Study of some Virulence Factors for *Clostridium perfringens* isolated from Clinical Samples and Hospital Environment and showing their Sensitivity to Antibiotics

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**ABSTRACT**

The study included taking 100 samples from different clinical sources, including wounds and burns, and from the hospital environment, in Kirkuk General Hospital and Azadi Teaching Hospital in the city of Kirkuk for the period from November 2017 to August 2018. The results of isolation and diagnosis showed the growth of 30 isolates that are positive for *Clostridium perfringens*, distributed between 15 isolates 37.5% from burns, 11 isolates 27.5% from wounds, and 4 isolates 20% from the hospital environment. These isolates were diagnosed based on microscopical, cultural and biochemical tests, in addition to being diagnosed with the Api 20A system. The sensitivity of isolates was tested toward a number of types of antibiotics, and all bacterial isolates showed a high sensitivity 100% against imipenem. As for the sensitivity to vancomycin, amikacin, tetracycline was 96.66, 90, and 66.66% respectively. While, all isolates showed a high resistance to metronidazole and colistin 100%, some virulence factors of *C. perfringens* isolates have been studied, and showed that all isolates (%100) have the ability to produce hemolysin, lecithinase, capsule, and spore, while 70% of the isolates produced DNAase.

**Keywords:** Antibiotic sensitivity, *Clostridium perfringens*, Virulence factors.

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**INTRODUCTION**

The contamination of wounds and burns with bacteria of the most important problems and challenges faced by people living with, and contamination with anaerobic bacteria, especially *C. perfringens*, is one of the indicators that pose a threat to the lives of infected persons. *C. perfringens*, formerly known as *Clostridium welchii*, is a gram positive, obligate anaerobic, encapsulated, spore forming, non-motile bacterium.\(^1\)

*C. perfringens* are found as a normal flora in the intestines of humans and animals, soil and marine sediments are a natural habitat for these bacteria,\(^2\) and they are opportunistic pathogens, where they cause a wide range of diseases such as wound infection, including gas gangrene, contaminated wound, and anaerobic cellulitis, also be responsible for the burn infection, bacteremia, and septicemia.\(^3\)

The virulence of bacteria is largely due to its ability to produce a variety of enzymes and toxins, as well as, capsules and spores.\(^4\) The production of toxins differs between strains of *C. perfringens* and its the basis of the classification system that relies on the production of four of the main toxins (alpha-beta-epsilontita), which divides the strains of *C. perfringens* into toxicity patterns from A to E.\(^5,6\)

**MATERIALS AND METHODS**

**Collection and Culturing of Samples**

100 samples of wounds and burns were collected from both sexes and different ages, in addition to the hospital environment in Kirkuk General Hospital and Azadi Teaching Hospital for the period from November 2017 to August 2018. These samples were taken by sterile cotton swabs and used to transfer them to the laboratory cooked meat broth medium, the medium containing the sample was boiled in a water bath at 100°C for 20 minutes and incubated anaerobically at 37°C for 24 hours using anaerobic jar, and gas pak, which provide the anaerobic conditions necessary for bacterial growth through reduced oxygen inside the container atmosphere, then a loopful from the apparent growth was subcultured onto (5–10%) human blood agar supplemented neomycin sulfate (100 μg/mL), and incubated aerobically at 37°C for 24 hours. Re-implant on the last medium for the purification procedure to perform other diagnostic tests.

**Identification of *C. perfringens***

Isolates were diagnosed based on microscopy examination and biochemical tests according to C. M. Kelly, *et al.*, P.M.

**Antibiotic sensitivity test**

The bacterial isolates sensitivity test for antibiotics was performed using a Kirby-Bauer disk diffusion method, as mentioned in J. G. Cappuccino, et al. by preparing a bacterial trap and comparing its turbidity with a McFarland standard tube (0.5) equivalent to $1.5 \times 10^8$ cells/mL, and then spreading the suspension onto the Columbia base agar medium using a sterile cotton swab. By planning in different directions to ensure equal stickiness, the dishes were allowed to dry for 5 minutes at room temperature, and then antibiotic tablets were distributed at 5 tablets per dish using sterile forceps, and incubated for 18 to 24 hours at $37°C$ in anaerobic conditions. Results recorded by measuring the diameter of inhibition zone around the disks and then compared with the standard global tables in CLSI.

