Epidemiological Features of *Clostridium difficile* Colonizing the Intestine of Jordanian Infants

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**1. Introduction**

*Clostridium difficile* is a major cause of nosocomial antibiotic-associated diarrhea due to the production of toxins A and B. *C. difficile* infection (CDI) can result in asymptomatic carriage, mild diarrhea, or pseudomembranous colitis (PMC). CDI can be associated with significant morbidity, mortality, and healthcare costs in hospitalized patients [1, 2]. Increased incidence of CDI is coupled with more serious clinical presentation; especially mortality among patients ranges from 24 to 50% [3–5].

The rate of *C. difficile* colonization among newborns varies widely (2.5–90%) [1], mainly during the first 2 years of life and usually in an asymptomatic manner [6, 7]. A recent study from USA reported increased rate of pediatric CDI-related hospitalization over the period 1997–2006 [8]. Several hypotheses have been reported to explain asymptomatic state of *C. difficile* in newborns. These include the competitive intestinal colonization by nontoxigenic strains, the immaturity of the immune system, possible absence of toxin receptors in the intestinal tract, modulation of toxin production by the infant microbiota, and toxin neutralization by maternal antibodies [6, 9].

Transmission of *C. difficile* occurs primarily in healthcare facilities, where exposure to antimicrobial drugs and environmental contamination by its spores are causing mostly nosocomial infection [10, 11].

This study investigated the occurrence rate of *C. difficile* intestinal colonization and most important epidemiological factors associated with its presence in intestine of Jordanian infants.

**2. Patients and Methods**

2.1. **Study Population.** This prospective convenience sampling study was conducted at the Pediatric Department/Jordan University Hospital, Amman, Jordan.
Table 1: Demographic characteristics of 287 infants with positive and negative C. difficile culture.

| Variables                  | Number (%) of positive C. difficile | Number (%) of negative C. difficile | P value |
|----------------------------|-------------------------------------|-------------------------------------|---------|
| **Age by group**           |                                     |                                     |         |
| 1 day–≤30 days             | 15 (8.7)                            | 157 (91.3)                          | 0.050   |
| >1 month–≤1 year           | 22 (19.5)                           | 93 (80.5)                           |         |
| **Gender**                 |                                     |                                     |         |
| Male                       | 18 (11.9)                           | 133 (88.1)                          | 0.605   |
| Female                     | 19 (14.0)                           | 117 (86.0)                          |         |
| **Hospital ward**          |                                     |                                     |         |
| NICU*                      | 0 (0.0)                             | 50 (100)                            | 0.001   |
| OPD                        | 37 (15.6)                           | 200 (84.4)                          |         |
| **Antibiotics treatment**  |                                     |                                     |         |
| Yes                        | 10 (10.5)                           | 85 (89.5)                           | 0.400   |
| No                         | 27 (14.1)                           | 165 (85.9)                          |         |
| **Presence of diarrhea**   |                                     |                                     |         |
| Yes                        | 3 (23.1)                            | 10 (76.9)                           | 0.262   |
| No                         | 34 (12.4)                           | 240 (87.6)                          |         |
| **Hospital length of stay**|                                     |                                     |         |
| 1–7 days                   | 14 (14.1)                           | 85 (85.9)                           | 0.062   |
| 8–30 days                  | 1 (2.0)                             | 49 (98.0)                           |         |
| >30 days                   | 2 (15.4)                            | 11 (84.6)                           |         |
| **Gestational age**        |                                     |                                     |         |
| <32                        | 0 (0.0)                             | 12 (100)                            |         |
| 32–36                      | 9 (14.3)                            | 54 (85.7)                           | 0.420   |
| 37–39                      | 16 (11.6)                           | 122 (88.4)                          |         |
| >39                        | 12 (16.2)                           | 62 (83.8)                           |         |
| **Birth weight**           |                                     |                                     |         |
| ≤2500                      | 16 (16.2)                           | 83 (83.8)                           | 0.230   |
| >2500                      | 21 (11.2)                           | 167 (88.8)                          |         |
| **Mode of delivery**       |                                     |                                     |         |
| Normal vaginal delivery    | 20 (13.8)                           | 125 (86.2)                          | 0.654   |
| Cesarean section           | 17 (12.0)                           | 125 (88.0)                          |         |
| **Type of feeding**        |                                     |                                     |         |
| Breast                     | 4 (5.1)                             | 74 (94.9)                           | 0.012   |
| Formula and mix            | 33 (15.8)                           | 176 (84.2)                          |         |

*Included 30 newborns which were admitted to neonatal intensive care unit (NICU). ** Mostly soft stools without any clinically significant gastrointestinal symptoms or diarrhea during the collection of the specimens.

