RESEARCH ARTICLE

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, which included 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-epsilon-iota), 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.

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Tille, *et al.,* C. R Mahon, *et al.,* J. G. Cappuccino, *et al.,* K. Steve, *et al.,* M. J. Leboffe, *et al.,* A. S. Sastry, *et al*.[7-13] Also, the diagnosis of bacterial isolates was confirmed using the Api 20A diagnostic system.

Antibiotic sensitivity test

The bacterial isolates sensitivity test for antibiotics was performed using a Kirby-Bauer disk diffusion method, as mentioned inJ. G. Cappuccino, *et al.*[10] by preparing a bacterial trap and comparing its turbidity with a McFarland standard tube (0.5) equivalent to 1.5×10^8 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.[14]

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.[13]

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.[13]

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.[15]

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 detect the presence of the capsule.[16]

Spore formation

The presence of the spore was detected by using malachite green stain and safranin stain, by placing a pure colony, and brushing it on the glass slide, and then fixation it by flame, and then put a malachite green stain (7.5%) on the slide was left for a period 10 minutes, then the slide was washed with sterile distilled water and submerged with safranin and left for one minute, then washed with water, and examined with a microscope using an oil lens (100X) to see the shape and location of the spore.[17]

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*.[18]

Which constituted (25%), as it approached from the results of K.K.A. Al-Qarawi, *et al.,*[19] 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*.,[20] which was 40%, but it was inconsistent with the result of M.R. Ali, *et al*.,[21] 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*.,[22] which was 41.6%, but it differed with the results conducted in the researcher's study,[23] 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*.,[18] which reached 22.5%, but it was inconsistent with the result of M.R.A. Al-Kazragi, *et el*.,[24] 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 1: Percentage of *C. perfringens* isolates according to the isolation sources.

Isolation sources	*The total number of samples*	*C. perfringens isolates*	
		Number	*Ratio%*
Burn	40	15	37.5
Wound	40	11	27.5
Hospital environment	20	4	20
Total	100	30	30

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

		C. perfringens isolates					
		Sensitive		*Intermediate*		*Resistance*	
Antibiotics	*Symbols*	*No*	*%*	*No*	*%*	*No*	*%*
Imipenem	IPM	30	100	-–	–	–	–
Vancomycin	VA	29	96.66	1	3.33	–	–
Tetracycline	TE	20	66.66	10	33.33	–	–
Colistin	CO	–	-–	–	–	30	100
Metronidazole	MET	–	–	–	–	30	100

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

Isolates	*Virulence factors*				
	Hemolysin %	*Lecithinase %*	*DNAase %*	*Capsule %*	*Spore %*
30	30 (100%)	(100%) 30	21 (70%)	(100%) 30	(100%) 30

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.[25]

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[26] 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.

REFERENCES

1. Jain A, Venkatesh V, Agarwal J. Microbiology Practical Manual E-Book: 1st ed. Elsevier .India. 2018.
2. Grass JE, Gould LH, Mahon BE. Epidemiology of foodborne disease outbreaks caused by Clostridium perfringens, United States, 1998–2010. Foodborne pathogens and disease. 2013 Feb 1;10(2):131-136.

3. Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clinical microbiology reviews. 2001 Apr 1;14(2):244-269.
4. Revitt-Mills SA, Rood JI, Adams V. Clostridium perfringens extracellular toxins and enzymes: 20 and counting. Microbiology Australia. 2015 Sep 17;36(3):114-117.
5. McDonel J.L. Toxins of Clostridium perfringens types A, B, C, D, and E.In Dorner F, Drews H (ed), *Pharmacology of bacterial toxins*. Pergamon Press, Oxford, England. 1986;477–517.
6. Petit L, Gibert M, Popoff MR. Clostridium perfringens: toxinotype and genotype. Trends in microbiology. 1999 Mar 1;7(3):104-110.
7. Kelly CM, Heidir S, Cowan MK, Smith H. Microbiology, 5th ed. *Aystems Approach, McGraw-Hill,* USA. 2018.
8. Tille PM. Baily and scott's Diagnostic Microbiology- E- Book . 14th ed . St. Louis, Missouri : Elsevier. China. 2017.
9. Mahon CR, Lehman DC, Manuselis G. Textbook of Diagnostic Microbiology.5th ed. Saunders, Elsevier.Inc, China. 2015.
10. Cappuccino JG, Welsh CT. Microbiology: A Laboratory Manual, Global edition. 11th ed. Pearson Education Limited. 2017.
11. Steve K, Dennis S, Mary J. Laboratory Exercises in Organismal and Molecular Microbiology .ASM. Washington. USA. 2004.
12. Leboffe MJ, Pierce BE. Microbiology: laboratory theory and application. 2th ed. Morton Publishing Company. USA. 2012.
13. Sastry AS, Bhat S. Essentials of medical microbiology. 2nd ed.Jaypee Brothers, Medical Publishers Pvt. Limited. 2018.
14. CLSI (Clinical and Laboratory Standards Institute). Methods for antimicrobial susceptibility testing of bacteria. Setting the standard for quality in medical laboratory testing around the world. M100,28th ed. 2018.
15. Leboffe MJ, Pierce BE. A photographic atlas for the microbiology laboratory. 4th ed. Englewood . Morton Publishing Company. USA. 2011.
16. Tille PM. Bailey and Scott's Diagnostic Microbiology-E-Book. 13th ed . Louis, Missouri : Elsevier. China. 2014.
17. Pommerville J.C. Alcamo's Laboratory Fundamentals of Microbiology. 8th ed. Jones and Bartlett .USA. 2007.
18. Al-Kanani H.Q.M. Isolation and Diagnosis of *Clostridium perfringens* from surgery and burn lobbies and revealing their toxicity within the body of the organism Invivo. Journal of al-qadisiyah for pure science (quarterly). 2010;3(15):83–88.
19. Al-Qarawi KKA, Al-Obaidi HMR. Investigation of *Clostridium perfringens* in inflamed wounds and gauze of the patients′ feet infected with diabetics. Journal of kerbala university. 2019;1(17):157–160.
20. Al-Kanani HQM. Bacteriological study of Gas gangerena among burns and surgery patients. Journal of al-qadisiyah for pure science (quarterly). 2016;2(21):73–81.
21. Ali MR, Mohamed Ali SS, Abdulrazzak AA. Isolation and Diagnosis of *Clostridium perfringens* from Clinical Samples and the Environments of some Baghdad Hospital and Detection of their Virulence Factors. Al-Mustansiriyah Journal of Science. 2011;4(22):37–55.
22. Roggentin P, Hobrecht R, Tirpitz D, Rothe B, Schauer R. Application of sialidase antibodies for the diagnosis of clostridial infections. Clinica chimica acta. 1991 Feb 15;196(2-3):97-106.
23. De A, Varaiya A, Mathur M, Bhesania A. Bacteriological studies of gas gangrene and related infections. Indian journal of medical microbiology. 2003 Jul 1;21(3):202–204.
24. Al-Kazragi MRA. Bacteriological and Genetic Study OF *Clostridium perfringens* Isolated From Clinical Specimens and Some of Baghdad Hospital Environments. PhD thesis. Department of Biology. College of Science. AL-Mustansiriyah University. 2007.
25. Kiffer C, Hsiung A, Oplustil C, Sampaio J, Sakagami E, Turner P, Mendes C. Antimicrobial susceptibility of Gram-negative bacteria in Brazilian hospitals: the MYSTIC Program Brazil 2003. Brazilian Journal of Infectious Diseases. 2005 Jun;9(3):216-224.
26. Chon JW, Seo KH, Bae D, Park JH, Khan S, Sung K. Prevalence, toxin gene profile, antibiotic resistance, and molecular characterization of Clostridium perfringens from diarrheic and non-diarrheic dogs in Korea. Journal of veterinary science. 2018 May 1;19(3):368-374.
27. Akhi MT, Asl SB, Pirzadeh T, Naghili B, Yeganeh F, Memar Y, Mohammadzadeh Y. Antibiotic sensitivity of Clostridium perfringens isolated from faeces in Tabriz, Iran. Jundishapur journal of microbiology. 2015 Jul;8(7).
28. Silva ROS, Junior FCF, Marques MVR, Junior CAO, Martins NRDS, Lobato FCF. Genotyping and antimicrobial susceptibility of Clostridium perfringens isolated from Tinamidae, Cracidae and Ramphastidae species in Brazil. Ciência Rural, Santa Maria, 2014;44(3):486–491.
29. Mader, J.T., Wang, J. and Calhoun, J.H. (2001). Antibiotic therapy for musculoskeletal infections. J. of Bone and joint surgery (American). 83(12), 1878–1890.
30. Abd El Tawab AA, El-Hofy FI, Ammar AM, Aideia HA, Hammad EA. Bacteriological and molecular studies on toxigenic Clostridium perfringens in milk and some milk products. Benha Veterinary Medical Journal. 2016 Dec 1;31(2):144-148.
31. Nelson ML, Levy SB. The history of the tetracyclines. Annals of the New York Academy of Sciences. 2011 Dec;1241(1):17-32.
32. Mehdi LY, Wannas NS. Isolation and Identification of Clostridium perfringens and its Enterotoxin in Food poisoning Patients. Journal of the Faculty of Medicine. 2017;59(2):145-150.
33. Land KM, Johnson PJ. Molecular basis of metronidazole resistance in pathogenic bacteria and protozoa. Drug Resistance Updates. 1999 Oct 1;2(5):289-294.
34. Howland RD, Mycek MJ. pharmacology . 3th ed. Lipincott Williams and Wilkins . USA. 2006; pp:77–79.
35. Milanov D, Petrović T, Todorović D, Aleksić N, Čabarkapa I. Toxin genotypes of Clostridium perfringens in animal feed and their role in the ethiology of enterotoxemia in domestic animals.2018;5(1): 67–76.
36. Malmarugan S, Boobalan A, Dorairajan N. Necrotic Enteritis in broiler and layer farms in Tamil Nadu, India. Intl. J. Agro. Vet. Med. Sci. 2012;6(4):241-249.
37. Rahman MS, Sharma RK, Borah P, Chakraborty A, Devi MR, Longjam N. Characterization of Clostridium perfringens isolated from mammals and birds from Guwahati city, India. Journal of Venomous Animals and Toxins including Tropical Diseases. 2012;18(1):83-87.
38. Dar PS, Wani SA, Wani AH, Hussain I, Maqbool R, Ganaie MY, Kashoo ZA, Qureshi S. Isolation, identification and molecular characterization of Clostridium perfringens from poultry in Kashmir valley, India. Journal of Entomology and Zoology Studies. 2017;5(5):409-414.