Virulence Factors and Antibiotic Profiles of *Bacillus cereus* Isolated from Stool and Vomitus of Inpatients with Acute Diarrhea

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ABSTRACT

Ninety five specimens were collected from inpatients suffering from diarrhea at Al-Zubair General Hospital, including 50 stool and 45 vomitus samples. *Bacillus cereus* was presumptively identified by the appearance of red-purple colonies surrounded by a halo of white precipitate after culturing samples on the selective medium Mannitol-Egg Yolk Polymyxin B Agar (MYPA). Identification was confirmed by characterization tests and resistance to penicillin. *B.cereus* was recovered from 19 samples (20%): 13 from stool (26%) and 6 from vomitus (13%).The highest recovery percentage was among children aged less than 10 years (30%) and the least was from adults aged above 40 years (14%) with significant difference($P<0.05$). *B.cereus* was recovered in stool and vomitus specimens of the same patients in 8 cases.

All recovered isolates from vomitus were able to produce hemlysin, casienase and gelatinase (100%) while only hemlysin and casienase were produced by all recovered isolates from stool. Only 83.3% of vomitus and 76.9% of stool isolates were able to lyse starch. Recovered isolates from both sources exhibit swarming motility with higher percentage shown by vomitus isolates (80% *VS* 66.6%).

Ability of *B.cereus* to grow at low temperatures was determined; 40% of each vomitus and stool isolates were able to grow and reproduce at $4^{O}C$, growth rate was raised to 80% and 60% at $6^{O}C$ respectively and reached up to 100% at $10^{O}C$. The mean time for survival of spores of stool and vomitus isolates at $100^{O}C$ was 4.1 and 4.25 min. respectively. The study has proved ability of stool isolate to produce enterotoxin when injected in the vein of mice tail. Isolates from both sources were almost equally highly resistant to ampicillin, carbencillin, tetracycline and streptomycin. The least resistance was toward gentamycin,

erythromycin and chloramphenicol, which makes them the drug of choice. Four patterns of antibiotic resistance were reported among isolates of *B. cereus.*

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Introduction:

Bacillus cereus is a gram-positive aerobic or facultatively anaerobic spore-forming rod, widely distributed in the environment (Kotiranta, *et al.*. 2000; Banerjee, *et al.*. 2011). Its pathogenic spectrum ranges from strains used as probiotics to human-lethal strains (Kamar, *et al.*. 2013). *Bacillus cereus* is a cause of food poisoning, which is frequently associated with the consumption of rice-based dishes. It causes two distinct food poisoning syndromes: Rapid-onset emetic syndrome which is food intoxication caused by emetic toxin and slow-onset diarrheal syndrome which is an infection caused by vegetative cells, ingested as viable cells or spores, thought to produce protein enterotoxins in the small intestine (Schoeni and Wong, 2005). A broad range of secreted cytotoxic factors, are produced during growth including at least four hemolysins (Sineva, *et al.* 2012; ; Ramarao and Sanchis 2013), several phosphor-lipases, proteases, an emetic toxin and a score of pore-forming toxins (Arnesen *et al.* 2008 , Bottone, 2010). Nonhemolytic enterotoxin also has been associated with the diarrheal syndrome (Heilkenbrinker, *et al.*. 2013). Toxins may contribute to the pathogenicity of *B. cereus* in non gastrointestinal disease including wound and eye infections, systemic infections and periodontitis, fatal pneumonia resembling anthrax (Hoffmaster, *et al.* 2006), a prostate wound (Turnbull, *et al.* 1979), endo-phthalmitis and meningitis (Bottone, 2010). It was also isolated from bronchial lavage fluid and transbronchial biopsy specimen, necrotizing pneumonia in immunocompromised hosts particularly in those developing transient gastroenteritis symptoms (Miyata, *et al.* 2013; Ramarao and Sanchis 2013).

Swarming is the fastest known bacterial mode of surface translocation and enables the rapid colonization of a nutrient-rich environment and host tissues. It has now become clear that many of these pathways also affect the formation of biofilms, surface-attached bacterial colonies (Verstraeten, *et al.*. 2008). Recent reports indicate that *Bacillus* species potentially

form biofilms and cause serious problems, such as antibiotic resistance, medical device-related infections and nosocomial bacteremia via catheter infection (Kuroki, *et al.*. 2009). It was reported that strains involved in gut colonization were better biofilms formers (Auger, *et al.*. 2009).