University Hospital (JUH) over a period of 8 months from March 2015 through October 2015. The study was approved by the School of Medicine and the School of Graduate studies at The University of Jordan. A total of 287 stool fecal samples were obtained from 151-male and 136-female infants aged one year or less.

Biographical and clinical data which were obtained from each infant included age, gender, name, duration of hospitalization, presence of diarrhea, hospital length of stay, mode of delivery, type of feeding, and antibiotics treatment at the time of sampling (Table 1).

2.2. Ethical Permission. A permission was also obtained from the Ethical Review Board (ERB) at the Jordan University Hospital (JUH), permission number 75/2015. Verbal consent was obtained from all mothers of infants after explaining the aim of the study.

2.3. Culture and Identification of C. difficile. One fecal sample from each patient was collected during investigation in outpatients’ clinic or directly after admission to hospital, using sterile prewetted cotton swabs in 0.85% normal saline. All fecal specimens were sent within 2-3 hrs to research microbiology labs. The specimens were first treated by absolute ethanol (v/v) for 1 hr before inoculation into Clostridium difficile moxalactam-norfloxacin agar plates (CDMN, Oxoid, England) which was supplemented with 7% (v/v) defibrinated horse blood. Culture plates were incubated for 48 hours at 37°C under anaerobic condition, and a control reference strain of C. difficile (NCTC 11204) was included. All suspected
colonies resemble C. difficile in their appearance which were used for Gram and spore stains and later confirmed by Remel RapID ANA II system (Remel Inc., Lenexa, KS, USA). All C. difficile isolates were frozen at −70°C in brain-heart infusion broth (BHIB) with 20% glycerol for further antimicrobial susceptibility test and characterization of their potential toxins genes.

2.4. Antimicrobial Susceptibility Testing. All C. difficile isolates were tested using E-tests (Oxoid, England) for vancomycin, metronidazole, ciprofloxacin, and erythromycin according to guidelines of CLSI (2015) [12]. A quality control C. difficile strain (NCTC 11204) was included.

2.5. Molecular Methods. C. difficile DNA was prepared using a single colony by boiling at 95°C for 10 min in water bath. The identity of C. difficile isolates was confirmed by amplification of the 16S rRNA gene using C. difficile specific primers (PG48 and B) [13]. PCR reactions were used for the detection of C. difficile toxin genes A and B (TcdA and TcdB) and to detect genes encoding the enzymatic (cdtA) and binding (cdtB) components of the binary toxin as described by Terhes et al. [14]. Mutation detection in gyrA and gyrB genes was carried out using PCR as reported by Dridi et al. [15].

2.6. Statistics. All data analyses were carried out using SPSS version 20. χ²-test was used for statistical analysis. P ≤ 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of Infants. Table 1 shows important characters from all examined infants with positive or negative C. difficile fecal isolates according to their age group, admission to intensive care unit (INCU) or outpatients department (OPD), sex, diarrhea, duration of hospitalization, type of feeding, birth weight (BWT), mode of delivery, and current antibiotic treatment.

3.2. Detection of C. difficile. A total of 37/287 (12.9%) of C. difficile isolates were recovered from infants aged ≤1 year, of these 20/37 (54.1%) were toxigenic strains. All isolates were confirmed using 16S rRNA gene-targeted PCR.

3.3. Detection of C. difficile Toxigenic Genes. Table 2 shows that 13/37 (35.1%) carried both genes of toxin A (TcdA) and B (TcdB), whereas 17/37 (45.9%) of isolates were negative for toxin A and B genes. Only one isolate (2.7%) was positive for binary toxin genes (TcdA and TcdB) (Table 2). Successful sequencing (99%) of the cdtA and cdtB genes was used to confirm the identity of one isolate that was positive for the binary toxin genes (Macrogen, Korea). The sequence reads were aligned manually using the online software NCBI BLAST.

3.4. Antimicrobial Susceptibility. All C. difficile isolates were 100% susceptible for both vancomycin and metronidazole as shown by their MICs breakpoint, whereas the MICs for ciprofloxacin and erythromycin showed that 29/37 (78.4%) and 11/37 (29.7%) of the isolates were resistant, respectively. Fluoroquinolone resistance-determining mutated gene (gyrA and gyrB) were present in 15/37 (40.5%) among the isolates (Table 3).

