

Evaluation of General Characteristics of Local Isolates *Helicobacter pylori* Bacterium that Causing Acute and Hemorrhage Peptic Ulcer in Adults

Zanab Sami Ali; Ali Shihab Ahmed and Nidhaal Sehail Zbar
College of Applied Biotechnology, University of Al Nahrain.

Abstract

This study was aimed to isolate, identify and study the genomic profile of hemorrhage and acute peptic ulcer infections by *Helicobacter pylori* bacterium in patients at the age (40- 60 year). Two techniques for identification of the infection by *H. pylori* were used, invasive and non invasive. Blood and faeces specimens were examined directly in hospital for specific antibody and antigen detection. One hundred and twenty biopsies specimens were used for the fast urea tests and. A new medium (ZAN-1) was prepared also other types of media were modified to be suitable for bacterial growth and isolation. Virulent factors such as time of color change in the fast urea solution by urease activity and ammonia production were studied, also specific shape was determined by Scanning Electron Microscope (SEM). Other tests such as lecithinase test, oxidase, catalase and blood hemolysis were done for identification of this bacterium. Two isolates were selected to represent the hemorrhage and acute infections. Hemorrhage isolate exhibited different characters especially in blood hemolysis and lecithinase activity. Antimicrobial susceptibility test indicated that acute isolate is more resistant than hemorrhage. The results of genomic profile indicated the absence of the plasmids and the virulent factors were of chromosomal source.

Keywords: *Helicobacter pylori*, Isolation, Identification, Ammonia, Nanchitosan delivery, Antibiotics.

Introduction

The discovery of *Helicobacter pylori* in 1982 was the starting point of a revolution concerning the concepts and management of gastroduodenal diseases [1]. The genus *Helicobacter* belongs to the subdivision of the Proteobacteria, order Campylobacterales, family Helicobacteraceae. To date, the genus *Helicobacter* consists of over 20 recognized species, with many species awaiting formal recognition [2]. *H. pylori* is a Gram-negative spiral bacteria measuring 2-4 μm in length, 0.5-1 μm in width and has 2-6 sheathed flagella 3 μm in length [3]. It was found that *H. pylori* growth at optimal range of: temperature 34-40°C, pH 5.5 -8.0 but can survive at pH 4 and the key feature of *H. pylori* its microaerophilicity. Growth at optimal level of: 2 -5% oxygen, 5-10% carbon dioxide and 85% nitrogen. Members of the genus *Helicobacter* in most cases are catalase and oxidase positive, and many but not all species are also urease positive [4]. The public health importance of the discovery of *H. pylori* and its role in stomach diseases was recognized in 2005 by the attribution of the Nobel Prize in Physiology or Medicine to Marshall and

Warren. In the history of Nobel prizes, this is only the third time that the discovery of a bacterium has been acknowledged [5]. It is now well accepted that the most common stomach disease, peptic ulcer disease, is an infectious disease, and all consensus conferences agree that the causative agent, *H. pylori*, must be treated with antibiotics [6,7]. Infection with *H. pylori* is one of the risk-factors for gastric neoplasm, including lymphomas and gastric cancer [8]. Infection with *H. pylori* is associated with an increase in gastric acid output and a reduction in the thickness of the mucous layer and in gastric mucosal hydrophobicity [9]. The most Bacterial virulence factors are the urease enzyme. The net effect of ammonia production is increased pH of the culture. Biopsy samples are placed in an agar gel or paper strip containing a pH indicator. If organisms are present in sufficient numbers in the antral biopsy sample, a color change will occur as a result of urea breakdown and ammonia production by *H. pylori* urease. Ammonia is a key component in nitrogen metabolism as well as acid resistance [10]. Other virulent factors are also important in infection and survival the

bacterium. Several invasive and non invasive tests are available to detect *H. pylori* infection. The invasive tests include biopsy, histology, rapid urease test, bacterial cultures and PCR [11,12,13]. Non – invasive tests include serum and whole blood antibody [14], stool antigen [15] and urea breath test [16].

The size of the two sequenced *H. pylori* genomes is approximately 1.7 Mbp, with a G+C content of 35% to 40% [17]. In contrast to other pathogenic bacteria which are highly clonal, *H. pylori* is genetically heterogeneous which is possibly an adaptation of *H. pylori* to the gastric conditions of its host [18]. Genetic heterogeneity is occur via several methods of DNA rearrangement and the introduction and deletion of foreign sequences [19,20]. Diversity is also seen at the nucleotide level via several mechanisms including translational and transcriptional phase variation and mutation [21].

