Determination the Pathogenicity of C. difficile Isolates among Mice

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Abstract
Clostridium difficile is the main causative agent of antibiotic-associated diarrhea and colitis in hospitalized patients, the disease is caused mainly by two exotoxins, TcdA and TcdB, produced by the bacteria. Recurrent C. difficile infection (CDI) constitutes one of the most significant clinical issues of this disease. This study aimed to Determination the pathological effects of C. difficile in mice, the results shown that bacterial dose 1x10^8 CFU/ml led to 50% death within (2-4) days post infection and 100% developed diarrhea, that would cause infection but not universal lethality. In conclusion, we have established a mouse CDI model that allows for future investigations of the role of the host immuneresponse in the disease’s pathogenesis and permits critical testing of new therapeutics targeting recurrent disease.

Keywords: C.difficile, dose, pathological effects, mouse.

Introduction
Clostridium difficile (C. difficile) infections (CDI) are a clinical concern and are one of the leading causes of antibiotic-associated diarrhea of nosocomial outbreaks. CDI is dramatically increasing in both the prevalence and clinical severity of cases, (McFarland, 2015).

C. difficile is a Gram positive, rod-shaped, sporforming, anaerobic and toxin producing bacteria, commonly isolated from soil, humans, and other mammals (Kuijper et al., 2006; McFee and Abdelsayed, 2009). Also is a multidrug-resistant pathogen, flourishes in the colon after the gut microbiota has been altered by antibiotic therapy (Darkoh et al., 2015). Acquired either from the environment or the fecal-oral route (Khanna et al., 2012; Julia et al., 2013). About (15-25)% of the episodes of antibiotic associated diarrhea (AAD) is linked with the pathogenic strains, also 86% of C. difficile isolates from the suspected cases of C. difficile associated diarrhea (CDAD) were characterized as toxigenic (Barbut et al., 2007; Cohen et al., 2010). The CDI were associated with two virulence factors potential toxins including enterotoxin A and cytotoxin B (linked to the tcdA and tcdB genes, respectively), producing from pathogenic strains of C. difficile (Sunenshine and McDonald, 2006; Kuehne et al., 2011). Another toxin known as C. difficile binary toxin (cdt) has been isolated from some certain strains that associated with severe infectious form of disease in human (McEllistrem et al., 2005; Songer, 2010; Lessa et al., 2012).

Clinically, there is a wide spectrum of C. difficile presentations ranging from asymptomatic carriage to severe, life threatening, fulminant colitis, and toxic megacolon (Bartlett and Cerding, 2008; Surawicz, 2013). The severity of CDI ranges from mild diarrhea to pseudo membranous colitis (PMC) and can result in death (Alcala, 2013), reaching (95-100)% among patients with documented antibiotic-associated colitis (Christina et al., 2013). Recurrent CDI occurs in more than 20% of patients (Sun et al., 2011), that become more frequent, more severe, more refractory to standard treatment, and more likely to relapse (Mattila et al., 2012; Khanna and Pardi, 2014). The aims of this study is to Determination pathological study of C. difficile in mice.

Materials and Methods
C.difficile isolate:
C. difficile were isolation and identification from stool samples were collected from hospital in Baghdad previously by 1.selective media. 2.Gram stain ,Malachite green for spore,API20A Kit(BioMerieux,USA). 3.detection of two toxins A&B in stool samples by ELISA Kit (primier toxin A&B from Meridian Bioscience ,USA). 4. Detection of toxins A&B genes by PCR method .(Mehdi and Al-Mossawei, 2015).

Preparation of C. difficile spores:
Five different dose of spores (1x10^4,1x10^5,1x10^6,1x10^7,1x10^8)spore/ml were used for challenge orally to determined the suitable concentration that will be used to experimental study in mice ,as following: Concentration of spore/ml=(10^6x4xB)/N were : B= spores number , N=chamber square counted .

