

Study of some virulence factors in *Proteus sp.* associated with diarrhea

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Abstract

Various virulence factors including protease, lipase, lecithinase, deoxyribonuclease, gelatin liquefaction, haemolysin, congo red binding and urease production were determined for 94 *Proteus* isolates recovered from stool specimens of 518 children suffering from diarrhea. Eighty six (91.5%) of these isolates were belong to *Proteus mirabilis* and 8 (8.5%) were *Proteus penneri*. The results showed that the distribution of virulence factors was variable among the tested isolates. All isolates 94 (100%) were urease producers' but have different ability for binding to Congo red, gelatin liquefaction, deoxyribonuclease, lipase, protease, lecithinase, and haemolysin production. In order to study the invasion character, mice (Balb/C) infants were sucked with the suspension of (1) selected virulent isolate of *Proteus mirabilis* (positive for all the virulence factors), histological sections were performed to study the mode of the entry of bacteria into the enterocytes, and the histological changes in the liver. Microscopical examination for histological sections showed pathological effect on two organs when compared with the control organs. The results give clear evidence that *Proteus mirabilis* more virulent than *Proteus penneri*.

Key word: *Proteus sp.*, virulence factors, diarrhea.

Introduction

At birth the baby's intestinal tract is sterile. Thus the enteric flora is derived exclusively from the environment; the enteric bacteria colonize the human infant in an a feco-oral direction ⁽¹⁾. The normal gastrointestinal aerobic flora, which includes *E. coli* and frequently other coliform and *proteus spp.* in small numbers, is important in preventing disease through bacterial competition, the microflora reinforce the barrier function of the intestinal mucosa, helping in the prevention of the attachment of pathogenic microorganisms and the entry of allergens⁽²⁾. *Proteus species*, members of the family *Enterobacteriaceae* are motile Gram negative enteric bacteria⁽³⁾, they are important pathogens of the urinary tract and are the primary infectious agent in patients with indwelling urinary catheters⁽⁴⁾. Pathogenic bacteria have developed numerous virulence factors to adapt to their host environment, studying the main *Proteus sp.* virulence factors would increase our understanding of how a microorganism from normal flora infects and colonizes other sites and establishes infection. Several potential virulence factors of *Proteus* had been studied in relation to its virulence and pathogenicity of urinary tract, including hydrolysis of urea by urease, cell invasiveness, cytotoxicity induced by hemolysins, cleavage of IgA and IgG by proteolytic enzyme⁽⁵⁾. The main pathogenic features of *P. mirabilis* rods are their urealytic, proteolytic, and hemolytic abilities, and swarming motility^(6, 7). Understanding of the common themes in microbial pathogenicity is essential to recognize the microbial virulence in order to develop novel vaccines and other therapeutic agents for the treatment and prevention of infectious diseases. Accordingly, the aim of this work was to study some potential virulence factors produced by some *Proteus spp.* isolated from children suffering from diarrhea.

Materials and Methods

Samples collection

The study was carried out on children (out and inpatient suffering from diarrhoea) attending Azadi teaching hospital and Pediatric hospital in Kirkuk

city, from June 2009 to June 2011, 518 Stool specimens were collected in disposable, clean screw-capped, used for this purpose. All the specimens were processed immediately or used Carry Blair transport media if delayed for 1-2 hours after their collection and then cultured ⁽⁸⁾.

Bacterial isolation and identification

Collected samples were cultured directly on MacConkey's agar; XLD agar, SS agar and EMB agar for primary isolation of the Enterobacteriaceae and blood agar to detect beta hemolytic isolate ⁽⁸⁾. All isolates were incubated aerobically at 35 °C for 24 hours, and suspicious colonies were selected for definitive microscopic examination, culture characteristics, biochemical testing and the usage of API 20E System (BioMérieux/France) for identifying *Proteus spp.* ⁽⁹⁾.