B. cereus produces beta-lactamases unlike *Bacillus anthracis*, and so is resistant to beta-lactam antibiotics; it is usually susceptible to treatment with clindamycin, vancomycin, gentamicin, chloramphenicol, and erythromycin (Brook, 2001; Bottone, 2010). The aim of the present study is to determine incidence of *B. cereus* in stool and vomitus sampled from inpatients with acute diarrhea, and to study some of virulence factors, antibiogram profiles and patterns of antibiotic resistance of recovered isolates.

Material and Methods:

Samples: Ninety five specimens were collected from inpatients suffering from diarrhea at Al-Zubair General Hospital / Basrah city / Iraq including 50 stool and 45 vomitus samples.

Culturing and identification: Swabs from stools and vomitus were cultured on the selective and differential medium mannitol egg yolk polymyxin blood agar. It consists of: tryptone(10gm), meat extract(1gm), D-mannitol(10gm), sodium chloride(10gm), phenol red(0.025gm), agar(12gm) suspended in distilled water(900ml). The medium was autoclaved and cooled to 45^{o}C. The medium was supplemented aseptically with 100ml egg yolk and 10mg polymyxin B (Mossel, *et al.* 1967). Colonies not utilizing mannitol, producing phospholipase C, were selected, purified and cultured on nutrient agar and subjected to the following characterization tests: Gram and spore staining, motility, production of acid and citrate and resistance to penicillin G (Harley and Prescott 1996, Wong, *et al.*. 1988; Banerjee, *et al.*. 2011).

Detection of virulence factors:

Enzyme production: Ability of recovered isolates to produce hemolysin, gelatinase, amylase and casienase was determined according to Harley and Prescott (1996).

Swarming motility: Surface swarming colonies of recovered isolates of *B.cereus* on nutrient agar was detected according to Kirov, *et al.* (2004). Loopfuls of normal saline (NaCl 0.85 %) containing grown

isolates at concentration of 10^4 were placed centrally on plates containing semisolid nutrient medium (0.5g Agar, 1.3g Nutrient broth in 100ml distilled water). Plates were incubated at 30^OC for 16-18hrs.

Growth at low temperature: Growth of recovered isolates at 4^oC, 6^oC and 10^o C was determined according to Jaquette and Beuchat (1998).

Resistance *of B.cereus* Spores to high temperature was determined according to Wong, *et al.* (1988).

Enterotoxin production: Three *B .cereus* isolates, from stool, soil and from rice were examined for their ability to produce enterotoxin by injecting 0.5ml cell filtrate in the vein of mice tail (Wong, *et al.*. 1988).

Resistance to Antibiotics: Disk-plate method using Mullar-Hinton agar was applied to detect antibiotic susceptibility of recovered isolates toward the following antibiotics: Erythromycin (15µg), Gentamycin (10µg), Tetracyclin (30µg), Streptomycin (10µg), Chloramphenicol (30µg), Cephalothin (30µg), Nalidixic acid (30µg), Ampicillin (10µg), Carbenicillin (10µg), Sufamethoxazote-trimethoprim (25µg). Inhibition zones was measured in millimeter and compared with standard tables (CLSI, 2008).

Results:

Figure (1) demonstrates streaks of purified selected colonies of *B.cereus* subcultured on MYP agar. Colonies are rough and dry with a bright pink background surrounded by an egg yolk precipitate. Identification of colonies was confirmed when cells were shown to be Gram positive, spore forming, motile bacilli producing citrate and acid from glucose with no gas and were resistant to penicillin G.

Fig. (1) *Bacillus cereus* on Mannitol Egg Yolk Polymyxin Agar

Incidence of *B.cereus* in stool and vomitus:

Table (1) illustrates that percentage recovery of *B. cereus* from stool samples was as twice as that recovered from vomitus samples. *B.cereus* was recovered from 19 samples (20%): 13 from stool (26%) and 6 from less vomitus (13%).

Table (1) Percentage recovery of *B. cereus* from stool and vomitus

Samples	No. of Samples	No. of Positive Samples (%)
Stool	**50**	**13(26)**
Vomitus	**45**	**6 (13)**
Total	**95**	**19 (20)**

Table (2) clarify that highest percentage recovery of *B. cereus* was among children under the age of 10 years (30%) and the least was from adults aged above 40 years (14%) with a significant difference($P<0.05$). It should be noted that *B.cereus* was recovered in the stool and vomitus specimens of the same patients in 8 cases.

