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Prevalence and Distribution of *Clostridioides difficile* among Breastfed Infants in Lagos State, Nigeria

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ABSTRACT

Clostridioides difficile colonization is common in infancy, yet factors influencing early-life carriage remain controversial, particularly in low and middle-income settings where epidemiological data are limited. This cross-sectional epidemiological study investigated the carriage rate of *C. difficile* among infants in four Local Government Areas of Lagos State, Nigeria, and evaluated the association between mode of feeding and colonization. A total of 134 infants aged between one day and 12 months were enrolled. Stool samples were processed for the isolation of *C. difficile* using alcohol shock treatment, selective and non-selective anaerobic culture, and isolates were identified by conventional biochemical testing, API 20A, and latex agglutination. Overall, *C. difficile* was isolated from 53 infants, giving a carriage rate of 39.6% (95% CI: 31.9–48.7%). Carriage varied by location, with the highest rate observed in Mushin (55.0%; 95% CI: 33.2–76.8%) and the lowest in Ikeja (30.2%; 95% CI: 16.6–43.8%). Exclusively breastfed infants had a significantly lower carriage rate (28.3%; 95% CI: 17.0–39.6%) compared with infants who were not exclusively breastfed (48.6%; 95% CI: 37.2–60.0%) ($p < 0.05$). Although colonization was frequent, no evidence of clinically significant disease was observed. These findings indicate that *C. difficile* colonization is common among infants in Lagos and that exclusive breastfeeding is associated with reduced carriage, supporting ongoing public health advocacy for exclusive breastfeeding in early life.

Keywords: *Clostridioides difficile,* breastfeeding, Infants, Carriage Rate, API 20A, Latex Agglutination, Lagos State.

Introduction

Clostridioides difficile (formerly *Clostridium difficile*) is a Gram-positive, spore-forming obligate anaerobe (Giles and Roberts, 2022) that colonizes the gastrointestinal tract and is a major cause of antibiotic-associated diarrhoea and pseudomembranous colitis. Studies by Cui *et al.,* (2021) and Tougas *et al.,* (2021) demonstrated high carriage rates in healthy neonates, suggesting that the organism could form part of the normal intestinal flora. Subsequent studies reported widely variable colonization rates in infants, ranging from 15% to over 60% depending on age, setting, and methodology (Drall *et al.*, 2019; Mahnic *et al*., 2025). Environmental acquisition, particularly in hospital settings, was later shown to be more important than maternal transmission (Mirowska *et al*., 2023; Monnier *et al*., 2026). Diet has been proposed as a modifying factor in infant colonization.

Some studies reported lower carriage rates among exclusively breastfed infants compared with formula-fed infants (Spigaglia, 2024; Fontaine *et al*., 2025), whereas others found no significant association (Nakagawa *et al.*, 1982). These inconsistencies likely reflect differences in study populations, environmental exposure, and microbiota development.

Although infants frequently harbour toxigenic strains without symptoms (Welch and Marks, 1982; Richardson *et al*., 1983; Matsuki *et al*., 2005), the long-held belief that *C. difficile* lacks pathogenicity in early life has been challenged. Studies have documented antibiotic-associated and *C. difficile*-associated diarrhoea (CDAD) in neonates and young children (Hyams et al., 1984; Karsch et al., 1989), with reported prevalence decreasing with age (Turck *et al*., 2003; Lacey *et al*., 2007).

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More recent epidemiological data suggest that while asymptomatic carriage remains common in infants, clinically significant infection can occur, particularly following antibiotic exposure (Sammons *et al*., 2013; Centers for Disease Control and Prevention , 2019). Age appears to play a critical role, as the intestinal microbiota does not reach adult-level complexity until approximately two years of age, potentially influencing susceptibility to colonization and disease (Milani *et al*., 2017; Nicholson *et al.*, 2026; Ricci *et al.*, 2026). High background rates of asymptomatic carriage in infancy complicate interpretation of positive toxin or culture results, making careful clinical correlation essential.

This study therefore aimed to determine the carriage rate of *Clostridioides difficile* among infants in four Local Government Areas of Lagos, to compare carriage between breastfed and formula-fed infants, and to assess the association between diet and colonization.

