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Non-invasive detection of *Helicobacter pylori* virulence genotypes *ureA, vacA, cagA* and *babA2* among asymptomatic Egyptian infants

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Helicobacter pylori **is a microaerophilic spiral-shaped Gram-negative bacterium that infects approximately 50% of the world's population, particularly in developing countries. Infections early in childhood are postulated to induce a low-grade chronic inflammatory condition. This study aimed to determine the prevalence of *H. pylori* virulence genotypes *ureA, vacA, cagA* and *babA2* among asymptomatic Egyptian infants and to define the possible infection associated risk factors. Non invasive test using polymerase chain reaction on stool samples was used for detection of these genes. Prevalence of *H. pylori* among those infants was 88.9%. Prevalence of *ureA, vacA, cagA* and *babA2* was 86.9, 98.8, 71.4 and 67.8%, respectively. Risk factors significantly associated with infection included bed sharing, premastication of food and nursery attendance (P<0.005). The prevalence of *H. pylori* infection among Egyptian infants is very high with high prevalence of virulence genotypes, so follow up of these infants and repetition of this study on a wider scale is recommended.**

Key words: *Helicobacter pylori*, virulence, Egypt, infants.

INTRODUCTION

Helicobacter pylori (H. pylori) is a microaerophilic motile Gram-negative spiral-shaped bacterium (Wen and Moss, 2009). Approximately, 50% of the world's population is infected with the organism (Torres et al., 2000). Poor socio-economic conditions were found to be associated with early colonization in children. High prevalence of *H. pylori* among people in low-income countries has been demonstrated by several studies (Hestvik et al., 2010). Socio-economic status includes not only income but also living conditions, sanitation and educational level (Khalifa et al., 2010). Fayoum Governorate, where this study was based, is a low socioeconomic area located in Upper Egypt. It is surrounded by many villages with limited services especially in the health sector. Total population of Fayoum Governorate is about 3 millions.

H. pylori causes chronic infection of the stomach for almost the entire lifetime of the individual. Chronic gastritis and peptic ulcers or gastric carcinoma can be associated

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with chronic *H. pylori* infection (Frenck and Clemens, 2003). These long term complications associated with *H. pylori* infection varies between developed and developing countries. The age at which an individual is infected with *H. pylori* may play a role in this difference (Khalifa et al., 2010).

Early infections in childhood are postulated to induce a low-grade chronic inflammatory condition which may develop into pre-malignant changes and eventually gastric carcinoma. In contrast, when infection is acquired later in life, it is more likely to induce a brief inflammatory response. Although, the prevalence of *H. pylori* infection in Egyptian population is very high and acquired early in life, the prevalence of gastric cancer in Egypt is very low ($3.4/10^5$) (Hussein, 2010).

Of those infected, disease development is influenced by *H. pylori* strain virulence. Bacterial virulence is the ability of some bacteria to cause disease. The major *H. pylori* virulence factors include the vacuolating toxin VacA, the blood group antigen binding adhesin (BabA) and the cag pathogenicity island (cagPAI). The cagPAI is a cluster of genes with seven of these genes, *virB4, virB7, virB8, virB9, virB10, virB11*, and *virD4*, homologous to the gene components of the type IV secretion system (T4SS) of *Agrobacterium tumefaciens* (Censini et al., 1996).

The cytotoxin associated gene A (cagA), being a marker for the presence of the cag pathogenicity island (cagPAI), was the first recognized virulence gene in the *H. pylori* genome. It is present in 60-70% of *H. pylori* strains and encodes a high molecular weight antigenic protein (120-140 kDa) (Atherton, 1998; Queiroz et al., 2000).

Vacuolating cytotoxin, VacA, induces cytoplasmic vacuolation in cultured epithelial cells. Unlike the cagPAI, the *vacA* gene is present in almost all strains. The vacuolating cytotoxin A (*vacA*) gene exists in different subtypes, varying in the signal (*s1 or s2*), the intermediate (i) and the middle (*m1 or m2*) regions. Polymorphisms among the VacA alleles result in different levels of cytotoxicity. All possible combinations from these regions have been identified and among them, vacuolating activity is highest in *s1m1* strains, less in *s1m2* strains and is absent in *H. pylori* expressing *s2m2* forms (Ko et al., 2008).