Materials and Methods

Culture media

a-Fast Urea solution: This solution was prepared by mixing 10g of urea and 0.002 g phenol red, then dissolved in 100 ml of distilled water. The pH was adjusted to 6.4. This solution was sterilized by sterile Millipore filter 0.22 µm and collected in sterile screw cupped tubes [22].

b-New Urea Broth (ZAN-1): This new medium was prepared from the following ingredients (g/l): Urea, 20; Dipotassiumphosphate (Na_2HPO_4), 9.5; Monopotassium phosphate (KH_2PO_4), 9.1; phenol red, 0.01; yeast extract, 0.1; Ampicilline, 0.1; Naldixic acid, 0.02; L-Cystein, 1. The ingredients were dissolved in 800 ml of distilled water. The pH was adjusted to 6.4, then sterilized through sterilized Millipore filter (0.22µm) and distributed into sterile screw cupped test tubes. The agar medium was prepared by adding sterilized 2% agar into prepared broth medium.

c-Modified Brain Heart Infusion Agar Medium (BHI Agar): The medium was prepared according to the manufacturer instruction (Hi-media). The modified BHI agar was prepared by adding the sterilized solution

of urea, 10 g/l; L-cystine, 1g/l; phenol red, 0.01 g/l; ampicilline, 0.1 g/l; naldixic acid, 0.02 g/l and 2,3,5-Triphenyltetrazolium chloride, 0.04 g/l after adjusting the pH to 6.4, then sterilized through sterilized Millipore filter (0.22µm) and distributed into sterile screw cupped test tubes.

d-Modified Tryptic Soyabean Broth (TSB Broth): The medium was prepared according to the manufacturer instruction (Hi-media). The modified TSB was prepared by adding beef extract, 5 g/l; urea, 10 g/l; L-cystine, 1g/l; ampicilline, 0.1; naldixic acid, 0.02 after adjusted the pH 6.4 and sterilized through Millipore filter (0.22 µm) and distributed in sterile screw cupped tubes.

e-Modified Tryptic Soyabean Agar Medium (TSB Agar): The medium was prepared according to the manufacturer instruction (Hi-media). The modified TSB was prepared by adding beef extract, 5 g/l; 2,3,5-Triphenyltetrazolium chlorid, 0.04 g/l; agar, 20 g/l. The pH was adjusted to 6.4 and sterilized in autoclave at 121° C for 15 min. Urea, 10 g/l; L-cystine, 1g/l; phenol red, 0.01 g/l;Ampicilline,0.1g/l; Naldixic acid, 0.02 g/l were sterilized by Millipore filter (0.22 µm) and added to the molting media.

Hospital tests: A total number of 120 specimens of biopsy were collected from gastroscopy department in *Alkadumia hospital*. Three types of specimens were collected normal, acute and hemorrhage infection during the endoscopy adult patients (40-60 years age). Care must be taken to insure that the patients have not received any antibiotics or antisecretory drugs especially proton pump inhibitors (Ombrasol). Part of specimen was inoculated in fast urea solution and transferred to the laboratory under cooling conditions. The tests were conducted in hospital included fast urea solution and blood antibody. The blood antibody test was achieved by using rapid anti - *H. pylori* test kit by taking blood samples from the suspected patients. This kit confirmed presence *H. pylori* infection.

Other parts of the same biopsy specimens were submerged in fixative solution of 10% formaldehyde according to the direction histological private laboratory. The fixation

preserved the morphology of bacteria and tissue for histological examination. Histological slide and staining with Giemsa stain were prepared. Antigen detection in faeces of the patients visiting the hospital was difficult to achieve, so it was done randomly on suspected cases outside the hospital.

Laboratory tests: These tests included the isolation of bacteria and studying their characteristics. Urea broth culture (ZAN-1) containing the biopsy was streaked on agar media (modified BHI, modified TSB and new urea agar ZAN-1) and incubated at 37°C for 18-72 hs under anaerobic conditions using anaerobic jar with gas generation kit (H₂, CO₂). Pure colonies were examined for morphological and confirmation tests. The confirmation tests Included oxidase, catalase, lecithinase in egg yolk emulsion agar (EGE), 2,3,5-Triphenyltetrazolium chloride hydrolysis, blood agar test, antibiotic susceptibility test, urease test and scanning electron microscope (Physics department / Al-Nahrain University).