Materials and Methods

Study Design

This study employed a laboratory-based cross-sectional design to evaluate method of feeding among infants. Samples were collected in primary health care centres in four randomly selected Local Governments in Lagos metropolis, Nigeria, including Ikeja, Agege, Mushin and Surulere. The study was explained to the parents or guardians of the children and upon obtaining their consent, were given sterile stool containers for faecal sample collection at their convenience. The age range of these children was between a day and 1 year. Information of the patients (registered infants for the study) was obtained through structured questionnaires which included age, sex, social status of parents, antibiotic history, and method of feeding.

Ethical Committee Approval

The research protocol, informed consent documents, and all related study materials were reviewed and approved by the Health Research Ethics Committee of Lagos University Teaching Hospital, Lagos, Nigeria (Ref: ADM/DCST/221), prior to the commencement of the study.

Reference Strains: The reference strains used in this study were *Clostridium difficile* strains: ATCC 9689 and *Pseudomonas aeruginosa:* ATCC 27853.

Culture Media

The media used for the study included: non selective media, fastidious anaerobe agar (FAA) (Lab M, UK), the selective and differential medium - cycloserine cefoxitin fructose agar (CCFA) (Oxoid, UK & BioMerieux, France), Robertson cooked meat medium, Wilkins Chalgren broth and agar (Oxoid, UK), Blood agar (Oxoid, UK), EMueller-Hinton agar (Oxoid, UK), Brain Heart Infusion broth (Oxoid, UK) Nutrient agar (Oxoid, UK).

Growth Condition and Storage

All incubations were carried out at 37ºC unless otherwise stated. Isolates were stored in Brain Heart Infusion Broth containing (BHIB) 20% glycerol at -80^{0}C and in Robertson cooked meat medium at room temperature.

Collection and Processing of Samples

Faecal samples were collected in sterile universal bottles and kept for not more than 18 h or refrigerated duringdelays. Direct plating of each specimen was done on fastidious anaerobe agar (FAA) which is a non-selective agar and cycloserine cefoxitin fructose agar (CCFA) which is a selective and differential medium for *C. difficile* (George *et al*., 1979) to obtain single colonies. All samples were then subjected to "alcohol shock" (Borriello and Honour 1981) by homogenizing the faecal specimens with an equal volume of 95% ethanol at room temperature for 1 hour to destroy all vegetative cells leaving only spores. They were then placed into bottles containing Robertson Cooked Meat Broth (RCMB) for enrichment and the lid tightly shut. All plates were incubated anaerobically in anaerobic jars containing gas kit (Oxoid, U.K/ BioMerieux, France) (Table 1). Made up of nitrogen (80%), carbon dioxide (10%) and hydrogen (10%) at 37ºC for 48 h. *C. difficile* control strain (ATCC 9689) and *Pseudomonas aeruginosa* control strain (ATCC 27853) were incubated along with the specimens. Anaerobiosis was monitored with *Pseudomonas aeruginosa* and resazurin indicator strips (Oxiod, UK/ BioMerieux, France).

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Clostridioides difficile Screening Test: Methylene Blue Stain for Faecal Leucocytes

One drop of fresh (<3 h since collection) stool was mixed with an equal proportion of Loeffler's methylene blue stain (0.2% methylene blue). The suspension was examined under high-dry power (HPF) (X100) for the presence of polymorphonuclear leukocytes (WBCs). Positive results were analyzed as either ≥1or ≥5 WBC/HPF (Guerrant *et al*., 1992).

Identification of _Clostridioides difficile_ isolates

In order to confirm purity and identity of *C. difficile*, isolates in Robertson Cooked Meat Broth (RCMB) were sub-cultured onto cycloserine cefoxitin fructose agar (CCFA) and incubated anaerobically at 37°C for 48 h. Isolates showing yellow fluorescence under ultraviolet light at a wave length 365nm were sub-cultured onto blood agar and identified on the basis of characteristic colony morphology, typical odour, production of acid or gas or both from glucose, fructose, dextrose, galactose, mannose, mannitol and xylose. Ability to ferment maltose, lactose, soluble starch, sorbitol and sucrose. Ability to Produce lipase, lecithinase, catalase, and indole; hydrolyse aesculin, gelatin and starch (Sagar, 2022). Species identity was confirmed using Gram stain, biochemical identification system API 20A (BioMerieux, France) and a latex agglutination test kit (Oxoid, UK) (Bowman *et al*., 1992).

Aero-tolerance Testing

Clostridium difficile suspected isolates were sub-cultured on chocolate agar and incubated at 37°C for 24 h aerobically (5%) to rule out possible contamination by facultative faecal flora. Any growth seen after 24 h incubation was not *Clostridium difficile* strain and hence discarded.

