 3 months.

	Quantity of maternal milk			
	n	Coefficient	CI 95%	p value
qPCR				
negative	110	0	-	
positive	5	-164.11	-234.45; -93.76	0.000
Age				
≥3 months	44	0	-	
<3 months	71	-140.88	-214.87; -66.89	0.000

CI : Confidence interval

Table 4: Relation between quantity of breastmilk intake and *Plasmodium falciparum* parasitaemia (qPCR positive) adjusted on age: a linear regression.

Relation between *Plasmodium falciparum* parasitaemia and breastfeeding regimens

Table 5 describe the relation between *Plasmodium falciparum* parasitaemia prevalence and the type of breastfeeding regimen, adjusted on age, mother's parity and IPTp used and LLIN.

<i>Plasmodium falciparum</i> parasitaemia		
Exclusive and predominant breastfeeding	Odds Ratio (CI 95%)	p value
No	1	
Yes	1.44 (0.11-17.58)	0.772
Age		
≥ 3 months	1	
<3 months	2.47 (0.18- 33.62)	0.496
Parity		

<2	1	
≥ 2	0.49 (0.042- 5.73)	0.573
IPTp one dose		
No	1	
Yes	0.034 (0.00- 1.05)	0.05
LLINs		
No	1	
Yes	0.017(0.00- 0.60)	0.04
CI=Confident interval		

Table 5: Relation between *Plasmodium falciparum* parasitaemia and breastfeeding regimens adjusted to others variables (age, parity, IPTp, LLINs): a logistic regression.

There is statistical significant with a low risk of *Plasmodium falciparum* when the mother had IPTp and used LLIN. Maternal parity was not significantly associated to malaria infection in this study.

Discussion

The aim of our study was to determine *Plasmodium falciparum* parasitaemia prevalence in children aged less than six month according to their breastfeeding regimen and the link between maternal milk dose ingested and *Plasmodium falciparum* parasitaemia in southern Benin. With the quantitative techniques used, we found a low *Plasmodium falciparum* parasitaemia prevalence and a significant relation between *Plasmodium falciparum* parasitaemia and the amount of milk ingested in our study. No study carried out *in vivo* had previously suggested a relationship between both.

In our study, the average age of infants was 2.3 months, similar to the Medoua research in 2007 which studied infants aged between 1.15 and 4.5 months with an average age of 3 months [12]. In Haisma study's, infants were 4 months old and an average age of 2.1 months [17]. In a study conducted in Cameroon, however, the population of children was 65.9% female. Beninese mothers had a normal average body mass index (BMI) (23.9 kg/m²). During pregnancy, they had taken at least one dose of SP, which would reduce serious placental parasitism. The use of LLIN was high during the study (92%) different from the frequency observed in the study conducted by combien in the OKT zone [8].

The overall prevalence of malaria among the 115 infants aged less than 6 months who were breastfed in our study was 4.35%. It is lower than that found by Brazeau in the Congo children (10.5%). It is necessary to stipulate that the techniques used have precisions. The deuterium oxide "dose to the mother" allowed us to determine the amount of breastmilk and the amount of fluid consumed from sources other than breastmilk, which makes it possible to define in a very precise way the breastfeeding regimens. These quantitative results are not affected by recalls, found during mothers' declarations [5,6]. This technique classify breastfeeding regimens into exclusive, predominant and partial types.

However, the classification of the type of breastfeeding using the deuterium oxide technique depends on the volume of non-dairy water consumed by the infant. This parameter should be standardized and

associated with the WHO classification of breastfeeding regimen [17]. We also identified the *Plasmodium falciparum* parasitaemia prevalence by qPCR. It appeared in our study that only 3.48% of mothers breastfed their babies exclusively. The majority was classified as mainly predominant (51.30%) or partial (45.22%).

Regarding the relationship between breastfeeding and *Plasmodium falciparum* parasitaemia there was no significant association. In fact, exclusive breastfeeding is not an essential method of providing protection [18]. In fact, it is possibly due to a dose-response effect [7]. By examining the relationship between the amount of milk ingested and malaria infection, the risk of malaria infection increased when the amount of breast milk decreased significantly (p=0.00, CI-234.45-93.76). The protection against malaria, therefore, depends on the amount of milk ingested by the infant. According to Lartey study, infants who consume a significant amount of non-dairy water and a large amount of food other than breast milk are more susceptible to infections [19].

The relationship between malaria infection and breastfeeding regimen adjusted for age, parity, IPT and LLIN covariates shows that IPTp (p=0.05) and LLIN (p=0.04) were significant a) with a reduced risk for the child to have *Plasmodium falciparum* parasitaemia when the mother has received at least one dose of SP during IPT or has slept under LLIN.

Appropriate statistical methods were used to account for the cross-sectional nature of the data. Our study reveals a potential protective effect of breast milk on *Plasmodium falciparum* parasitaemia. Predominant breastfeeding supplemented with small amounts of water, porridge or herbal tea does not eliminate the protection offered by breast milk.

Obviously, this is not meant to discourage exclusive breastfeeding, but in our society where the mother had to take on many responsibilities in addition to feeding their babies, exclusive breastfeeding is not always practiced. Finally, breastfeeding does not obey to the law of "all or nothing". The more breastmilk an infant receives in the first six months of life, the better protected he will be.

The limits of our study are related to the size of our sample. Due to financial limitations, the size of the sample could not be increased. Thus, breastfeeding practices reflect the inherent differences between mothers. Finally, the sample size of this study did not allow us to examine the effect of exclusive breastfeeding regimen alone because the size of the population of this group does not allow such a study. We therefore, grouped the exclusive and predominant breastfeeding group during the analysis. Exclusive breastfeeding may provide breastmilk protection against malaria because of the duration of breastfeeding and the amount of milk ingested by the infant in exclusive regimen.

Conclusion

The prevalence global prevalence of *Plasmodium falciparum* parasitaemia was 4.35%. Our findings suggest that exclusive breastfeeding may reduce the risk of *P. falciparum* in southern Bénin, in addition to the use of LLIN in young infants and IPTp in mothers. Study with larger numbers of patients, conducted for a longer time would be necessary to confirm this relationship between malaria and quantity of ingested breastmilk.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Contributions

All authors participated in the design and planning of the study. YSSDT, AOH, JMA, AWH, GD, AM, DK, PB, AA, CF, DA, KK, CA, NGM participated to the collect of data in the field and laboratory work. YSSDT, GC and LA analysed data. All authors reviewed the manuscript and approved the final version.

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