Detection of some virulence factors

For all isolates Urease activity ⁽¹⁰⁾. Gelatin liquefaction test ⁽¹⁰⁾. Hemolysin production⁽¹⁰⁾. Binding to Congo red ⁽¹⁰⁾. Extracellular protease ⁽¹⁰⁾. DNase production ⁽¹⁰⁾. Lecithinase production ⁽¹⁰⁾. lipase⁽¹¹⁾. Were done. Mouse infection was done by culturing bacterial isolates in brain heart infusion broth (BHI) for 24 hours at 35 °C, (3) suckling Balb/C mice aged 4-6 days old were gavaged with 0.1ml of 2×10⁶cell/ml of *Proteus mirabilis* isolates number 7 (possessed all tested virulence factors) through a feeding needle, and (3) mice ingested with 0.1ml of sterile normal saline (used as diluent's) and left as control. After 24 hours post-infection, mice were sacrificed with chloroform; the liver and intestine were taken and fixed in 10% buffered formalin for histopathological study ^(12, 13). The preparation of histological sections depended on standard method of Baker *et al.*, (1975) the sections were examined by light microscope under magnification power 100X and 400X. Photographs were taken by digital-camera ⁽¹⁴⁾.

Results and discussion

Isolation and identification

The results of identification were showed that among 433 positive samples 94 *Proteus spp.* (Table-1) isolated from children with diarrhea. Among 94 isolates, 86 (91.5%) belonged to *Proteus mirabilis* and 8 (8.5%) belonged to *Proteus penneri*, and the both species isolated in male more than female and among (2-12 months) age group more than other age groups (Table-2) and (Table-3). The increased incidence in this age is most likely the result of greater exposure to enteropathogens (as the use of solid food starts at this age), plus the presence of susceptible host⁽¹⁵⁾ due to the immaturity of the immune system of the child, as well as the decline of the passive immunity after its 6 months of age⁽¹⁶⁾.

Table 1: Identification of *Proteus spp.* using API 20E system.

Isolates species	<i>Proteus mirabilis</i> (86)	<i>Proteus penneri</i> (8)
ONPG	—	—
ADL	—	—
LDC	—	—
ODC	+	—
CIT	—	—
H ₂ S	+	+
URE	+	+
TDA	+	+
IND	—	—
VP	+	—
GEL	+	+
GLU	+	+
MAN	—	—
INO	—	—
SOR	—	—
RHA	—	—
SAC	—	+
MEL	—	—
AMY	—	—
ARA	—	—

Table 2: Distribution of *Proteus spp.* according to patient's sex

Isolates	Gender	Male	Female	Total
<i>Pr. mirabilis</i>		44 (51.2%)	42 (48.8%)	86 (100%)
<i>Pr. penneri</i>		5 (62.5%)	3 (37.5%)	8 (100%)
total		49 (52.1%)	45 (47.8%)	94 (100%)

Table 3: Distribution of *Proteus spp.* according to patient's age groups

Isolates	Age group/year				Total
	0.2- 1	1.1-5	5.1-10	10.1-13	
<i>Pr. mirabilis</i>	60 (69.8%)	19 (22.1%)	6 (6.9%)	1 (1.2%)	86 (100%)
<i>Pr. penneri</i>	4 (50%)	4 (50%)	0 (00%)	0 (00%)	8 (100%)
total	64 (68%)	23 (24.5%)	6 (6.4%)	1 (1.1%)	94 (100%)

Table 4: Virulence factors produced by *Proteus spp.*

Virulence factors	<i>Pr. mirabilis</i> (n=86)		<i>Pr. penneri</i> (n=8)	
	No.	%	No.	%
Urease	86	100	8	100
Gelatin liquefaction	86	100	5	62.5
Congo red binding	56	65.1	5	62.5
Deoxyribonuclease	52	60.5	4	50
Lipase	50	58.1	6	75
Lecithinase	50	58.1	2	25
Haemolysin	41	47.7	3	37.5
Protease	36	41.9	2	25

Virulence factors of *Proteus spp.*

Many evidence indicated that markedly different microbial pathogens use common strategies to cause infection and disease⁽¹⁷⁾. Many diverse bacterial pathogens share common mechanisms in terms of their abilities to adhere, invade, and cause damage to host cells and tissues,⁽¹⁸⁾ as well as to survive host defenses and establish infection. Many of these infections appeared to be related to the acquisition of large blocks of virulence genes from a common microbial ancestor, which can be disseminated to other bacteria⁽¹⁹⁾. In the present study, a total of 94 *Proteus spp.* were isolated from children suffering from diarrhea.