Biochemical Test

The identification of isolates was by conventional methods as described below and API 20A method (BioMerieux, France) was employed for isolates confirmation (Bowman *et al*., 1992).

Sugar Fermentation Test

These were performed according to the method of Phillips (1976). The following sugars were used: Glucose, dextrose, fructose, mannitol, xylose, mannose, sucrose, lactose, maltose, sorbitol and galactose.

Procedure

One ml of a 20% solution of each sugar was pipetted onto dried carbohydrate free medium plates. The plates were dried in an incubator at 37°C for 30 min. Each plate was then divided into six sections using a sterile disposable scalpel.

Using sterile swabs, five isolates were spot inoculated (each spot about 10 mm) on five segments leaving one as control. All plates were incubated in an anaerobic jar (Oxoid, UK) at 37°C for 48 h in an anaerobic atmosphere. An agar plug was removed from the centre of growth of each strain and placed in a labelled microtitre well. Two drops of bromophenol blue were added to each well as an indicator. A colour change from blue to yellow or yellow green was considered positive while no colour denotes a negative reaction.

Esculin Hydrolysis

Principle

The esculin molecule is hydrolysed to glucose and esculetin by a bacterial enzyme. Released esculetin then reacts with iron salt (ferric ammonium citrate) to form a dark brown or black complex, indicating a positive result.

Procedure

One ml of 20% aesculin was pipetted onto a dried agar plate and isolates were spot inoculated and grown as previously described. Plates were viewed under long wave ultraviolet light (365nm) after 48 h incubation. A blackening of the medium around and beneath the growth was indicative of aesculin hydrolysis while no colour development was considered negative.

Starch Hydrolysis

Isolates were grown on a starch medium at 37°C for 48 h under anaerobic conditions. Aliquots (2-3mls) of Gram's Iodine were added and observed immediately. No colour change around the colonies indicated a positive reaction. A blue-black discolouration of the medium was a negative result (Sagar, 2022).

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Gelatin Hydrolysis

Isolates were grown on gelatin plates under anaerobic conditions for 48 h. A drop of 17% mercuric chloride in 1 N hydrochloric acid (HCl) was pipetted onto each plate in order to detect gelatinase production. A clearing of the medium around or beneath the growth is indicative of positive result and no clearing was a negative result (Sagar, 2022).

Lecithinase and Lipase Production

Lecithinase Principle

Lecithinase mediates the breakdown of lecithin to diglyceride and phosphorylcholine. On egg yolk medium (EYA), the formation of insoluble diglyceride causes a visible opacity around the colony.

Procedure

Isolates were grown on Egg yolk medium at 37°C for 48 h under anaerobic conditions. Opacity of agar surrounding colonies due to precipitation of complex fats was indicative of lecithinase reaction. No reaction on the agar was a negative result (Sagar, 2022).

Lipase Principle

Bacterial liapases hydrolyze the breakdown of triglycerides into glycerol and free fatty acids. Fatty acids are mostly insoluble and cause opacity on EYA, producing an iridescent sheen on the colonies and surface of EYA. Unlike lecithinase, lipase is not diffusible, and the reaction occurs only on the surface of the agar in the immediate vicinity of the colony

Procedure

Isolates were grown on Egg yolk medium at 37°C for 48 h under anaerobic conditions. An iridescent sheen on the surface of bacterial growth and on the agar surface around the colonies and no reaction on the agar indicated a negative reaction (Sagar, 2022).

Spot Indole Test

Each colony of isolate was taken from a 48 h culture and smeared on a water moistened filter paper. A drop of para-dimethylaminocinnamaaldehyde was added to the smear and allowed to react for 1 min before the results were read. The development of a dark pink to red colour on the disc in which the inoculum was placed indicated a positive reaction and no colour change signified no reaction (Sagar, 2022).

Catalase Production Test

A colony of each isolate was taken from a 48 h culture with a wooden stick and smeared on a clean grease-free slide. A drop of 15% hydrogen peroxide (v/v) was then placed on the smear. The development of effervescence was an indicative of a positive reaction and no effervescence, negative reaction (Sagar, 2022).