**Detection of Some Virulence Factors of C. perfringens**

**Hemolysin Production**

Bacterial colonies were cultured on the neomycin blood agar incubated at $37°C$ in anaerobic conditions for 24 hours, the appearance of double zone of hemolysis around the colonies evidence of the positive result.

**Lecithinase Production**

This enzyme was detected by inoculation the egg yolk agar with bacterial isolates and incubated for 24 hours at $37°C$, the appearance of a turbid white area around the colony is evidence of the production of lecithinase.

**DNAase Production**

The DNAase agar medium was inoculated with bacterial isolates in a straight line in the middle of the dish and incubated at $37°C$ for 24 hours, then added the HCl (1 M), the result was considered positive by the appearance of a halo clear around the growth line.

**Capsule Production**

The presence of the capsule was detected using the India ink stain by placing a drop of it on the surface of a clean glass slide, then using the loop, a pure colony was taken and placed on the glass slide, and mixed with the stain, and after the slide was left on the air to dry, it was examined by optical microscopy using an oil lens (100X) to see the shape and location of the spore.

**RESULTS AND DISCUSSION**

The diagnosis of bacterial isolates on the cultures media was carried out according to their phenotypic characteristics, as they gave on the neomycin blood agar, a circular, convex, and glossy brown colonies surrounded by two zone of the hemolysis the first is complete by the theta toxin and the second incomplete by (alpha toxin), while on the tryptone glucose yeast extract agar it gave creamy colonies. It was also shown through a microscopic examination of the isolates that they are a gram positive, bacillus-shaped with flat ends, which may be slightly swollen due to the presence of the spore, and are arranged in the form of single or double. As for the biochemical tests, all isolates were positive for methyl red and litmus milk reaction, while they were negative for oxidase catalase, motility, indole test, voges-proskauer, and simmon citrate. Also, confirmed the diagnosis of the isolates by using the Api 20A system.

The results of the current study showed that the total percentage of C. perfringens isolates reached isolate with a ratio of (30%) out of 100 samples collected from different clinical sources, including wounds and burns, and from the hospital environment (Table 1), this result was close to H.Q.M. Al-Kanani, et al. Which constituted (25%), as it approached from the results of K.K.A. Al-Qarawi, et al., which formed 20%. The results also showed that the highest percentage of C. perfringens isolates were among the burn samples, which reached 37.5% (Table 1), and this result was close to P. Roggentin, et al., which was 40%, but it was inconsistent with the result of M.R. Ali, et al., which reached 1.2%, while the percentage of isolates were taken from wounds reached 27.5% (Table 1), this result was approached from the results of P. Roggentin, et al., which was 41.6%, but it differed with the results conducted in the researcher's study, which constituted 81.25%. As for the isolates obtained from the hospital environment, was 20% (Table 1), this ratio approached from the result of H.Q.M. Al-Kanani, et al., which reached 22.5%, but it was inconsistent with the result of M.R.A. Al-Kazragi, et al., which was 8.6%. The reason for the high percentage of C. perfringens isolates in burns samples is due to the physiological state of the burned tissues, which provides the appropriate environmental conditions for the growth of Streptococcus bacteria and other optional anaerobic bacteria.

<table>
<thead>
<tr>
<th>Isolation sources</th>
<th>The total number of samples</th>
<th>C. perfringens isolates Number</th>
<th>Ratio%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burn</td>
<td>40</td>
<td>15</td>
<td>37.5</td>
</tr>
<tr>
<td>Wound</td>
<td>40</td>
<td>11</td>
<td>27.5</td>
</tr>
<tr>
<td>Hospital environment</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>
Study of some virulence factors for *Clostridium perfringens* isolated from clinical samples and hospital environment