Concerning the production of urease enzymes, all *Proteus spp.* (*Pr. mirabilis* and *Pr. penneri*) showed high ability to produce urease enzymes. This was similar to previous reports by Gendlina *et al.* (2002) who demonstrated that *Proteus spp.* produced high amount of urease compared with other bacteria. Urease enzyme was known to hydrolyzes urea into ammonia and ultimately carbon dioxide^(20, 21). As a result of ammonia production, an increase in local pH causes precipitation of normally soluble calcium and magnesium ions. These salt crystals can grow to remarkable size to produce bladder and kidney stones⁽²²⁾.

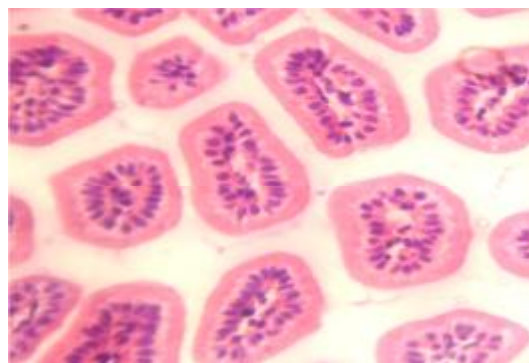
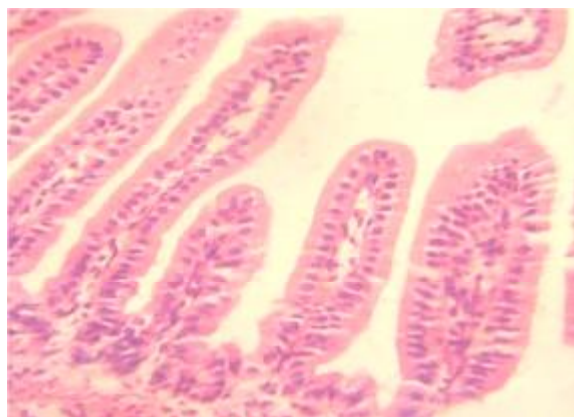
For hemolysin 41 (47.7%) of *Pr. mirabilis* strains and 3(37.5%) of *Pr. penneri* demonstrate hemolytic activity. some isolates possessed strong activities than other which showed weak hemolytic activity⁽²¹⁾.

Congo red (CR) dye agar test was first used by Surgalla and Beasley for differentiation of virulent and a virulent *Yersinia pestis*⁽²³⁾. Subsequently, it was used by other researchers as phenotypic marker to differentiated invasive and non- invasive *E. coli* and *Shigella spp.*⁽²⁴⁾.

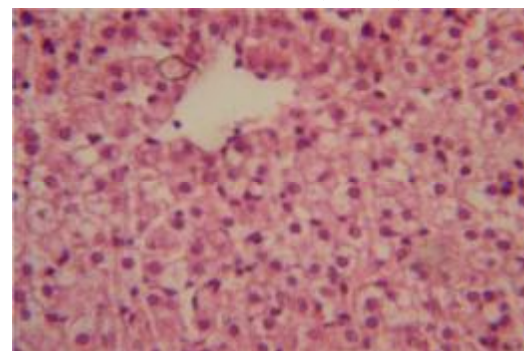
In the present study, the results showed that from 94 *Proteus spp.* 56 (65.1%) *Pr. mirabilis* 5 (62.5%) *Pr. penneri* were positive (red colony) (Table -4). The results of El-Baghdady, *et al.* (2009) study showed that all *Proteus spp.* isolated from different clinical samples exhibited both high invasion capability and epithelial cell cytotoxicity⁽²⁰⁾. This was also reported by Jacobsen *et al.* (2008) who reviewed invasion, cytotoxicity and biofilm formation of *Proteus mirabilis* and *Escherichia coli*⁽²⁵⁾.

All tested isolates showed variable ability to produce extracellular protease, gelatin liquefaction, lecithinase, lipase and DNase production (Table-4). Metalloproteases are a major group of proteolytic enzymes in *Proteus* strains⁽²⁶⁾. Using PCR, Stankowska *et al* (2008) found that genes protease *zapA* were found in all studied isolates⁽²⁷⁾. More than half or two third of the isolates showed ability to produce lecithinase, lipase and DNase production. However they proved that the virulence factors in *Pr. mirabilis*, were strain-specific. This was also demonstrated in this study that only 42% of the collected samples were able to produce elastase activities⁽²¹⁾.