Table (2) Percentage recovery of *B. cereus* from stool and vomitus according to age and sex

Age Category	Stool N (%)	Vomitus N (%)	Stool & Vomitus N (%)	Total N (%)
3mon.-10yrs Male: N=20 Female: N=20	 3(15) 2 (10)	 1 (5) -	 2+2 (20) 1+1 (10)	 8 (40) 4(20)
11-20yrs. Male: N=10 Female: N=10	 1 (10) 1 (10)	 1 (10) -	 1 (10) -	 2 (20) 1 (10)
21- 40 yrs. Male: N=10 Female: N=10	 1 (10) -	 - -	 - 1 + 1 (20)	 1 (10) 2 (20)
Above 40 yrs. Male: N=10 Female: N=10	 - 1 (14)	 - -	 - -	 - 1 (14)

All recovered isolates from vomitus were able to produce hemlysin, casienase and gelatinase (100%) while only hemlysin and casienase were produced by all recovered isolates from stool. Only 83.3% of vomitus and 76.9% of stool isolates were able to lyse starch (Fig.2)

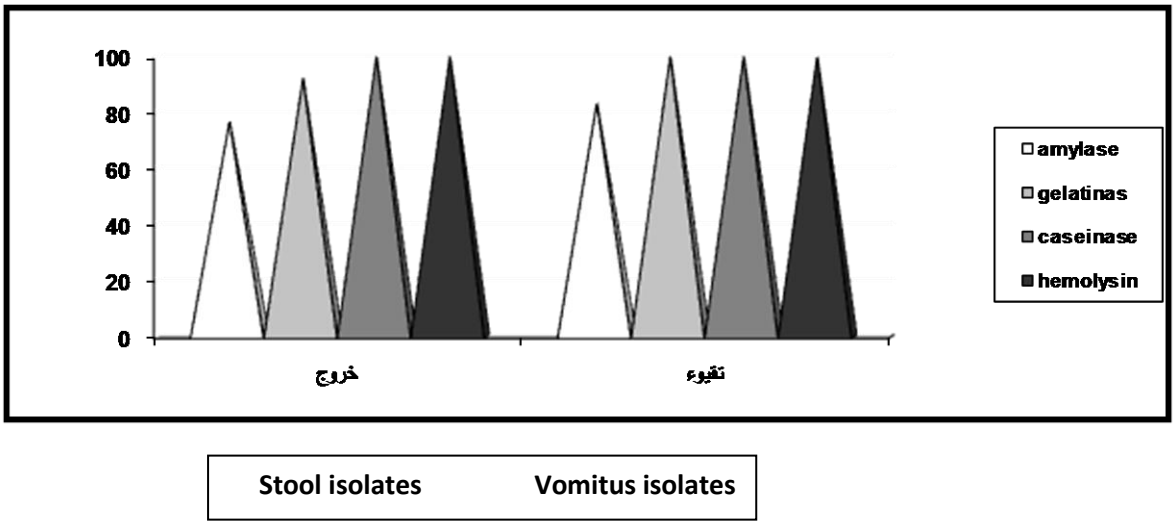


Fig. (2) Percentage production of lytic enzyme by isolates of *B. cereus*

Bacilli isolated from vomitus showed higher potential for swarming (Fig.3) motility (80%) as compared to those isolated from stool (66.6%).

Fig. (3) Surface swarming colonies of *B. cereus* on nutrient agar

All *B.cereus* isolates (100%) were able to grow at 10^{o}C, though vomitus isolates showed higher potential than stool isolates to grow at 6^{o}C (80% *VS* less than 60%). However, less than 40% of isolates from both sources were able to grow at 4^{o}C.