Genomic profile: Maxwell ® 16 Tissue DNA purification kit and Maxwell device for DNA extraction were used for DNA extraction. DNA extract was migrated into gel electrophoresis using agarose, 1%, 100 V and 70 minutes.

Results and Discussion

Results of fast urea solution test was appeared within 2-6 days indicating the presence of *H.pylori* infection in biopsy specimens. Color of this solution was changed from yellow to pink. This type of bacteria have multi copies of urease enzyme and could be hydrolysed the urea into ammonia and CO₂ during few hours Fig.(1).

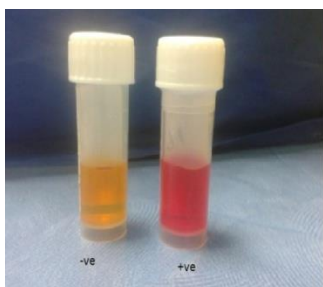


Fig.(1): Fast urea solution test.

Fig.(2) illustrated the results of anti-*H.pylori* test conducted in the hospital.

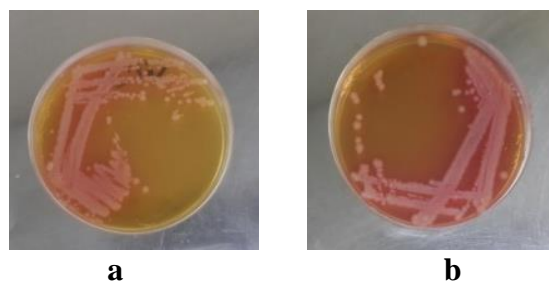


**Fig.(2): Rapid anti *H. pylori* test kit for human blood
(Down, +ve; Uper slide, -ve test).**

New urea broth cultures enhanced the growth of bacteria and gave fast result in the presence of this type of bacteria Fig.(3). Clear and recognized colonies of *H. pylori* were obtained after culture on modified BHI and TSB. They are surrounded by red zone due to reduction the 2,3,5-Triphenyltetrazolium chloride pigment by unique hydrolysis activity of this bacterium. The hydrolysis of urea during this short period was the most important indicator for isolation and identification of this because of their ability to produce highly active and urease enzyme which regarded as an important virulent factor [23]. The results of culture are shown in Figs.(3,4). Two isolates were selected, acute (A-1) and hemorrhage (H-1) for other tests.



Fig.(3): Growth of *Helicobacter pylori* in urea broth medium ZAN-1 (Red color indicates positive result).



**Fig.(4): Colonies of *Helicobacter pylori*.
(a- modified BHI agar;
b - modified TSB agar).**

Microscopic examination of the stained slide showed Gram negative spherical bacteria Fig.(5:a,b). Scanning electron microscope (SEM) examination showed the presence of flagellated curved cells of bacterium Fig.(5,c, d). Figs. (4) and (5), exhibited no morphological differences between acute a hemorrhage isolates. The results of another confirmation testes were the oxidase (+), catalase (+) and lecithinase (+) for acute and hemorrhage. The difference between two isolates was in blood hydrolysis as shown Fig.(6,d). It was found that hemorrhage isolate (H-1) produced the strange colonies with some alpha hemolysis.

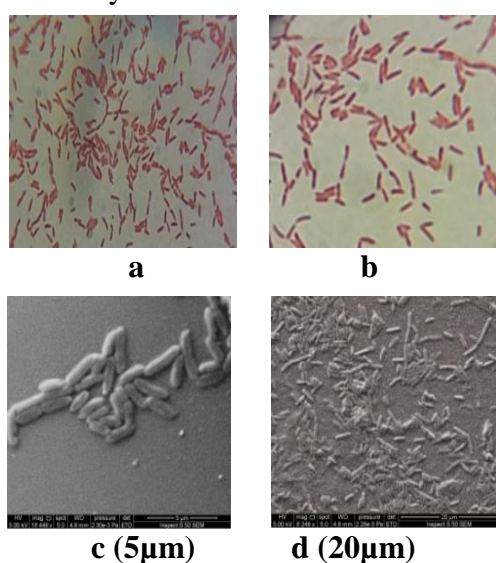


Fig.(5): Morphological characteristics of *H.pylori* (a: Acute isolate; b: hemorrhage isolate. c and d, *H.pylori* under SEM).