The blood group antigen binding adhesin (BabA), encoded by the *babA2* gene, binds to Lewis b antigens and ABO antigen. There are two distinct *babA* alleles (babA1 and babA2). Only the *babA2* allele is functionally active (Ilver et al., 1998).

The combined presence of *babA, cagA* and *vacAs1* ‘‘triple-positive strains”, was reported to be associated with duodenal ulcer and gastric adenocarcinoma in Western populations (Zambon et al., 2003).

Virulence-associated gene, *ureA,* is one of the genes of the urease operon encoding the urease subunit A (Blom et al., 2000). The *ureA* is widely used for identifying *H. pylori* by PCR (Clayton et al., 1992; Espinoza et al., 2011). Other targets of PCR amplification methods include the 26-kDa species-specific antigen (*SSA*) gene and the phosphosamine mutase (*glmM*) gene (Smith et al., 2004). Other noninvasive *H. pylori* diagnostic tests include: culture, the ^{13}C urea breath test, antigen enzyme immunoassay (EIA) for detection of *H. pylori* in faeces and ELISA serology (Monteiro et al., 2001).

This study aimed to determine the prevalence of *H. pylori* virulence genotypes *ureA, vacA, cagA* and *babA2* among asymptomatic Egyptian infants using stool samples as a non invasive screening test. Determination of the possible infection associated risk factors was also an objective of the study.

MATERIALS AND METHODS

This is a cross-sectional study of virulent *H. pylori* prevalence in asymptomatic Egyptian infants in Fayoum Governorate, a low socio-economic governorate. Virulence genes of *H. pylori* were detected by polymerase chain reaction of stool samples, as a non invasive test.

Egyptian infants included in the study should fulfilled the following criteria (a) aged ≤24months (b) asymptomatic regarding gastro-intestinal (GIT) symptoms (c) no antibiotics received within the 2 months before stool collections.

One hundred and eighty nine infants, with an average age (16.1 m±5.6) were included in the study. This cohort included 126 males (66.66%) and 63 females (33.33%). Before sample collection, verbal consent was taken from the parents. A detailed history and physical examination was done for each infant included in the study.

Specimen collection and DNA extraction

Stool, as a noninvasive sample, was used for screening. Stool samples were kept in buffered phosphate saline (BPS) at -20°C until DNA extraction to keep DNA intact as long as possible. DNA was extracted from stool samples by the EZ-10 Spin Column Soil, fecal samples DNA Mini-Preps Kit (Biobasic, Canada) following the manufacturer’s instructions. Extracted DNA was stored at -20°C until use.

The *ureA, cagA, vacA and babA2* genotyping

Stool samples were collected from 189 asymptomatic infants. Detection of *H. pylori* disease-causing genotypes was performed on all samples by PCR using 5 primer sets to amplify *ureA, cagA,* vacA and babA2 specific bands. Oligonucleotide primers used in the amplifications were obtained from Biobasic, Canada. In all cases, separate PCR reactions were performed for detection of each gene. A final reaction volume of 25 µl was used. Reaction mixture contained 12.5 uL, 2X ready to use PCR master mix (PCR-EZ D-PCR Master Mix, Biobasic, Canada) consisted of 1 Unit of Taq Polymerase, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris HCl (pH 8.75), 0.1% Triton X-100, 0.1 mg/ml BSA, 2 mM $MgCl_2$ and 200 mM dNTPs. Also, 0.6 mM sense and antisense primers, 100 ng genomic DNA and a PCR grade water was added upto the final reaction volume. Used primers and its cycling conditions are shown in Table 1.

Statistical analysis

Collected data were computerized and analyzed using Statistical Package for Social Science (SPSS) version 16. Descriptive statistics

Table 1. Oligonucleotide primers used for the amplification of *ureA, cagA, vacA and babA2* genes and its cycling conditions.

