University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

Isolation and Characterization of Lytic, Morpholoical and pH Resistanece Properities of Two *Escherichia coli* O111 - Specific Phages from Sewage Samples

Hekmat K. Ateya

Microbiology Department, College of Veterinary Medicine, University of ThiQar, Iraq.

hekmatkadhum@utq.edu.iq

https://doi.org/10.32792/utq/utj/v

Abstract

In recent years, bacteriophages have piqued interest as a potential alternative antimicrobial therapy for bacterial infections. Because phage therapy is the most promising biological remedy of this period, its therapeutic success is a source of concern. Hence, the present study was aimed to isolate the potential bacteriophages against *Escherichia coli* O111 fromt sewage samples and to analyze their lytic and morphological traits. Bacteriophages were isolated from swage samples using the double agar overlay method. Isolated phages were purified by diluting in the SM-buffer and filtering through 0.22 μm filter. After purification through glycerol gradient The two phages (named P1 and P2) were visualized by transmission electron microscope , both phages

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

appeared to belong to *Myoviridae* characterized by icosahedral heads, necks and contractile tails with tail fibers. The two phages were resistant to pH from 5 to 9 to an acidic and alkaline environment. Therefore, these phages may be utilize as biocontrol agents alternative for antibiotic but certainly further studies (especially whole sequencing and bioinformatic analysis of the phage genomes) are required as well as assessment of their activity in animals particularly cattle which are major reservoirs of this pathogens.

Keywords: Phage, *Escherichia coli* O111, Plague, TEM

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), a diverse group of foodborne bacteria that cause severe diarrhea and epidemics of hemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS) in human, has lately emerged as a serious zoonotic infection in humans, with domestic ruminants carrying the most bacterial serotypes. (Johnson *et al.*, 2006; Fan *et al.*, 2019) . More than 400 non-O157 STEC serotypes have been associated with sporadic human illness and outbreaks, with approximately 50 of these serotypes associated with human bloody diarrhea or HUS. (Johnson *et al.*, 2006). For the majority of human STEC diseases, including food borne outbreaks, seven non-O157 STEC serogroups, O26, O45, O103, O111, O121, O145, and O157, called 'top-7,' are responsible for (Brooks *et al.*, 2005; Scallan *et al.*, 2011;Valilis *et al.*, 2018).

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

Between 1987 and 1994, E coli O111 accounted for 50% of non-O157 STEC recovered from HUS patients in an Australian laboratory. (CDC,1995) In Japan, the O111 serotype was found to be the etiological agent in about 4% of EHEC infections (NIID,2011). Between 2006 and 2010, there were 83 EHEC outbreaks in Japan, each with ten or more EHEC-positive cases. Six of these outbreaks were caused by EHEC O111 strains (NIID, 2013): three by EHEC O111 stx1 strains and the other three by EHEC O111 stx1 stx2 strains. In August 2008, the largest known Escherichia coli O111 serotype outbreak in the United States occurred in Oklahoma, resulting in 341 illnesses, including hemolytic uremic syndrome (HUS). HUS is not well understood in non-O157 E coli outbreaks, However, it has happened in 2% to 15% of O157 infections, mostly in children.(Piercefield, *et al*., 2010)

Bacteriophages are viruses present in nature and have the ability to intrude bacteria. Beneficial effects of using bacteriophages to treat bacterial infections have been confirmed by many studies conducted on the humans and animals (Sulakvelidze *et al.*, 2001;Summers, 2001), as well as in treating food-borne bacteria prevalent in agriculture (Au *et al*.,2021).The safety and effectiveness of phage treatment comparing to antibiotics attributed to the bacteriophages specificity for certain bacteria, manifested as their ability to infect only one serotype, species or strain. This mode of action does not destroy the normal intestinal flora (Loc-Carrillo and Abedon,2011; Singh *et al*.,2022).

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

The goal of this research was therefore to isolate phages that were active against *E. coli* O111 and to characterize them with respect to morphology, lytic and pH stability properties, suggest that they could be used (after DNA genomic analysis) in food protection / medicine as an alternative to antibiotics.