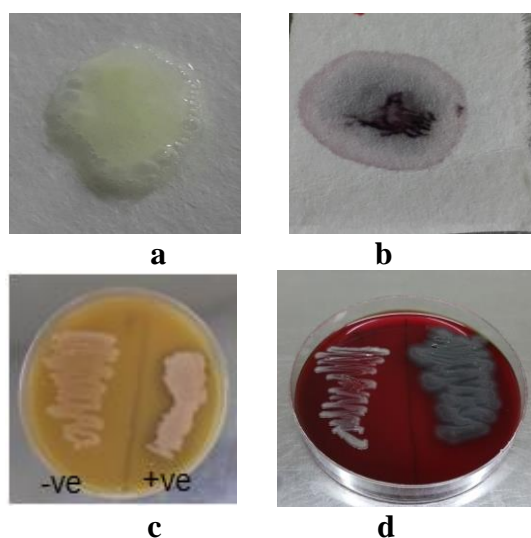


Fig.(6): Confirmation tests for *H.pylori* isolates [(a: Catalase +; b: Oxidase +; c: Lecithinase +; d: Beta hemolysis for blood agar (Left, A-1; Right, H-1)].

Susceptibility tests in Table (2) and Fig.(7) indicate that these isolates have different responses against the antimicrobial agents (Ciprofloxacin, Tetracycline, Levofloxacin, Amikacin). It was found that A -1 isolate more resistant than H-1. The most effective antimicrobial agent was the ciprofloxacin for both acute and hemorrhage isolate.

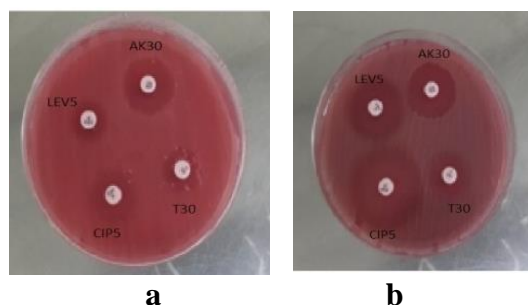


Fig.(7): Susceptibility tests for *H. pylori* isolates in modified TSB media (a: acute infection, b: hemorrhage infection).

**Table (2)
Susceptibility tests for acute and hemorrhage isolates of *H. pylori*.**

Antibiotic	Isolate	
	Acute (A-1)	Hemorrhage (H-1)
Ciprofloxacin (cip5)	18mm	25mm
Tetracycline (T30)	17mm	14mm
Levofloxacin (Lev5)	22mm	24mm
Amikacin (Ak30)	23mm	25mm

Histological examination showed differences in stomach tissue level between the normal and infected ones. Fig.(8,a-1,2) showed that gastric body mucosa appeared normal looking gastric gland which contains the oxyphiles cells and normal looking lamina propria. Tissue infected with *H. pylori* section showed the gastric body mucosa with increased lamina propria inflammatory cells with disorganization of gastric glands. Atrophic change were not seen Fig.(8:b-1,2).

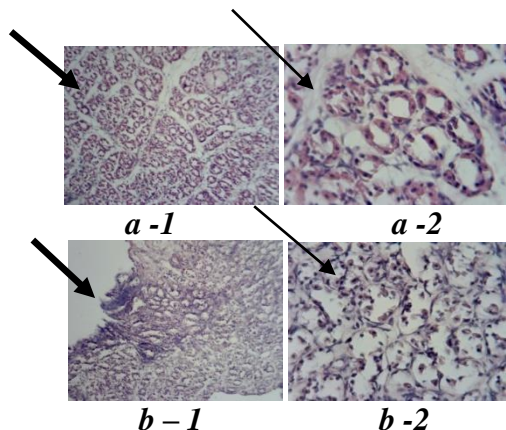


Fig.(8) a: 1 and 2 Sections, The gastric body mucosa with normal looking gastric gland that contain oxyphiles cells and normal looking lamina propria). b: 1and 2 sections, The gastric body mucosa with increased lamina propria inflammatory cells with disorganization of gastric glands , no atrophic changes).

Genomic profile exhibited that the acute and hemorrhage isolates (A-1, H-1) of *H. pylori* have only chromosomal DNA with the absence of plasmids Fig.(9). These results confirmed that virulent genes and other characteristics are carried on the DNA chromosomal of *H. pylori* and this may be result into more explanations about the peptic ulcer infection and their complications in the patients.