Primer designation	Sequence (5'-3')	PCR product size (bp)	Cycle Conditions*	Reference
ureA F ureA R	GCCAATGGTAAATTAGTT CTCCTTAATTGTTTTTAC	411	(35 cycles) 94°C, 1 min; 45°C, 1 min; 72°C, 1 min	Clayton et al. (1992)
cagA F cagA R	GATAACAGGCAAGCT TTTGA CTGCAAAAGATTGTTTGGCAGA	349	(35 cycles) 94℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min,	Atherton et al. (1995)
vacAs F vacAs R	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAA	s1-259 s2-286	(35 cycles) 94℃ for 1 min, 52℃ for 1 min and 72℃ for 1 min,	Atherton et al. (1995)
vacAm F vacAm R	CAATCTGTCCAATCAAGCGAG GCGTCAAAATAATTCCAAGG	m1-567 m2-642	(40 cycles) 94℃ for 1 min, 60℃ for 1 min and 72℃ for 1 min,	Torres et al. (2009)
babA2 F babA2 R	AATCCAAAAAGGAGAAAAAGTATGAAA TGTTAGTGATTTCGGTGTAGGACA	832	(40 cycles) 94℃for 1 min, 60℃ for 1 min and 72℃for 1 min,	Mizushima et al. (2001)

*The PCR had an initial step at 94℃ for 1 min, a final extension at 72℃ for 5 min. A thermal Cycler (Bio-Rad, USA) was used. PCR products were analyzed on 1.5% agarose gel electrophoresis with ethidium bromide.

Table 2. Prevalence of *H. pylori* according to age groups (<12 m and ≥12m).

Case	Age (months)		Total	P value
	<12 m	≥12 m		
Negative	2 (3.8%)	19 (11.9%)	21 (11.1%)	0.162
Positive	50 (96.2%)	118 (88.1%)	168 (88.9%)	
Total	52 (100%)	137 (100%)	189 (100%)	

were used to describe variables; percent, proportion for qualitative variables. Mean, standard deviation and range were used to describe quantitative variables. Chi-square test was done for comparing qualitative variables between groups. P values of less than 5% were considered statistically significant.

RESULTS

The study included 189 asymptomatic Egyptian infants. Sixty three were females and 126 were males (females: males = 1:2). Age range was 6-24 months (16.1 m±5.6). Overall prevalence of *H. pylori* was 88.9% (168/189). No significant difference in prevalence between boys and girls was found (P= 0.999). We examined the effect of age on *H. pylori* infection by dividing the children into 2 groups: those <12 months, and ≥12 months. We found no significant difference in the prevalence between these age groups (P= 0.162). Data are shown in Table 2.

Risk factors assessment

Bed sharing, premastication of food and nursery attendance were found to be significantly associated with *H. pylori* infection (P<0.005). Breast feeding didn't appear to be protective, but bottle feeding and usage of cow milk as a feeding supplement appeared to be significantly associated with infection (P=0.01). No significant association was observed with order between siblings, number of siblings, bath sharing, sewage disposal and clean water supply (P>0.05). A summary of results pertaining risk factors positively associated with infection is presented in Table 3.

Prevalence of *ureA, cagA, vacA and babA2* in positive samples

The prevalence of *H. pylori* in the stool samples tested was 88.9% (168/189) of which 41.7% were triple positive *"cagA, vacAs1 and babA2"*. The prevalence of virulence genes *ureA, cagA, vacA* and *babA2* in the positive stool samples for *H. pylori* is presented in Table 4.

Genes were identified according to sizes of PCR product bands in agarose gel. The ureA gene, visualized as 411 bp band, was detected in 86.9% of cases. Amplified *cagA* gene was visualized as a band of 349 bp in 71.4% of the positive strains and *vacA* subtypes were detected in 98.8% of strains. The *babA2* gene was visualized as a band of 832 bp in 67.8% of the positive strains.

The most virulent *vacAs1* genotype was the predominant

Table 3. A summary of results pertaining risk factors positively associated with infection.

Parameter	Positive		Negative		P value	Odds ratio or (CI 95%)
	Yes	No	Yes	No		
Bed sharing	106 (56.1%)	62 (32.8%)	3 (1.6%)	18 (9.5%)	0.000	13.68 (1.19-1.4)
Nursery attendance	84 (44.45%)	84(44.45%)	0	21 (11.1%)	0.000	2.0 (1.72-2.33)
Premastication of food	38 (20.1%)	130 (68.8%)	0	21 (11.1%)	0.000	1.29 (1.19-1.4)
Breast feeding	98(51.9%)	73 (38.6%)	8(4.2%)	10(5.3%)	0.000	1.4 (1.2-1.6)

Table 4. Prevalence of disease causing *H. pylori* genotypes.