Procedure for API 20A Method of Identification

Each isolate obtained after 48 h incubation at 37°C on FAA was suspended into an ampoule of API medium to obtain a final turbidity of 3 McFarland. Sterile pipette was used to dispense the suspension into each cupule of the strip provided by the manufacturer. The Indole (IND) microtube was slightly under filled and overlaid with mineral oil. The strips were then sealed with adhesive tape at both ends, bent so as to fit into the anaerobic jar and incubated anaerobically at 37°C for 48h. For viability and purity of the isolates, two sets of strains inoculated on FAA were incubated anaerobically and aerobically each. After incubation, required reagents were added according to the manufacturer's directions and the reactions recorded on the numerical identification sheet. The numerical codes obtained were then compared with the API Index Table.

Rapid Confirmatory Latex Agglutination test

Latex agglutination Test was used to further confirm *C. difficile* isolates from selective CCFA media. The test kit [Oxoid *C. difficile* Test Kit (DR1107A)] was allowed to reach room temperature before the commencement of the test. A drop of normal saline was dropped within one circle on the reaction card placed on the work bench. *C. difficile* isolates were emulsified in the drop of saline to produce a heavy smooth suspension. Latex reagent was mixed with *C. difficile* suspension using a clean mixing stick for 30 s and any agglutination observed within 2 min was recorded as positive. Positive and negative controls were run concurrently with the test.

Data Analysis

For epidemiological study, data was analyzed by using SPPS statistical package (version 15, Chicago 2006).

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Stepwise Multiple logistic regression using chi square (χ^2) was employed to analyze the variables at 95% confidence interval and to show the relationship between one respondent (CDAD) and several independent variables. Probability value ≤ 0.05 was considered statistically significant.

Results

The occurrence of *Clostridioides difficile* among infants was analyzed in relation to breastfeeding status across the four Local Government Areas (LGAs). A total of 134 infants were studied for the carriage of *Clostridioides difficile* (Table 1). Of these, 60 infants were exclusively breastfed, 2 were exclusively formula-fed, and 72 infants received a combination of breast milk and formula. The carriage of *C. difficile* was found to vary according to feeding method. *C. difficile* carriage was higher among infants who were not exclusively breastfed compared to those who were exclusively breastfed (Table 2). Among the breastfed infants 17 were positive for *C. difficile*, giving an overall carriage rate of 28.3%. In contrast, among the infants who were not exclusively breastfed, 36 out of 74 infants sampled were positive for *C. difficile*, resulting in a significantly higher carriage rate of 48.6%. When analyzed by location, the carriage rate among breastfed infants was 23.5% in Ikeja (4/17), 42.9% in Mushin (3/7), 23.8% in Agege (5/21), and 33.3% in Surulere (5/15).

Among infants who were not exclusively breastfed, the carriage rates were higher across all locations: 34.6% in Ikeja (9/26), 61.6% in Mushin (8/13), 52.9% in Agege (9/17), and 55.6% in Surulere (10/18).

Overall, the results indicate that infants who were not exclusively breastfed had a markedly higher carriage rate of *C. difficile* compared to exclusively breastfed infants across all the study locations. The difference in carriage rates between exclusively breastfed and non-exclusively breastfed infants was statistically significant, indicating a strong association between breastfeeding and reduced *C. difficile* colonization in infants in Lagos, Nigeria. These findings suggest that breastfeeding may play a protective role against colonization by *C. difficile* in infants.

Table 1: Distribution of *Clostridioides difficile* Carriage in Infants by Local Government Area or Location

Location	Number of infants sampled	Carriage (%)
Ikeja	43	13(30.2)
Mushin	20	11(55.0)
Agege	38	14(36.8)
Surulere	33	15(45.5)
Total	134	53(39.6)

Table 2: Correlation between breastfeeding and *C. difficile* occurrence in the four Locations (LGA)

Location	Breastfed Infants			Not breastfed Infants		
	Number of infants sampled	Number of infants with *C. difficile*	Carriage rate (%)	Number of infants sampled	Number of infants with *C. difficile*	Carriage rate (%)
Ikeja	17	4	23.5	26	9	34.6
Mushin	7	3	42.9	13	8	61.6
Agege	21	5	23.8	17	9	52.9
Surulere	15	5	33.3	18	10	55.6
Total	60	17	28.3	74	36	48.6

Significant difference between the carriage rates of exclusive breastfed not exclusive breastfed. $P < 0.05$

Discussion

This present study has revealed the carriage rate of *Clostridioides difficile* among infants in four (Ikeja, Mushin, Agege and Surulere) Local Government Areas of Lagos. The study successfully compared carriage between breastfed and formula-fed infants and assessed the association between diet and colonization. In this study, *C. difficile* was not isolated from any newborn.