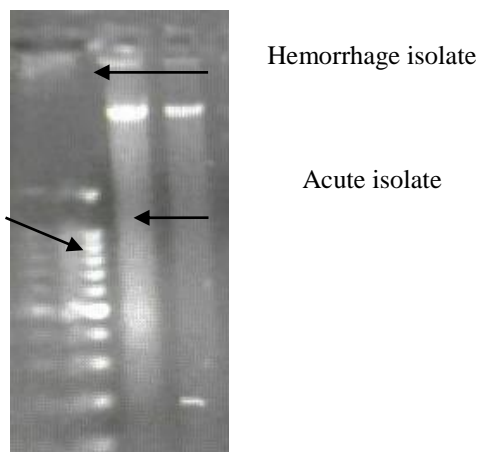


Fig.(9): DNA profile for different acute and hemorrhage infectious cases of *H.pylori* (Left line: hemorrhage infection ; Right: acute infection).

Conclusion

H. pylori is a microaerophilic spiral bacterium that can be easily cultivated in new and modified media. The indicator for the virulence factor (urease enzyme) was phenol red that change from yellow to pink due to

hydrolysis urea to ammonia by the function of this enzyme. The difference between acute and hemorrhage infection depends on the type of virulent genes, patient diet, mental and psychical state of the patient. Acute isolate was more resistant than hemorrhage. Genetic profile showed that the genes responsible for virulent factors were chromosomal origin.

References

- [1] Warren J. R., and B. Marshall. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet*, I, 1273-1275, 1983.
- [2] Fox J. G. The non-*H. pylori* helicobacters: their expanding role in gastrointestinal and systemic diseases. *Gut*, 50, 273-283, 2002.
- [3] Nedrud J.G. and Czinn S.J. Host heredity and *Helicobacter*. *Gut*, 45, 323-332, 1999.
- [4] Han S. W., Flamm R., Hachem C. Y., Kim C., Evans J., Beyer J., Drnec, D. and Graham Y. Transport and storage of *Helicobacter pylori* from gastric mucosal biopsies and clinical isolates. *Eur. J. Clin. Microbiol. Infect. Dis.*, 14, 349-352, 1995.
- [5] Megraud F. A humble bacterium sweeps this year's Nobel Prize. *Cell*, 123, 975-976, 2005.
- [6] European Helicobacter pylori Study Group. Current European concepts in the management of *Helicobacter pylori* infection. The Maastricht consensus report. *Gut*, 41, 8-13, 1997.
- [7] Lee J., and O'Morain C. Who should be treated for *Helicobacter pylori* infection? A review of consensus conferences and guidelines. *Gastroenterology*, 113, S99-S106, 1997.
- [8] Asante M. Ahmed, H. Patel, P. Davis, T. Finlayson, C.Mendall, M and Northfield, T. Gastric mucosal hydrophobicity in duodenal ulceration:role of *Helicobacter pylori* infection density and mucus lipids. *Gastroenterology*, 113, 449-454, 1997.
- [9] Ernst PB. and Gold BD. The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu. Rev. Microbiol.*, 54, 615-640, 2000.
- [10] Stingl K., Altendorf K. and E. P. Bakker. Acid survival of *Helicobacter pylori*: how does urease activity trigger cytoplasmic pH homeostasis. *Trends Microbiol.*, 10, 70-74, 2002.