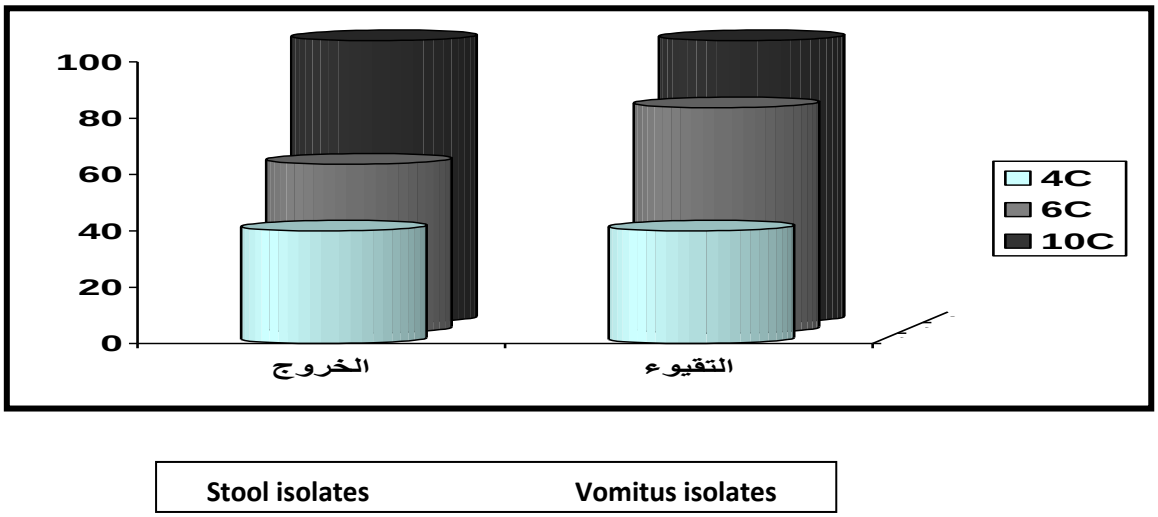


Fig. (4) A comparison of percentage capabilities of stool and vomitus isolates of *B.cereus* to grow at low temperatures

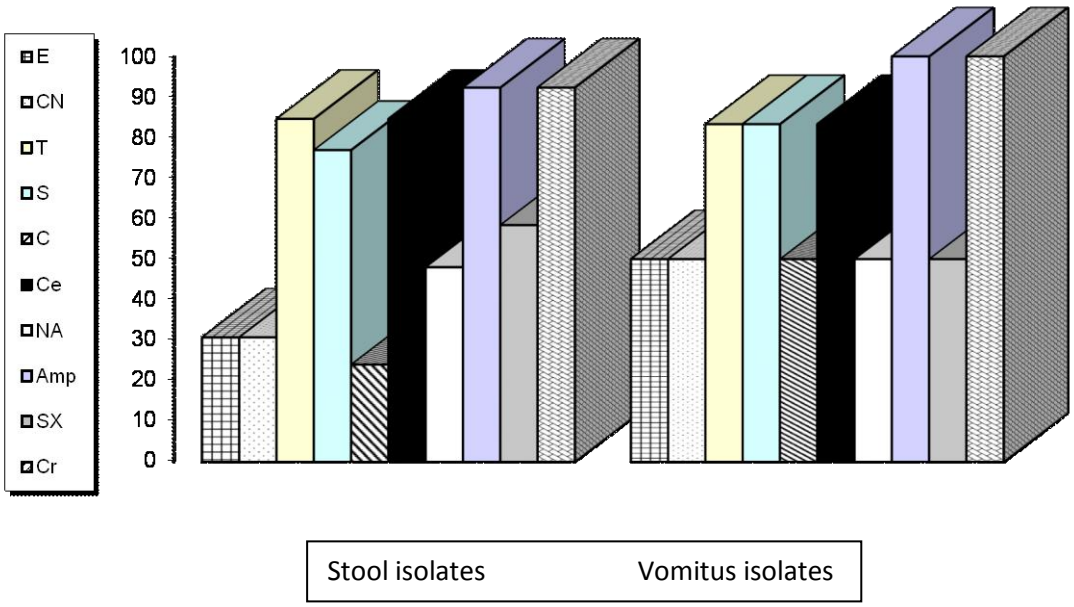
Average time for spores of *B. cereus* isolates from both sources to resist boiling temperature (100^{o}C) was 4.21 minutes (Table 3).

Table (3) Resistance of *B.cereus* spores to high temperatures(100^{o}C)

Source of Isolates	NO. of Isolates	Time	Av. In Min.
Stool	5	3-5	4.17
Vomitus	4	3-5	4.25

Enterotoxin production: Only stool isolate produced enterotoxin, while isolates from rice and stool were found negative.

Antibiograms and patterns of antibiotic resistance : Figure (5) illustrates that although vomitus isolates of *B.cereus* showed higher percentage of resistance against all antibiotics under study, nevertheless isolates from both sources were almost equally highly resistant to ampicillin, carbencillin, tetracycline and streptomycin. The least resistance was toward gentamycin, erythromycin and chloramphenicol.