Parameter	Positive	Negative	Total
ureA	146 (86.9%)	22 (13.1%)	168 (100%)
vacA	166 (98.8%)	2 (1.2%)	168 (100%)
cagA	120 (71.4%)	48 (28.6%)	168 (100%)
babA2	114 (67.8%)	54 (32.2%)	168 (100%)

Table 5. Relationship between *vacA* subtypes, *cagA, ureA and babA2* genes.

Parameter	*cagA*			*ureA*			*babA2*			
vacA	**positive**	**negative**	**P value**	**positive**	**negative**	**P value**	**positive**	**negative**	**P value**	**Total**
vacAs1m1	38	10	0.047	46	2	0.007	28	20	0.369	48 (28.9%)
vacAs1m2	64	20	0.009	78	6	0.000	68	16	0.000	84 (50.6%)
vacAs2m1	4	0	0.29	4	0	0.29	2	2	0.699	4 (2.4%)
vacAs2m2	18	12	0.24	22	8	0.14	22	8	0.341	30 (18.1%)

Significant association between *ureA* and *cagA* (p=.000) and between *ureA* and *babA2* (p=.002) was found. Relationship between *ureA, cagA and babA2* is shown in Table 6.

Table 6. Relationship between *ureA, cagA and babA2.*

Parameter		*cagA*			*babA2*		
		Positive	**Negative**	**P value**	**Positive**	**Positive**	**P value**
ureA	Positive	114	32	0.00	98	48	0.002
	Negative	6	34		16	24	

genotype in *H. pylori* isolates, and was visualized as a band of 259 bp on agarose gel electrophoresis in 79.5% of positive cases, whereas 20.5% of isolates had the *vacAs2* genotype. The middle *(m2)* region of the *vacA* gene predominated in positive samples (68.7%), while *m2* genotypes were 31.3%. On the other hand, *s1m2* genotype was the most common combination of the *vacA* subtypes in the current study. Prevalence of other *vacA* subtypes and its distribution among *ureA, cagA and babA2* positive and negative cases is summarized in Table 5. A significant association between *ureA, cagA* and *vacAs1m1, vacAs1m2* genotypes was found ($p<0.05$) (Table 5). An association between babA2 and vacAs1m2 was also found (Table 6).

DISCUSSION

To our knowledge, this is the first study to evaluate the prevalence of disease associated virulent *H. pylori* genotypes among asymptomatic Egyptian infants in Fayoum Governorate, a rural low socioeconomic area with restricted economical activities, limited social and medical services. In this study, a non-invasive assay for screening and early detection of *H. pylori* disease-associated genotypes in stool samples was used as recommended by Sicinschi et al (2012), who used PCR for amplification of *H. pylori* virulence genes from stool DNA. The study included 189 infants (63 females and 126 males) aged 16.1 m±5.6 with overall prevalence of 88.9%.

In the current study, no significant difference was found in prevalence between males and females. This supports results obtained by meta-analysis of 10 studies conducted over the last 20 years which found no sex difference regarding prevalence of *H. pylori* among children (de Martel and Parsonnet, 2006). Higher prevalence in males or in females was reported in different other studies (Klein et al., 1994; Ndip et al., 2004; Dube et al., 2009).

An association was found between nursery attendance and *H. pylori* infection in the current study, which can be explained by crowding, lack of proper hygiene and may be mix of infant feeding utensils. Another significant association between *H. pylori* infection and bed sharing was found in this study ($p < 0.05$) and this was in concordance with reports from the developing world; Nairobi, China and Bangladesh (Brown et al., 2002; Langat et al., 2006) as well as the developed world, USA (Staat et al., 1996), as direct transmission through over-crowding was suggested as a mode of transmission of *H. pylori* infection.

A strong association between poverty-related factors and increased risk of acquiring *H. pylori* has been demonstrated by different earlier studies (Malaty et al., 1996; Khalifa et al., 2010). This can explain the high prevalence of infection in the studied community.