Isolate number 7 of *Proteus mirabilis* was possessed all virulence characters was selected for studying the histopathological changes. Suckling mice infection was done through a feeding needle with 0.1 ml of 2×10^6 cell/ml *Proteus mirabilis*. Suspension, after 24 hours post-infection, the liver and intestine of tested mice and control (mice sucked normal saline that used in preparation of bacterial suspension) were investigated for pathologic changes. Pathologic changes induced in the intestine of suckling mice by *Proteus mirabilis* infection were studied by conventional histological sections. Many polymorphonuclear leukocytes were seen in the lamina propria. There was villous atrophy, with considerable degeneration. The control mice's intestine histology section showed normal villous architecture with uniform microvillus brush border and no inflammatory changes. Also the control mice's liver histology section showed normal liver architecture with normal cell and no inflammatory changes. The pathologic changes induced in the liver of suckling mice by *Proteus mirabilis* included swelling hepatocytes and distribution of polymorphonuclear. There was hemorrhage, with considerable fatty degeneration, also tissue damage with necrotic and vaculation was seen in the liver tissues (Figure-1, 2, 3, 4, 5, 6) .



Intestine section of treated suckling mouse with normal saline after 24 h. (control)



Liver section of treated suckling mouse with normal saline after 24 h. (control)

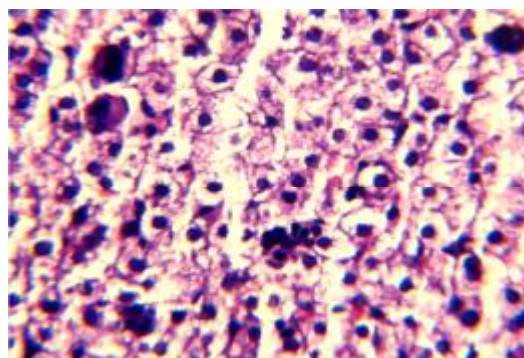


Figure 1: Liver section of treated suckling mouse with 2×10^6 of *Proteus mirabilis* suspension / ml, after 24 h. has and hemorrhage (H), Lymphocyte Infiltration (LI), karyolysis (KL), pyknotic nuclei (PN), giant cell (GC) and hypertrophy of kupffer cell (HKC) (H & E, 400 X).

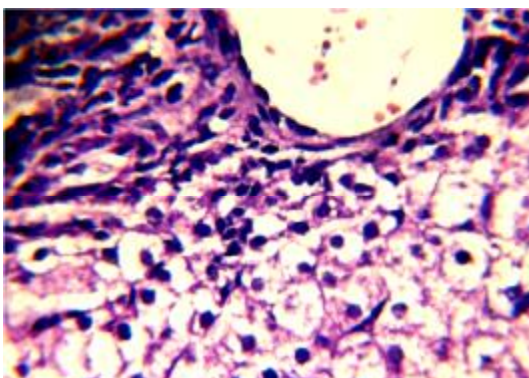


Figure 2: Liver section of treated suckling mouse with 2×10^6 of *Proteus mirabilis* suspension/ml, after 24 h. has vaculation in the cytoplasm of hepatocytes (VC), lymphocyte infiltration (LI), hypertrophy of kupffer cell (HKC) and karyolysis (KL), (H & E, 400X).

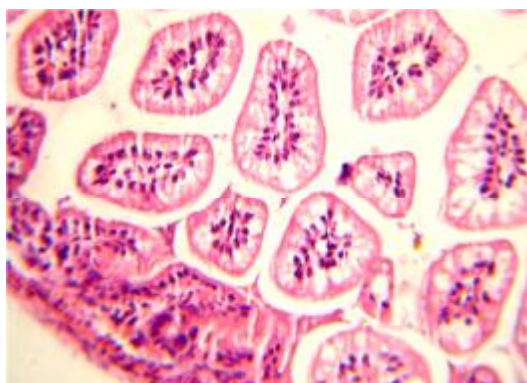


Figure 3: Intestine section of treated suckling mouse with 2×10^6 of *Proteus mirabilis* suspension/ml, after 24 h. has karyolysis (KL) and hemorrhage (H), (H & E, 400 X).