Materials and methods

Phage isolation and titration. *E coli* O111 isolates (Ten *E coli* O111 isolates were kindly provided by Dr. Mukallad A. Ramadan , these were isolated from human, animals and meat sources and confirmed by PCR and Sequencing techniques) were used as bacterial host to isolate specific lytic phage from raw sewage (Sewage treamment unit, Basrah University campus). Phage was isolated by a standard enrichment procedure (Seeley *et al.*, 2001). Briefly, 15ml of raw sewage were centrifuged at speed 3,500 *g*, temperature at 10°C and time for 30 minutes. Millipore filter (0.45 µm-pore-size) was used to filterate the supernatants, then adding of this filtrate to Luria Bertani (LB) broth (10 ml), and 100 µl contain 10^8 CFU of *E. coli* 011 isolate was also added. This mixture was incubated for overnight at 37°C. This mixture was centrifuged at 10,000 *g* for 10 minutes to remove the bacteria and debris then 0.45 µm-pore-size filter was used to filterate the supernatants. Spot assay was used to test the coliphage activity of the supernatant was tested by put 5µl of coliphage on LB agar inoculated with a lawn of *Escherichia coli* (O111). After 5 hours at 37°C incubation, the plates were tested for plaques formation. Serial dilution was done to

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

supernatants which give lytic result, then by using a technique of top agar overlay with *Escherichia coli* O111 the plaques were isolated and purified (Sambrook and Russel,2001). The coliphage that gave plaques on all *E. coli* (O111) isolates lawn was selected for further studies.

Purification of Phage Lysates through Glycerol Gradient.

Glycerol gradient protocol was used to yield phage lysate with good purity to be suitable for the subsequent electron microscopy (Sambrook and Russel,2001).

Electron microscopy of coliophages

The morphology of coliphages was examined by transmission electron microscopy. A 10 µl drop of each phage suspension was negatively stained with 2% phosphotungstic acid and added on a copper grid surface then tested by transmission electron microscope (Zeiss EM10C electron microscopy located in Khajeh Nasir Toosi University of Technology, IRAN). Phages have been classified depending on their respective families as set out in the International Committee on Virus Taxonomy guidelines (Walker *et al*., 2019).

Resistance for the acid & alkali environments

A procedure described by Jamalludeen et al (2007) was used to test the ability of phages to survive at different pHs. Phage suspensions were exposed to adjusted pH values 1 to 11 (using NaOH or HCl solution) over 16 h of incubation at 37 °C, and then examined for viability.

University of Thi-Qar Journal

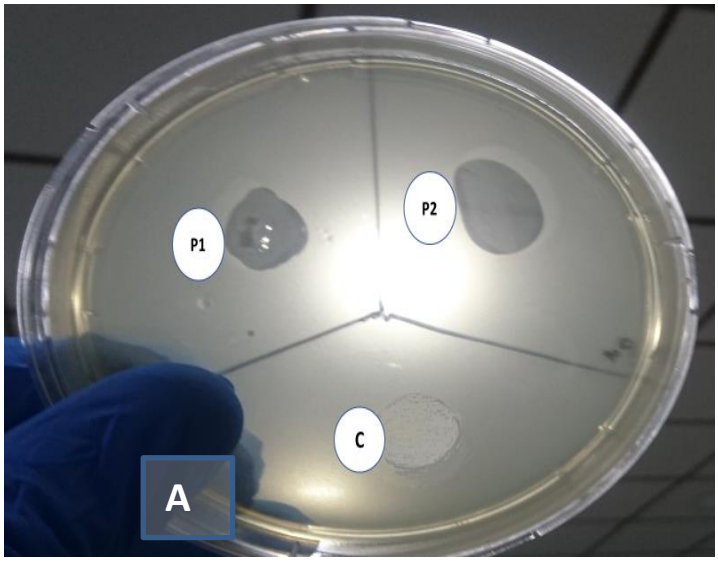
ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