Also, pre-mastication of food, which is a habit of many Egyptian mothers, has been found in both Bangladesh and Ethiopia as well as in the current study to be associated with an increased prevalence of *H. pylori* in babies, supporting the possibility of oral-oral transmission (Lindkvist et al., 1998).

Another cause of oral-oral transmission is maternal infection, as an infected mother may play a key role in the transmission of *H. pylori* within the family (Drumm et al., 1990). The high prevalence of *H. pylori* infection in asymptomatic infants can be explained by the high prevalence of *H. pylori* infection in their mothers (Bassily et al., 1999). Bassily et al. (1999), found that 82% of the children born to mothers infected with *H. pylori* also became infected compared to 14% infection in children of non-infected mothers. Ceylan et al. (2007), observed that *H. pylori*-associated infection was (69.2%) and (8%) among mothers in the *H. pylori*-infected and non-infected groups, respectively ($p<0.0001$). Transmission may occur by using common spoons, the licking of teats of feeding bottles, or for chewing or tasting children's food (Rothenbacher et al., 1999).

Although breast feeding has been shown to decrease the risk of infections in infants, especially faeco-orally transmitted infection, no protective effect of breast feeding was reported in this study. Findings of this study agree with other studies that were unable to demonstrate a protective effect of breast-feeding and one study even found that breast feeding increased the risk of childhood infection (Kitagawa et al., 2001; Dore et al., 2002; Rothenbacher et al., 2002). A study performed at USA found that breast-feeding play a protective role against acquisition of *H. pylori* (Malaty et al., 2001). Generally, the relationship between breast-feeding and infection is difficult to be realized as breast-feeding is almost present in the developing world until at least the first year of baby's life.

Contaminated food or water sources have been recognized as important risks for *H. pylori* infection especially in the developing world (Lu et al., 2002). Surprisingly and despite the high prevalence of *H. pylori* infection, all infants included in this study lived in houses with a clean water supply. Lu and colleagues, (2002), findings were in concordance with these findings. These unexpected finding was thought to be due to sub-optimal water treatment or breaks in the municipal pipes allowing for surface contamination of the water. Literature presented some cases, mainly in developing countries, where monitoring showed failures to establish the safety of the water consumed with presence of contaminating microorganisms in drinking water. This was because of limited resources and sanitation standards (Emiliano and André, 2012).

Previous studies on Egyptian children with age ranging from 6 months to school age reported that *H. pylori* prevalence ranged from 15-75.38% (Bassily et al., 1999; Naficy et al., 2000; Mohammad et al., 2007). In most cases this was lower than findings of this study. This discrepancy between results could be due to the difference in studied group ages economic standards or in testing methodology. Also, in Turkey different results were found with respect to the prevalence of *H. pylori* among different pediatric age groups (Us and Hasçelik, 1998; Selimoğlu et al., 2002).

Lower infection rates among Egyptian adults compared to infants can be explained by the results of many studies which found that infection among infants and young children can be transient. In a serology study of Egyptian children between 6 and 36 months of age during the period of observation, 42% of the children had sero-reversion between the first and second blood test, suggesting a spontaneous clearance of the infection (Naficy et al., 2000). Similar findings were reported in Peru, which found an overall prevalence decreased from 71.4% to 47.9% when children were between 6 and 18 months of age (Klein et al., 1994). Thus, while *H. pylori* infections in children appears to have repeated cycles of acquiring and losing the infection until the infection eventually becomes chronic, in adults it is chronic (Goodman et al*.,* 2005).

This decrease in the prevalence with age (Rothenbacher et al., 2002; Broussard et al., 2009), suggested spontaneous eradication, better attention to health issues in older children, or use of antibiotics for other common diseases (Malaty et al., 2002; Rothenbacher et al., 2002). Another explanation of this finding could be an increasing in antibody production with increasing age that may lead to decline of the prevalence rate in older children (Rothenbacher et al., 2002). Other suggested explanations for this decrease in prevalence are differences in types of

H. pylori in adults compared to children and differences in special gastric receptors (Granstrom et al., 1997).

The high prevalence among asymptomatic infants observed in this study (88.9%) parallels that reported in asymptomatic Colombian children (80.2%) (Sicinschi et al., 2012) and an overall prevalence of 86.8% in asymptomatic subjects in South Africa (Dube et al., 2009).