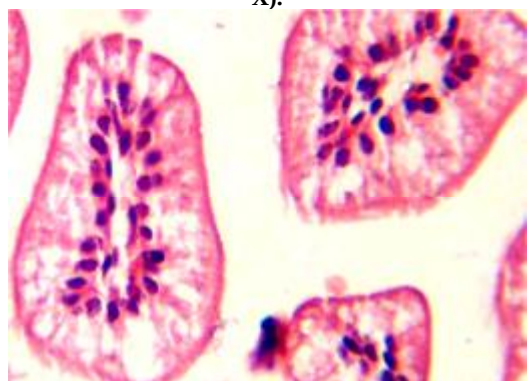


Figure 4: magnified figure of Figure 3

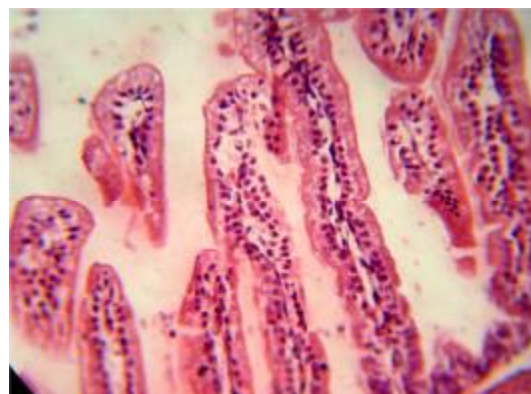


Figure 5: Intestine section of treated suckling mouse with 2×10^6 of *Proteus mirabilis* suspension/ml, after 24 h. the villous lack its normal cytoarchitecture (H & E, 400 X).



Figure 6: Intestine section of treated suckling mouse with 2×10^6 of *Proteus mirabilis* suspension/ml, after 24 h. has villous atrophy (VA) (H & E, 400 X).

References

1. Skinner FA, Car JG. The normal microflora of man. Academic Press. London, New York. 1976; p.188.
2. Collins MD, Gibson GR. Probiotics, Prebiotics and synbiotics: approaches for modulating the microbial ecology of the gut. *Am J Clin Nutr* 1999; 69 (5): 10525-10575. [Abstract]
3. Brooks GF, Butel JS, Morse SA. "Medical Microbiology". Jawetz, Melnick and Adelbergs. 25th ed., McGraw-Hill Companies. Appleton and Lange, California. 2010.
4. Warren JW, Tenney JH, Hoopes JM, Muncie HL, Anthony WC. (1982). A prospective microbiologic study of bacteriuria in patients with chronic indwelling urethral catheters. *J. Infect. Dis.* 146:719-723.
5. Coker C, Poore CA, Li X, Mobley HL. (2000). Pathogenesis of *Proteus mirabilis* urinary tract infection. *Microbes Infect.* 2:1497- 1505.
6. Brandenburg K, Wiese A. Endotoxins: relationships between structure, function, and activity. *Curr Top Med Chem.* 2004;4:1127-46.
7. Mayeux PR. (1997). Pathobiology of lipopolysaccharide. *J Toxicol Environ Health.* 51:415-35.
8. Baron EJ, Peterson LR, Tenenbaum SM. Baily & Scott's Diagnostic microbiology. 9th ed. Mosby Company. USA. 1994.
9. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's manual of determinable bacteriology 9th ed . William and Wilkins, Baltimore. 1994.
10. Branson D. Methods in clinical bacteriology manual of tests and procedures. Springfield. Illinois. USA. 1972.
11. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's manual of determinable bacteriology 9th ed . William and Wilkins, Baltimore. 1994.
12. Al-Qas MA. A study of *Providencia alcalifaciens* isolated from infantile diarrhoeal cases. M. Sc. Thesis. University of Mosul. College of Science. 2002. (In Arabic).
13. Adlard PA, Kirov SM, Sanderson K, Cox GE. *Pseudomonas aeruginosa* as a cause of infectious diarrhea. *Epidemiol Infect.* 1998; 121: 237-241.
14. Baker FJ, Silverton RE, Luckcock ED. An introduction to medical laboratory technology. 4th edition. Butterworth. 1975.
15. Pickering LK, Evans DG, Dupont HL, Vollet JJ, and Evans DJ. Diarrhea caused by Shigella, Rotavirus

and Giardia in day-care centers: Prospective study. The Journal of Pediatrics. 1981; 99(1); 51-56.