Results

Bacteriophage isolation

Phages were successfully isolated through classical amplification from samples of sewage (Figure 1). These phages were showing a strong lytic activity against *E. coli* 111). P1 and P2 were purified by repeated plating and picking of single isolated plaques from the lawns of target host (*E. coli* O11). The titre of the two phages P1 and P2 was $1.7x10^{10}$ and $2.6 x10^{10}$ respectively. A stock from two phages was prepared and purified. Both phages produced tiny clear plaques with no halo on a lawn of *E. coli* O111. The isolated phages were tested by spot test against all ten *E. coli* O111, Both phages showed lytic activity and clear zone of lysis (Figure 1).



University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

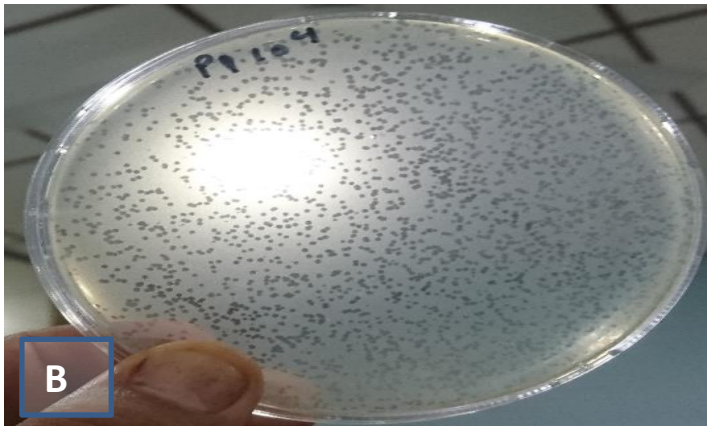


Figure 1 **A**. Bacteriophage isolation B. Spot test of the two phages (P1,and P2) against *E. coli* O111 strain showing a clear inhibition zone. C = control without phage (Only SM buffer). **B**: isolated phages showing tiny clear plaques on double agar overlay

Characterization of coliphage

Morphology of coliphage. The morphological characteristics of the three phages by transmission electron microscopy was revealed as in Figure 2. The phage P1 had icosahedral head and long thin flexible non-contractile tail with fibres. Depend on their morphological charcteristics, these phages under the family *Siphoviridae* (order *Caudovirales*), while Phage P2 had icosahedral head and less rigid, long and relatively thick tail with tail fibres suggesting that this phage under the family *Myoviridae* (order *Caudovirales*).

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

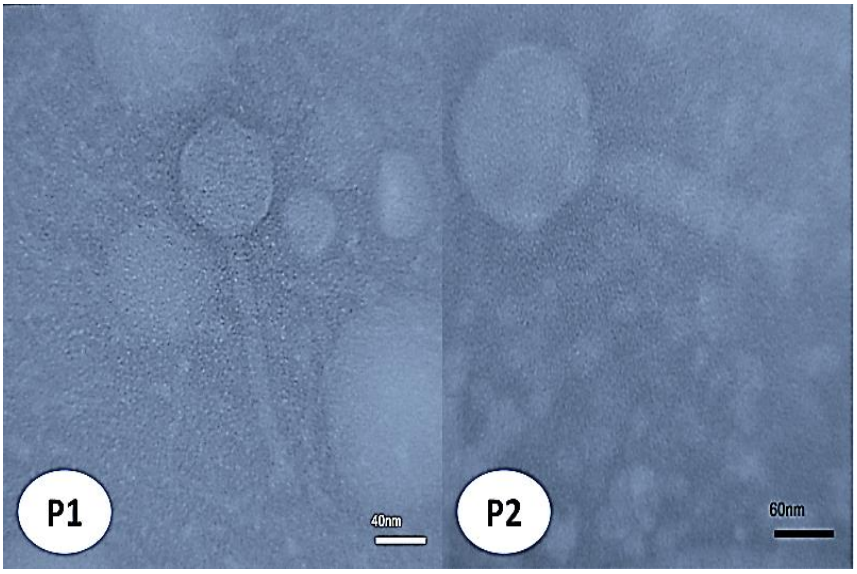


Figure 2. Morphological charateristics of two isolated phages by TEM , the phage P1 has long non contractile flexible of tail. Bar= 40nm, while P2 has less long, rigid, and relatively thick tail Bar=60.