Sporulation of the C. difficile was induced on brain heart infusion (BHIS) agar, culture were diluted in fresh media to turbidity equal McFarland (0.5), 0.1ml of this suspension was spread on BHIS agar and anaerobic incubation for seven days, the spores were washed off the plate with phosphate buffered saline, and stored at 4°C.
C, and concentration determined by serial dilution and using cytometer chamber and McFarland (0.5) (Sorg et al., 2010). Each mouse was inoculated intragastrically by sterile 1 ml gavage syringe with 200 µl of the spore suspension containing (1x10^4, 1x10^5, 1x10^6, 1x10^7, 1x10^8) CFU/ml (Sun et al., 2011) for challenge.

**Experimental infection in mice:**
Thirty six (n=36) albino male mice, Mus musculus BALB /C strain aged (5-6) weeks and weighing (20-25) g. were obtained from AL-Nahrain research center, AL-Nahrain university.

**Determination the pathogenicity of C. difficile isolates among mice:**
Thirty six (n=36) mice were randomly divided into six groups designated as 1, 2, 3, 4, 5 and 6. Each group consisted of 6 mice, and subjected to the following treatments according to (Sun et al., 2011). Scheme (1).

To establish C. difficile, mice were treated at day (0-3) with an antibiotic mixture in drinking water: Clindamycin (0.1mg/ml Pharma, USA), Metronidazol (0.215mg/ml India), Vancomycin (0.045mg/ml Julphar, U.A.E), followed, at day 4, administration 200µl of Clindamycin concentration (0.2mg/ml) orally, and at day 5, administration 500 µl of Clindamycin concentration (0.2mg/ml) intraperitoneal injection (i.p.) (Chen et al., 2008). These drugs have been used to disrupt intestinal microbial to establish of C. difficile infection.

**Results and Discussion**
**Pathogenicity of C. difficile among mice:** results shown in table (1) bacteria dose of 1x10^6 CFU/ml led to 50% death within (2-4) days post infection and 100% developed diarrhea, that would cause infection but not universal lethality. Figure (1) that was selected as suitable dose.
Table (1): The suitable bacteria dose concentration CFU/ml that would cause severe infection in experimental mice (each group No = 6)

<table>
<thead>
<tr>
<th>Bacterial Dose CFU/ml</th>
<th>Total No. of mice in each group</th>
<th>Death of mice No. within (2-4) day post infection(%)</th>
<th>No. of Developed diarrhea(%)</th>
<th>Chi-square($\chi^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^5$</td>
<td>6</td>
<td>0 (0.00%)</td>
<td>2 (33.33%)</td>
<td>9.715 **</td>
</tr>
<tr>
<td>$1 \times 10^6$</td>
<td>6</td>
<td>2 (33.33%)</td>
<td>4 (66.67%)</td>
<td>9.715 **</td>
</tr>
<tr>
<td>$1 \times 10^7$</td>
<td>6</td>
<td>3 (50.00%)</td>
<td>6 (100%)</td>
<td>11.569 **</td>
</tr>
<tr>
<td>$1 \times 10^8$</td>
<td>6</td>
<td>5 (83.33%)</td>
<td>6 (100%)</td>
<td>6.723 **</td>
</tr>
<tr>
<td>$1 \times 10^9$</td>
<td>6</td>
<td>6 (100%)</td>
<td>6 (100%)</td>
<td>0.00 NS</td>
</tr>
</tbody>
</table>

Chi-square($\chi^2$) = 15.248 ** 11.804 ** ---

** (P<0.01), NS: Non-significant.

There were highly significant differences between each concentration groups.

C. difficile is responsible of AAD in humans and animals, and is one of the most common nosocomial pathogens. Pathogenic C. difficile produces two exotoxins, toxin A and toxin B which induce intestinal inflammation, fluid secretion and mucosal injury (Pothoulakis, 1996).

In conclusion, we have established a mouse CDI model that allows for future investigations of the role of the host immuneresponse in the disease’s pathogenesis and permits critical testing of new therapeutics targeting recurrent disease.

References