16. Al-Sekait MA. A study of factors affecting incidence of diarrheal disease in children under 5 years in Saudi Arabia. Saudi Medical Journal. 1988; 9(5): 491-497.

17. Tang, H., Kays M. and Prince, A. (1995). Role of *Pseudomonas aeruginosa* pili in acute pulmonary infection. *Infect. Immun.* 63:1278-1285.

18. Finlay, B.B. and Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microb. Mol. Biol. Rev.* 16:136-169.

19. Hentzer, M. and Givskov, M. (2003). Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J. Clin. Invest.* 112:1300-1307.

20. Gendlina I., Gutman, D.M., Thomas, V., Collins, C.M. (2002). Urease-dependent signal transduction by the virulence regulator UreR. *J Biol Chem.* 277:37349-37358.

21. El-Baghdady Kh Z, Abooulwafa M M, Ghobashy M O, Gebreel H M. (2009). Plasmid mediated virulence factors of some *Proteus* isolates. Egypt. Acad. J. biolog. Sci., 1(1):7-22

22. Li, X., Zhao, H., Lockatell, C.V., Drachenberg, C.B., Johnson, D. E. and Mobley, H.L. (2002). Visualization of *Proteus mirabilis* within the matrix

of urease-induced bladder stones during experimental urinary tract infection. *Infect. Immun.* 70:389-394.

23. Surgalla, MJ, Beasley ED. Congo red medium for detecting pigmentation in *Pasteurella pestis*. *Appl. Microbiol.* 1969; 18, 834-837.

24. Berkhoff HA, Vinal AC. Congo red medium to distinguish between invasive and non invasive *Escherichia coli* pathogenic for poultry. *Avian Dis.*, 1986; 30, 117-121. Cited by Sharma *et al.* 2006.

25. Jacobsen, S.M., Stickler, D.J. Mobley, H.L.T. and Shirliff, M.E. (2008). Complicated Catheter-Associated Urinary Tract Infections Due to *Escherichia coli* and *Proteus mirabilis* *Clin. Microbiol. Reviews* 21: 26-59.

26. Belas R, Manos J, Suvanasuthi R. (2004). *Proteus mirabilis* ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides. *Infect Immun.* 72: 5159-67.

27. Stankowska, D., Kwinkowski, M., and Kaca, W. (2008). Quantification of *Proteus mirabilis* virulence factors and modulation by acylated homoserine lactones. *J. Microbiol. Immunol. Infect.* 41:243-253.

28. Jones BD. Host responses to pathogenic *Salmonella* infection. Cold Spring Harbor Laboratory Press. USA. 1997.

دراسة بعض عوامل ضراوة بكتريا *Proteus mirabilis* و *Proteus penneri* ذات العلاقة بالإسهال

سهام شكور عبيد الصالحي

الكلية التقنية ، كركوك ، العراق

الملخص:

تم تحديد بعض عوامل الضراوة المتضمنة إنتاج بعض الإنزيمات المحللة البروتين، اللايباز، الليسيثيناز، الأنزيم المحلل للـ DNA، تحلل الجيلاتين، إنتاج الهيموليسين، الإختراقية لخلايا العائل (الارتباط بصبغة الكونغو الاحمر)، اليوريز لـ 94 عزلة *Proteus* منه 86 (91.5%) تعود للنوع *Proteus mirabilis* و 8 (8,5%) تعود للنوع *Proteus penneri*. وقد اظهرت الدراسة بان جميع العزلات كانت منتجة لأنزيم اليوريز ولكنها متفاوتة في إنتاجها للعوامل الأخرى. ولدراسة خاصية الغزو والأمراضية تم تجريب صغار الفئران بمعلق جرثومة *Proteus mirabilis* والتي تمتلك عوامل الضراوة أعلاه التي أجريت في الزجاج وحضرت مقاطع نسيجية لمعرفة طريقة اختراق هذه الجراثيم للخلايا المعوية والتغيرات النسيجية في الأمعاء والكبد. بينت نتائج الفحص المجهرى لهذه المقاطع النسيجية وجود آفات مرضية في نسيج الأمعاء الكبد للفئران المجرعة بمعلق العزلات المختارة مقارنة مع الاعضاء السليمة.