The dimensions of head and tail of these two phages were revealed in Table 1. Five images of each phage were measured, and the mean values were recorded.

Table 1 Estimated dimensions of P1, and P2. Each value was the mean of five independent measurements.

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

Phage name	Dimensions of head (nm)		Dimensions of tail (nm)	
	Length	Width	Length	Width
P1	73	66	174	11
P2	138	127	211	28

pH Resistance

The two phages were resistant to pH 5-9. Phage P1 and P2 were more resistant to acidic and alkaline environments with high survival rate (Table 2).

Table 3. Survival of phages P1 and P2 following exposure to pH 1-11

pH	Titre of surviving, viable phages (pfu/mL)	
	P1	**P2**

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

1 and 2	ND	ND
3	1.9×10^8	2.4×10^8
4	4.7×10^8	5.8×10^8
5-9	$\geq 10^8$	$\geq 10^8$
10	2.9×10^8	3.7×10^8
11	3×10^7	3.6×10^7
control	$\geq 10^8$	$\geq 10^8$

*ND=not detected

Discussion

Resistant pathogens are ever increasing and it is anticipated that those pathogens would emerge as a substantial global problem. These emerging MDR pathogens and unavailability of newer antibiotics has reintroduced the use of phages cited to its specificity and novel mode of

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

action. Hence, treatment of these menacing pathogens with the lytic bacteriophage and researches on it is gaining spotlight in this era (Yang et al.,2010)

The bacteriophages are widely spread and can present in many various environments but mainly in waste water treatment plants and sewage (Ribeiro *et al.*, 2018; Yildirim *et al.*, 2018).

Phages have been used by many researchers to biocontrol *E. coli* and others types of bacteria. In all cases, none of the phages reported have been able to lyse all strains (Hyman., 2019). The present study describes a new bacteriophage against *E. coli* O111, including a description of its morphology, lytic activity and ph resistance that could be used as an alternative to antibiotics.

In this study, newely isolated P1 and P2 revealed strong activity against local *Escherichia coli* O111. These phages were isolated from sewage treamment unit, Basrah University campus as wastewater has always been the main sources of phage isolation. Raw sewage was considered as the unique source for the isolation of phages in this study with high phage titre $\geq 10^{10}$ because the phage belongs *Siphoviridae*, to lesser extent *Myoviridae*, are known for their ability to resist adverse conditions due to their morphology (Muniesa *et al.*, 1999).

Huff et al (2002) also identified phages, designated SPRO2 and DAF6, which were only active against the O2 serotype of *E. coli* in chickens. Mohammed-Ali *et al*., (2015) and Jamalludeen., (2021) also isolated

phages against the pathogenic *Staphylococcus aureus* which were considered good candidates for eradication of MRSA infection.

From this study, phages were isolated against strains of EHEC O111 and targeted isolates from Basrah that have broad activity against *E. coli* isolates.

Acknowledgments

Most of this work was carried out in the Department of Microbiology, College of Veterinary Medicine, University of Basrah, Iraq. I really grateful to the management staff for the kind support.

Conflicts of interest

There are no conflicts of interest.

References

Au, A., Lee, H., Ye, T., Dave, U., & Rahman, A. (2021). Bacteriophages: Combating Antimicrobial Resistance in Food-Borne Bacteria Prevalent in Agriculture. *Microorganisms*, *10*(1), 46. https://doi.org/10.3390/microorganisms10010046.

Brooks, J. T., Sowers, E. G., Wells, J. G., Greene, K. D., Griffin, P. M., Hoekstra, R. M., et al. (2005). Non-O157 Shiga toxin-producing Escherichia coli infections in the United States, 1983-2002. J. Infect. Dis. 192, 1422–1429. doi: 10.1086/466536.