Findings of this study are comparable to findings from other African and Asian countries in which prevalence of *H. pylori* infection ranged from 37.5 to 74.6% (Klein et al., 1994; Kawasaki et al., 1998; Rahman et al., 1998; Thomas et al., 1999; Wizla-Derambure et al., 2001; Hoang et al., 2005; Langat et al., 2006; Hestvik et al., 2010). The reason for the discrepancy between the results is not certain but is likely multi-factorial, including different study methodology, as well as host and environmental factors.

Very few studies have researched the prevalence of virulent *H. pylori* genotypes among Egyptian population and no studies have researched this among asymptomatic Egyptian infants or children. Although, it has been reported that the sensitivity and specificity of *ureA* is more than 90% (Sugimoto et al., 2009), the *ureA* gene was detected in 86.9% of positive cases in this study. This is consistent with results reported by Lu et al. 2002, who found that the sensitivity of *ureA* gene PCR was unsatisfactory and only 75% of specimens were amplified. This low sensitivity may be due to sequence polymorphism.

In the current study, the *cagA* gene was found in 120 of the 168 positive cases (71.4%). No previous trials to study the prevalence of cagA in Egyptian children or infants were done. The prevalence of *cagA* in Egyptian adults, according to earlier studies, ranged from (11.1 - 89%) depending on the clinical presentation of *H. pylori* (van Doorn et al., 1998; Said Essa et al., 2008). The prevalence of the *cagA* gene in children among European countries varies from 22.4 to 76% (Karhukorpi et al., 2000; Oleastro et al., 2003).

The vacA genes were detected in 166/168 (98.8%) of the infected cases, the most predominant type was *s1m2* (50.6 %). The *vacA s1m1, s2m1, and s2m2* genotypes were found in 28.9%, 2.4%, and 18.1%, respectively. It has been demonstrated that the geographic distribution for *vacA* alleles differs in many countries around the world (van Doorn et al., 1998). The *vacA s1m1, s1m2*, and *s2m2* genotypes were found in 34.7%, 57.1%, and 8.2%, respectively with no *s2m1* genotype was detected, in Turkey (Ozbey et al., 2013). The current data is consistent with the results reported in Poland (Maciorkowska et al., 2007) and Shanghai (Zhou et al., 2010) where the *s1m2* was the most prevalent genotype. In contrast, other predominant *vacA* genotypes were reported in Brazil, Slovenia, the Mid-western United States *(s1m1),* and Spain *(s2m2)* (Podzorski et al., 2003; Homan et al., 2009; Agudo et al., 2010; Garcia et al., 2010).

The *babA2* gene was detected in 114/168 (67.8%) of samples. This rate agrees with results obtained from Brazil, and Bulgaria but was higher than that obtained in Portugal and United States (Oleastro et al., 2003; Podzorski et al., 2003; Garcia et al., 2010; Boyanova et al., 2011). The fact that *H. pylori* strains exhibit different patterns of adherence to gastric mucosa cells in adults and children can be an explanation of the low prevalence of *babA2* in children (Blom et al., 2000).

Prevalence of *H. pylori*, as a human pathogen, in the developing world is high. As an objective of this study; prevalence of *H. pylori* virulence genotypes among asymptomatic Egyptian infants (a poorly studied group) was researched. Also, associated risk factors especially those related to human poverty were studied. The high prevalence of infection reported by the current study was alarming. With the high Egyptian birth rate, decline of economic resources after the Egyptian revolution health awareness among many Egyptian mothers, the prevalence of this infection may increase in the future with a possibility of more serious complications. So, more attention towards Egyptian infants' healthcare, improving standards of livings and maternal health education is required.

Conclusion

The prevalence of virulent strains of *H. pylori* among Egyptian infants, based on non-invasive test, was found to be very high in the current study. High prevalence of disease associated genotypes was detected with 41.7% of positive cases were triple positive for *cagA, vacAs1 and babA2*. Bed sharing, premastication of food, bottle feeding, cow milk feeding and nursery attendance were found to increase risk of *H. pylori* infection. Health education and environmental sanitation are recommended to lower prevalence *H. pylori* infection in developing countries. Follow up of those infants is recommended to study the dynamics and complications of infection at this early age.

Conflict of Interests

The authors have not declared any conflict of interests.

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