E: Erythromycin, CN: Gentamycin, T: Tetracyclin, S: Streptomycin , C: Chloramphenicol, Ce:Cephalothin, NA: Nalidixic acid, Amp: Ampicillin, Cr: Carbenicillin, SXT: Sufamethoxazote-trimethoprim

Fig. (5) Antibiotic resistance of *B. cereus* Isolates recovered from stool and Vomitus

Four patterns of antibiotic resistance were reported among isolates of *B. cereus* (Table3). Stool isolates were included in the four patterns, whereas vomitus isolates were included in two patterns only

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Table (4) Patterns of antibiotic resistance of *B. cereus* isolates from

Patterns of Resistance	No. of Antibiotics Resistant to	Antibiotics	Resistant Isolates
1	10	Resistant to all antibiotics under study	St2, St4, Vo2, Vo4, Vo5,
2	8	E, T, S, Ce, NA, Amp, Cr, SXT	St3
3	5	T, S, Ce, Amp, Cr	St8, St9, St11, Vo1, Vo3,
4	3	Amp, Ce, Cr	St7, St10

stool and vomitus

E: Erythromycin, CN: Gentamycin, T: Tetracyclin, S: Streptomycin , C: Chloramphenicol, Ce:Cephalothin, NA: Nalidixic acid, Amp: Ampicillin, Cr: Carbenicillin, SXT: Sufamethoxazote-trimethoprim

Discussion:

Mannitol Egg Yolk Polymyxin Agar MYP Agar is a selective and differential medium developed by Mossel, *et al*.. (1967). The diagnostic features of the medium rely upon the failure of *Bacillus cereus* to utilize mannitol and the ability of most strains to produce phospholipase C. The medium is made selective by the addition of Polymyxin B which inhibits Gram-negative bacteria. MYP Agar has proved to be very effective for detecting *B. cereus* even for ratios as challenging as one cell of *Bacillus cereus* to 106 cells of other organisms (Downes and Ito, 2001).

Incidence of *B. cereus* was significantly higher in individuals aged less than 10

years than adults aged above 40 years which disagree with the results of Al-Khatib *et al.*. (2007) study in Amman/ Jordan. It could be due to the utilization of dried milk products and infant food as they are known to be frequently contaminated with *Bacillus cereus* (Becker, *et al.*. 1994). However, contaminated materials such the dishes, spoons and the dishcloth were reported to be the cause of contamination with *B. cereus* for both adults and children (*Choi, et al.*. 2011).

The unique properties of *B. cereus* such as heat resistance, endospore forming ability, germination and outgrowth capacity of *Bacillus cereus* spores in processed foods (van der Voort and Abee 2013), toxin production including enterotoxins, emetic toxin (cereulide), hemolysins, and phoshpolipase C as well as many enzymes such as beta-lactamases, proteases and collagenases and the psychrotrophic nature give sufficient capacity for this organism in causing the emetic type of gastrointestinal disease and to be a prime cause of public health hazard ((Schoeni and Wong, 2005; Arnesen, *et al.*. 2008, Bottone, 2010; Sineva, *et al*.. 2012, Ramarao and Sanchis , 2013). Many of these virulence factors were detected in the present study (Figs: 2 and 4 and Table3).

Swarming motility was detected among 80% and 66.6% of vomitus and stool isolates respectively (Fig. 3). An association between swarming and hemolysin BL secretion was observed by Ghelardi, *et al.*. (2007) in a collection of 42 *Bacillus cereus* isolates. The highest levels of toxin were detected in swarmers suggesting that swarming *B. cereus* strains may have a higher virulence potential than nonswarming strains (Ghelardi, et al.. 2007). Swarming and biofilm formation are strongly related to disease as swarm cells undergo rapid and coordinated population migration across solid surfaces via a phenomenon known as quorum sensing (Daniels, *et al.*. 2004). Manag-ement between swarming cells and biofilm formation is central to bacterial survival among competitors (Verstraeten, *et al.*. 2008). Strains involved in gut colonization were reported to be better biofilms formers (Auger, *et al.*. 2009).