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

Centers for Disease Control and Prevention (CDC). Community outbreak of hemolytic uremic syndrome attributable to *Escherichia coli* O111:NM: South Australia 1995. *MMWR Morb Mortal Wkly Rep*. 1995;44(29):550-551, 557-558.

Fan, R., Shao, K., Yang, X., Bai, X., Fu, S., Sun, H., ... & Xiong, Y. (2019). High prevalence of non-O157 Shiga toxin-producing Escherichia coli in beef cattle detected by combining four selective agars. *BMC microbiology*, *19*(1), 1-9.

Huff, W. E., Huff, G. R., Rath, N. C., Balog, J. M., Xie, H., Moore,Jr, P. A., and Donoghue, A. M. (2002). Prevention of *Escherichia coli* respiratory infection in broiler chickens with bacteriophage (SPR02). Poult. Sci. 81: 437-441.

Hyman P. (2019). Phages for Phage Therapy: Isolation, Characterization, and Host Range Breadth. *Pharmaceuticals (Basel, Switzerland)*, *12*(1), 35. https://doi.org/10.3390/ph12010035

Jamalludeen N.M,. (2021). Nasal Carriage of *Staphylococcus aureus* in Healthy Children and its Possible Bacteriophage Isolates in Basrah, Iraq. *Biomed. & Pharmacol. J*, Vol. 14(1), 467-475.

Jamalludeen, N., Johnson, R. P., Friendship, R., Kropinski, A. M., Lingohr, E. J., and Gyles, C. L. (2007). Isolation and characterization of nine bacteriophages that lyse O149 enterotoxigenic *Escherichia coli*. Vet. Microbiol. 124: 47-57.

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

Johnson, K. E., Thorpe, C. M., and Sears, C. L. (2006). The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. *Clin. Infect. Dis.* 43, 1587–1595. doi: 10.1086/509573.

Loc-Carrillo C, Abedon ST. (2011). Pros and cons of phage therapy. Bacteriophage.;1:111–4.

Mohammed-Ali, MN and Jamalludeen, NM. (2015). Isolation and Characterization of Bacteriophage against Methicillin Resistant Staphylococcus aureus. J Med Microb Diagn 5:1.

Muniesa, M.; Lucena, F. and Jofre, J., (1999). Comparative survival of free Shiga toxin 2-encoding phages and *Escherichia coli* strains outside the gut. *Applied and Environmental Microbiology*. 65(12):5615–5618.

National Institute of Infectious Diseases. 2011. Enterohemorrhagic *Escherichia coli* infection in Japan as of April 2011. IASR **32:**125–126. http: //idsc.nih.go.jp/iasr/32/375/tpc375.html

National Institute of Infectious Diseases. 2013. Enterohemorrhagic *Escherichia coli* infection in Japan as of April 2013. IASR **34:**123–124. http: //www.nih.go.jp/niid/en/iasr-vol34-e/865-iasr/3570-tps399.html.

Piercefield EW et al. (2010). Hemolytic uremic syndrome after an *Escherichia coli* O111 outbreak. *Arch Intern Med* Oct 11; 170:1656.

Ribeiro, K. V. G.; Ribeiro, C.; Dias, R. S.; Cardoso, S. A.; de Paula, S. O.; Zanuncio, J. C. and de Oliveira, L. L., (2018). Bacteriophage

isolated from sewage eliminates and prevents the establishment of *Escherichia coli* biofilm. *Advanced Pharmaceutical Bulletin*.,8(1):85–95.

Sambrook, J. and Russel, D. W., **(2001).** Molecular Cloning : A Laboratory Manual. (Sambrook, J. & W. Russel, D, Eds.)*Cold Spring Harboc Laboratory Press*. Thid. Cold Sprong Harbor, New York 3.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., and Roy, S. L. (2011). Foodborne illness acquired in the United States—major pathogens. Emerg. Infect. Dis. 17, 7–15. doi: 10.3201/eid1701.P11101.