- [11] Palframan S.L., Kwok T. and Gabriel K. Vacuolating cytotoxin A(VacA) a key toxin for *Helicobacter pylori* pathogenesis. *Cellular and Infection Microbiology*, 2 (92), 1-8, 2012.
- [12] Elitsur Y., Hill I. and Lichtman SN. Prospective comparison of rapid urease tests (Pyloritek, CLO test) for the diagnosis of *Helicobacter Pylori* infection in symptomatic children: a pediatric multicenter study. *Am J Gastroenterol.*, 93, 217-9, 1998.
- [13] vander RW., van der E.A. and Homan A. Influence of metronidazole resistance on efficacy of quadruple therapy for *Helicobacter pylorie* radication. *Gut*, 42, 166-9, 1998.
- [14] Han FC. Li XJ., Jian, H., Qin LP., Li D., Gu YH., Liu ZG., Zhang L. and Yan XJ. Detection of *H. pylori* antibody profile in serum by protein array. *World J Gastroenteol.*, 12, 4044-8. 2006.
- [15] Vaira, D., Malfertheiner, P., and Megraud, F. Diagnosis of *Helicobacter pylori* with a new non-invasive antigen-based assay. *Lancet*. 354: 30-3 (1999).
- [16] Bode, G., Rothenbacher, D. and Brenner, H. Variation in the ¹³Curea breath test value by nationality in *Helicobacter pylori*-infected children. *Scand J Gastroenterol*. 33:468-72 (1998).
- [17] Boneca, I. G., H. de Reuse, J. C. Epinat, M. Pupin, A. Labigen, and I. Moszer. A revised annotation and comparative analysis of *Helicobacter pylori* genomes. *Nucleic Acid Res*. 31:1704-1714 (2003).
- [18] Kuipers, E.J., D. A. Israel, J. G. Kusters, M. M. Gerrits, J. Weel, A. van Der Ende, R. W. van Der Hulst, H. P. Wirth, J. Hook-Nikanne, S. A. Thompson, and M. J. Quasispecies development of *Helicobacter pylori* observed in paired isolates obtained years apart from the same host. *J. Infect. Dis*. 181:273-282 (2000).
- [19] Falush, D., T. Wirth, B. Linz, J. K. Pritchard, M. Stephens, M. Kidd, M. J. Blaser, D. Y. Graham, S. Vacher, G. I. Perez-Perez, Y. Yamaoka, F. Megraud, K. Otto, U. Reichard, E. Katzowitsch, X. Wang, M. Achtman, and S. Suerbaum. Traces of human migrations in *Helicobacter pylori* populations. *Science*, 299:1582- 1585 (2003).
- [20] Suerbaum, S., and Achtman, M. *Helicobacter pylori*: recombination, population structure and human migrations. *Int. J. Med. Microbiol*. 294:133-139 (2004).
- [21] Falush, D., Kraft, C., Taylor, N. S., P. Correa, J. Fox, G., Achtman, M. and Suerbaum, S. Recombination and mutation during long-term gastric colonization by *Helaicobacter pylori*: estimates of clock rates, recombination size, and minimal age. *Proc. Natl. Acad. Sci. USA* 98: 15056-15061 (2001).
- [22] Berry, V. and Sagar, V. Rapid Urease Test to Diagnose *Helicobacter Pylori* Infection. *J. Med. Edu. And Resear*. 8 (2), 86-88 (2006).
- [23] Al- Mofarji, S.T.K. The Relationship between Thyroid Disorders and *Helicobacter pylori* Infection. *Thesis*. College of Science, Al-Nahrian University (2014).

الخلاصة

هدفت هذه الدراسة الى عزل، تشخيص ودراسة النسق الوراثي لبكتريا الملوية البوابية (*Hlicobacter pylori*) المسببة لقرحة المعدة الحادة والنزفية في المرضى باعمار (٤٠-٦٠ سنة). استخدمت تقنيتان لتشخيص الاصابة ببكتريا *Helicobacter pylori* هما الفحوصات المجتاحة وغير المجتاحة. تم فحص عينات من الدم والغائط بصورة مباشرة في المشفى باستخدام كشف الضد والمستضد التخصصي. تم استخدام ١٢٠ عينات وخزات لفحوصات اليوريا السريعة. تم تحضير وسط زرعي جديد (ZAN-1) وكذلك تحويل العيدي من الاوساط للتنمية المناسبة وعزل البكتريا. تم دراسة عامل الضراوة مثل تغيير لون في محلول اليوريا السريع بواسطة فعالية انزيم اليوريز وانتاج الامونيا، وايضا تحديد الشكل الخاص بالبكتريا بواسطة المجهر الماسح الالكتروني (SEM). تم تنفيذ فحوصات اخرى مثل فحص انزيمات اللستينيز والاكسيديز والكتاليز وتحلل الدم لغرض تشخيص البكتريا. تم انتخاب عزلتين لتمثل الاصابات النزفية والحادة. اظهرت العزلة النزفية صفات مختلفة خاصة في تحلل الدم وفعالية اللستينيز. اشارت نتائج دراسة النسق الوراثي الى عدم وجود بلازميدات وان عوامل الضراوة هي ذات منشأ كروموسومي.