4. Discussion

This study demonstrates relatively low colonization rate of C. difficile (12.9%) in stools of hospitalized and nonhospitalized infants aged ≤1 year. Toxigenic C. difficile accounted for 54.1% of the isolates (Tables 1 and 2). A prospective study performed in France over 18-month period has found that the C. difficile colonization of French infants aged between 0 to 2 years was 33.7%, and the colonization rate by a toxigenic strain was 71% which is slightly less than that in our study [9]. A recent study from USA reported that the rate of pediatric CDI-related hospitalizations increased from 7.2 to 12.8% from 1997 to 2006; the lowest rate was observed in newborns (0.5%), while incidence for children aged <1 year and those aged 5–9 years were 32% [8]. A study in Japan reported that the carriage rate of toxigenic C. difficile in neonates was very low (2.5%) but was increased to 84.4% in infants under 2 years of age [16].

The reason why the incidence rates of C. difficile among infants differ widely between countries remains questionable. However, the rate of colonization in infants may be due to the low capacity of the infant gut flora to suppress growth of C. difficile or due to the absence of toxin receptors in the infantile gut mucosa [17]. Additionally, infants are more frequently colonized than adults, but they rarely develop C. difficile disease during the first year of their life [18–20].

The present study shows that infants receiving formula mixed milk were significantly more associated (P < 0.012) with C. difficile colonization than breast milk (Table 1). This finding was similar to other recent studies [6, 7, 19]. Otherwise, this study showed that there was no significant correlation (P > 0.05) between C. difficile colonization and certain neonatal conditions related to gender, gestational age, birth weight, mode of delivery, hospital length of stay, presence of soft stools, and antibiotic treatment.

In Jordan, most previous studies on C. difficile infection and colonization have involved mostly adult patients. A study performed at the Jordan University Hospital in 2007 found 13.7% prevalence rate of toxigenic C. difficile isolates among adult hospitalized patients as proved by the presence of positive culture/toxin genes or both and in association

| Toxin profile | Number of isolates (%) |
|---------------|------------------------|
| A+ B+         | 13 (35.2)*              |
| A+ B−         | 1 (2.7)                 |
| A− B+         | 6 (16.2)                |
| Negative A & B| 17 (45.9)**             |
| Total (%)     | 37 (100)**              |

*Only one isolate was positive for binary toxin genes as confirmed by sequencing; **54.1% of the C. difficile isolates were toxigenic strains; ***37/287 (12.9%) were intestine colonized with C. difficile.
with diarrhea, but there was no single case found among children [21]. The current study indicates that there was no correlation between positive colonization of toxigenic C. difficile in infants and presence of soft stools, previous antibiotic treatment, or inflammatory bowel disease, and overall our findings were similar to that of other investigators [18, 19]. A recent multicenter study done in three different private Jordanian hospitals in Amman over a period of 8 months showed high prevalence rate (92.4%) of positive C. difficile toxins among adults as well as older patients with a prolonged hospital stay and comorbidities [22]. Additionally, a recent study indicated that infants are widely colonized by nontoxigenic C. difficile strains and their early intestinal colonization with toxigenic strains originated from adults [9].

This study shows that all 37 C. difficile isolates were susceptible to vancomycin and metronidazole and were highly resistant to ciprofloxacin (78.4%) and moderate resistant to erythromycin (29.7%) (Table 3). These results are similar to some extent to a previous study published from our hospital [21] which showed that all C. difficile isolates were highly susceptible to vancomycin and metronidazole, while moderate resistant rate was found to ciprofloxacin. These findings are also much related to those of other European studies [15, 23, 24]. It is also important to mention here that fecal E. coli isolates from Jordan infant and adults were highly resistant to ciprofloxacin, and it is well known that Jordan physicians are extensively using fluoroquinolones in treatment of urinary and respiratory tract infections [25–27].

In conclusion, this study presents important epidemiological data about occurrence of toxigenic C. difficile in intestines of hospitalized and nonhospitalized infants living in a Middle East country.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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### Table 3: Antimicrobial MIC results of 37 C. difficile isolates.

| Antimicrobial agents | MIC50 (µg/ml) | MIC90 (µg/ml) | Resistance breakpoint (µg/ml) | MIC (µg/ml) range | Number (%) of resistance |
|----------------------|--------------|--------------|-------------------------------|-------------------|--------------------------|
| Ciprofloxacin*       | 5.3          | 9.6          | 8                             | 3.0–16.0          | 29 (78.4)                |
| Erythromycin         | 0.42         | 0.75         | 8                             | 0.25–2.0          | 11 (29.7)                |
| Vancomycin           | 0.89         | 1.60         | 32                            | 1.0–4.0           | Null                     |
| Metronidazole        | 0.09         | 0.16         | 32                            | 0.023–0.25        | Null                     |

* A total of 40.5% of isolates were positive for both mutated GyrA and GyrB genes.
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