However, varied carriage rates of between 2 to 75% of *C. difficile* in neonates and infants have been recorded by different investigators in Western countries

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(Boenning et al., 1982; Al-Jumail et al., 1984; Bacon et al., 1988; El-Mohandes et al., 1993; Matsuki et al., 2005; Lacey et al., 2007). Recent studies have also confirmed that *C. difficile* colonization is common in early infancy and is influenced by gut microbiota composition, feeding practices, antibiotic exposure, and environmental factors (Jolivet et al., 2023; Spigaglia, 2024). Surprisingly, no isolate was obtained from any newborn in this study. The reason for this is unknown, but a common practice was observed in all the Primary Health Centres whereby all the neonates were exclusively breastfed and were also given ampiclox (ampicillin and cloxacillin) at birth.

The breast milk of the mothers could have protective effects on the neonates from being colonised by *C. difficile* as demonstrated by previous studies where breastfed children had *C. difficile* colonization less frequently than formula-fed children (Tullus et al., 1989). Recent studies have also confirmed that breastfeeding promotes the growth of beneficial gut microbiota such as *Bifidobacterium* and *Lactobacillus*, which inhibit colonization and toxin production by *C. difficile* (Ho et al., 2022; Spigaglia, 2024). Breast milk also contains immunological components such as secretory IgA, lactoferrin and human milk oligosaccharides which help protect infants against gastrointestinal pathogens and influence early gut microbial colonization (Stewart et al., 2022). The correlation between the use of ampiclox and colonization resistance in this study is unknown. Ampiclox use has been reported to be a risk factor for development of CDAD; however, neonates were given this agent and yet were not colonised by *C. difficile*, calling for further investigation.

It has been reported that breastfed children have *C. difficile* colonization less frequently than formula-fed children. Tullus et al. (1989) found 39% carriage among formula-fed children and 19% among breastfed children less than 6 months. This observation is consistent with recent studies which have shown that feeding practices significantly influence the composition of the infant gut microbiota and susceptibility to colonization by *C. difficile* (Ho et al., 2022; Jolivet et al., 2023). In this study, only 2 infants were formula-fed and the two (100%) were positive for *C. difficile* while 18 out of 64 (28.1%) infants who were exclusively breastfed and 37 out of 70 (52.8%) who received a combination of breastfeeding and formula-feeding yielded *C. difficile*. This supports the documented evidence that breastfeeding is not only beneficial for normal growth and infant development but also provides protection against a number of infections through modulation of gut microbiota (Wilson and Colquhoun, 1998; Engevik et al. 2021).

This study shows that there is a high colonization rate of *C. difficile* among the different age groups especially in infants and adults in all the study groups. These colonized patients showed significantly low risk or no risk at all for *C. difficile* associated disease, suggesting a protective or preventive effect from such colonization. Earlier studies suggested that colonization with non-toxigenic strains of *C. difficile* may prevent disease caused by toxigenic strains (Wilson and Sheagren, 1983; Borriello and Barclay, 1985). Recent studies have supported this observation and reported that colonization with non-toxigenic *C. difficile* strains may provide colonization resistance against toxigenic strains and reduce the risk of infection (Gerding et al., 2018; Khanna et al., 2022).

Use of antibiotics in more non-colonized patients than colonized patients could have accounted for these findings, but it was observed that a higher proportion of colonized patients in each study group received antibiotics. Antibiotic exposure is known to be the most important risk factor for *C. difficile* colonization and infection because antibiotics disrupt the normal gut microbiota and allow *C. difficile* to proliferate (Smits et al., 2016; McDonald et al., 2022; Johnson et al., 2023). This observation raises the possibility of deliberate colonization of patients at risk of CDAD with non-toxigenic (and presumably harmless) strains of *C. difficile* to prevent CDAD caused by toxigenic *C. difficile* in the environment.

The study shows that there seems to be a relationship between diet and *C. difficile* colonization. Infants exclusively breastfed are less colonized than those breastfed in combination with formula. Finally, *C. difficile* is not a major factor in acute diarrhoea in children in the Lagos Metropolis, Nigeria.

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This observation is consistent with recent reports indicating that *C. difficile* colonization in infants is common but rarely associated with clinical disease due to developmental and microbiological factors in early life (Jolivet et al., 2023; Spigaglia, 2024).

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