Table 2: Antimicrobial sensitivity test of *C. perfringens* isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Symbols</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>Imipenem</td>
<td>IPM</td>
<td>30</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>VA</td>
<td>29</td>
<td>96.66</td>
<td>1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>TE</td>
<td>20</td>
<td>66.66</td>
<td>10</td>
</tr>
<tr>
<td>Colistin</td>
<td>CO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td>MET</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Virulence factors of *C. perfringens* isolates.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Hemolysin %</th>
<th>Lecithinase %</th>
<th>DNAase %</th>
<th>Capsule %</th>
<th>Spore %</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>30</td>
<td>(100%)</td>
<td>21</td>
<td>(100%)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(70%)</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

which paves the physiological state of the infected tissues for the growth of *C. perfringens*. Also, wrong health practices through winding the burn area with gauze and isolating it from the air for long periods of time and the conditions of contamination in hospitals can lead to an increase the infection of *C. perfringens*. While, the reason for the difference in the ratios of bacterial isolates from one researcher to another, it is due to a group of different reasons, which includes the number of samples taken for study by researchers, the time of collection, the place they were taken, the environment, the number of isolates obtained, in addition to the health status of the injured and attention to hygiene in hospitals lobbies and types of materials used for sterilization.

It was observed through the results of sensitivity test for antibiotics (Table 2) that isolates of *C. perfringens* showed a high sensitivity to imipenem (100%), this result was agreed with the result of the study in which, the sensitivity ratio was (100%), while this ratio decreased to 62% in the study of M.T. Akhi, *et al.*, 27 the ratio of the sensitivity for vancomycin, has reached (96.66%) this result was close to R.O.S. Silva, *et al.*, 28 which constituted 100%. Thus, vancomycin can be chosen as an alternative treatment when the bacteria resistance to penicillins, as it affects the cell wall of bacteria that is sensitive to it by inhibiting the peptidoglycan found in the wall cell of bacterial. 29 The ratio of the sensitivity of the isolates for tetracycline was (66.66%), and thus this percentage was an approach to the study of A.A. Abd El-Tawab, *et al.*, 30 which constituted 83.3%, tetracycline works to inhibit the synthesis of proteins through its association with (30S) subunit of the ribosome and preventing the aminoacyl-tRNAs from the engagement, thereby stopping the translation process. 31 While, none of the isolates showed sensitivity to metronidazole and colistin and the resistance percentage (100%) for each of them, the resistance ratio for colistin agreed with L.Y. Mehdi, *et al.*, 32 which formed (100%), while the resistance ratio for metronidazole was different and did not agree with the of others, it was 32% in M.T. Akhi, *et al.*, 27 the reason for the resistance of isolates to metronidazole is due to the decrease in the permeability of the drug or an increase in its flow, the decrease in the activation of the drug and the enhancing of the activity of enzymes involved in DNA repair. 33 The results of the sensitivity test showed that imipenem was considered an effective treatment against *C. perfringens*, which is from the group of carbapenems that have a wide effect on the gram positive and gram negative bacteria, and the reason for the high sensitivity by these isolates toward imipenem comes from being resistant to beta-lactamase secreted by bacteria, and, therefore has a lethal effect on bacteria. 34

The results of detection of some of the virulence factors owned by *C. perfringens* isolates (Table 3) showed that 100% of the isolates had the ability to produce double zone of hemolysis, this result was agreed with D.S. Milanov, *et al.*, 35 Who recored 100% in this study. The results also showed the ability of all isolates to produce lecithinase and capsule, this result was agreed with a number of studies, where the researcher 36 showed that all the isolates in his study were producing lecithinase 100%. The results also showed that (70%) of the isolates were producing the DNAase, this result was close to the result of M.S. Rahman, *et al.*, 37 whom found that 62.5% of the isolates showed their ability to produce the DNAase, while all isolates showed their ability to form a Spore 100%, this result was agreed with P.S. Dar, *et al.*, 38 who found that all isolates were able to produce this enzyme.

CONCLUSION

The results of the study showed that infection with *C. perfringens* was more in the isolation of burns compared with the isolation of wounds and the hospital environment.

All bacterial isolates showed a high sensitivity (100%) to imipenem, while a high resistance (100%) to colistin and metronidazole.

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