Seeley, H. W.; VanDemark, P. J. and Lee, J. J. (2001). Microbes in action: a laboratory manual of microbiology, 4th ed. W. H. Freeman and Company, New York;N.Y.

Singh A, Padmesh S, Dwivedi M, Kostova I. (2022). How Good are Bacteriophages as an Alternative Therapy to Mitigate Biofilms of Nosocomial Infections. *Infect Drug Resist*. 15:503-532 https://doi.org/10.2147/IDR.S348700

Sulakvelidze, A.; Alavidze, Z and G.M., J., **(2001).** Minireview, Bacteriophage Therapy. *Antimicrobial Agents and Chemotherapy*. 45(3):649–659.

Summers, W. C., **(2001).** Bacteriophage Therapy. *Annu. Rev. Microbiol.*,55437–51.

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

Valilis, E., Ramsey, A., Sidiq, S., and DuPont, H. L. (2018). Non-O157 Shiga toxin- producing Escherichia coli-A poorly appreciated enteric pathogen: systematic review. Int. J. Infect. Dis. 76, 82–87. doi: 10.1016/j.ijid.2018.09.002.

Walker PJ, Siddell SG, Lefkowitz EJ, Mushegian AR, Dempsey DM, Dutilh BE, et al. (2019). Changes to virus taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses .Arch Virol [Internet]. 2019;(0123456789).

Yang, H. L; Liang, S; Lin, and S. Jia, (2010). Isolation and characterization of a virulent bacteriophage AB1 of Acinetobacter baumannii," *BMC Microbiology*, 10(1) : 131.

Yildirim, Z.; Sakin, T. and Çoban, F., (2018). Isolation of Anti-*Escherichia coli* O157:H7 Bacteriophages and Determination of Their Host Ranges. *Turkish Journal of Agriculture - Food Science and Technology*.,6(9):1200.

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.17 No.4 Dec 2022

عزل وتوصيف الخصائص التحللية والمظهرية ومقاومة الحموضة لنوعين من العاثيات المحللة للنوع المصلي O111 لبكتريا الايشرشيا القولونية

الخلاصة

ازداد في السنوات الاخيرة استخدام العاثيات كعلاج بديل عن المضادات الحياتية للاحياء المجهرية المسببة للأمراض المعدية ولكون ان الفعالية العلاجية بالعاثيات أمرًا مثيرًا للاهتمام اذ أنه العلاج البيولوجي الواعد في هذا العصر لذلك صممت الدراسة الحالية ، اذ هدفت هذه الدراسة إلى عزل العاثيات المحتملة المضادة لبكتريا الايشرشيا القولونيةO111 من عينات مياه الصرف الصحي وتحليل سماتها التحليلية والشكلية باستخدام المجهر الالكتروني . اذ عزلت عاثيات البكتيريا من عينات انابيب الصرف الصحي باستخدام طريقة تحميل الأجار المزدوج و تمت تنقيتها بواسطة بالتخفيف في المحلول المتعادل , SMورشحت باستخدام مرشح 0.22 ميكرومتر. اظهرت النتائج بان هناك نوعين من العاثيات (سميت P1 و P2) والتي تنتمي إلى Myoviridae اذ تمتاز برؤوس متعدد الوجوه ، واعناق وذيول قابلة للتقلص مزودة بألياف الذيل بنهايتها. كما اظهرت الدراسة الحالية بان العاثيتين مقاومة لدرجة الحموضة من 5 إلى 9 في بيئة حمضية وقلوية. لذلك يمكن استعمال هذه العاتيات كعوامل سيطرة بيولوجية كبدائل للمضادات الحيوية لكن رغم ذلك نحتاج دراسات اخرى مثل تحليل تسلسل الجينوم الكلي والتحليل البيولوجي بالاضافة الى تقييم فعالياتها في الحيوانات الحقلية مثل الابقاروالتي تعتبر خازن رئيسي لهذه البكتريا.

الكلمات المفتاحية: العاثي, البكتريا الايشرشيا القولونيةO111 , البقعة التحللية, المجهر الالكتروني النافذ