It is confirmed in the present study, that spores of recovered *B. cereus* isolates from both sources were able to resist boiling

temperature (100^{o}C) for about 4.21 minutes in average (Table 3). Wjman, *et al.*. *(*2007) have proved that spores constituted up to 90% of the total biofilm counts, which indicates that *B. cereus* biofilms can act as a cavity for spore formation and subsequently can release them into the environments. They co-ordinate their virulence in order to escape the immune response of the host to be able to establish a successful infection. Moreover, van der Voort and Abee (2013) have confirmed that sporulation in complex conditions such as biofilms and surface swarming colonies increases heat resistance and dormancy of spores. Furthermore, bacterial populations use Quorum sensing (A process of cell–cell communication) that allows bacteria to share information about cell density, superior access nutrients and increases heat resistance and dormancy of spores that enables them to out-compete non-biofilm-producing neighbours (Nadell and Bassler 2011) in addition to adjustment of gene expression accordingly(Guillemet, *et al.*. 2013).

High percentage of resistance was detected among recovered isolates against ampicillin, carbencillin, tetracycline and streptomycin (Table3). This trend seems common in other regions as well (Whong and Kwaga, 2007; Banerjee, *et al.*. 2011) Isolates were allocated in four patterns of resistance. The first pattern which included five isolates from both sources (Table4) showed resistance against all isolates under study. It is reported that *B. cereus* produces a potent beta-lactamase that confer marked resistance to beta-lactam antibiotics (Bottone, 2010).

In conclusion, our results showed the importance of *B. cereus* among hospitalized patients with acute diarrhea. The study showed diverse virulence factors exhibited by isolates with no significant differences between isolates from either source. Knowledge of spectrum of antibiotic susceptibility will possibly become a guide to empirical therapy to shorten the morbidity in acute stage.

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عوامل الضراوة والمقاومة للمضادات الحيوية لجرثومة Bacillus cereus المعزولة من الخروج للمرضى المصابين بالاسهال الحاد والتقيؤ

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الخلاصة

جمعت 95 عينة من المرضى الراقدين في مستشفى الجمهوري العام / الزبير / مدينة البصرة / العراق يعانون من الإسهال، شملت الخروج (50 عينة) والتقيؤ (45 عينة). شخصت جرثومة ***Bacillus cereus*** بعد زرع العينات على الوسط الزرعي الإنتقائي (Mannitol-Egg Yolk Polymyxin B Agar (MYPA وظهور المستعمرات باللون الأحمر الوردي والمحاطة بهالة من الراسب الأبيض وكانت الخلايا عصوية موجبة لصبغة كرام مكونة للأبواغ ،منتجة للكاتليز، مخمرة للكلوكوزوغير منتجة للغاز، مستهلكة للسترات ومقاومة للبنسلين. عزلت جرثومة ***Bacillus cereus*** من19 عينة (20%) ، 13 من الخروج (26%) و6 من التقيؤ (13%) وكانت أعلى نسبة لظهور ***Bacillus cereus*** عند فئة الأطفال > 10 سنوات (30%)، وأدناها عند فئة البالغين < 40 سنة (14%) وبفارق معنوي (P) 0.05. ظهرت الجرثومة في خروج وتقيؤ نفس المريض عند 8 حالات.

تمكنت جميع عزلات التقيؤ من انتاج الإنزيم الحال للدم والكازائين والجيلاتين وتمكنت 83.3% من عزلات التقيؤ و76.9% من عزلات الخروج من حل النشا . استطاعت 80% من عزلات التقيؤ و66.6% من عزلات الخروج من الحركة الجماعية(Swarming Motility) .وعند اختبار قدرة العزلات على النمو في درجات الحرارة الواطئة ، تمكنت 40% من العزلات من النمو والتكاثر في 4 ^{O} م وارتفعت النسبة الى 80% و 60% لعزلات التقيؤ والخروج على التوالي في 6^{O}م حتى وصلت الى 100% في 10^{O}م لكلا المصدرين. كان المعدل الزمني لبقاء ومقاومة العزلات للحرارة العالية (100م) 4.1 و 4.25 دقيقة على التوالي . أثبتت الدراسة قدرة الجرثومة على انتاج السم المعوي الإسهالي عند الحقن داخل الوريد الذيلي للفأر .كانت جميع العزلات من المصدرين مقاومة تماما للمضادات الحيوية ampicillin, carbencillin, tetracycline and streptomycin واكنها كانت اقل مقاومة للمضادات الحيوية gentamycin, erythromycin and chloramphenicol مما يجعلهم الفضل للعلاج. تضمنت العزلات في اربعة انماط للمقاومة الحيوية .

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