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Bacteriological and Genetic study of some gram negative bacteria isolated from different infections

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Abstract

There are 30 of bacterial samples were isolated from 45 samples of different infections. These isolates were diagnosed microscopically and by using biochemical tests. ability of their resistance was tested against the following antibiotics: (amoxicillin, tetracycline, cephalexin, nitrofurantoin, ciprofloxacin, clarithromycin, erythromycin, ampicillin, rifampin), and for detection the sites of genes encoding for antibiotic resistance , bacterial conjugation experiments was done for some of bacterial isolates that contain plasmids. Results of bacterial conjugation has revealed that antibiotic resistance genes in isolates *(P. aeruginosa* (14), *E.coli* (4)) were present on DNA plasmid , not on chromosome. But the other isolates, we could not find conjugated bacterial colonies for them which indicate that there DNA plasmids do not have auto transmission.

Introduction

The DNA composition of microorganisms can be remarkably fluid .DNA can be transferred from one organism to another, and that DNA can be stably incorporated in the recipient, permanently changing its genetic composition [1]. This process is called lateral or horizontal gene transfer to differentiate it from the inheritance of parental genes, a process called vertical inheritance [2].

Three broad mechanisms mediate efficient movement of DNA between cells:

1- Conjugation- Plasmids are the genetic elements most frequently transferred by conjugation. Genetic functions needed for transfer are encoded by the *tra* genes, which are carried by self- transmissible plasmids. Some self-transmissible plasmids can removed other plasmids or portions of the chromosome for transfer. In some cases mobilization is achieved because the *tra* genes provide functions necessary for transfer of an otherwise non-transmissible plasmid. In other cases, the self-transmissible plasmid integrates with the DNA of another replicon and, as an extension of itself, carries a strand of this DNA into a recipient cell [3].

2- Transduction- Transduction is phage-mediated genetic recombination in bacteria. In simplest terms, a transducing particle might be regarded as bacterial nucleic acid in a phage coat. Even a lytic phage population may contain some particles in which the phage coat surrounds DNA derived from the bacterium rather than from the phage. Such populations have been used to transfer genes from one bacterium to another [4].

3- Transformation- Direct uptake of donor DNA by recipient bacteria depends on their competence for transformation. Natural competence is unusual among bacteria, and some of these strains are transformable only in the presence of competence factors, produced only at a specific point in the growth cycle. Other strains readily undergo natural transformation, and these organisms offer promise for genetic engineering because of the ease with which they incorporate modified DNA into their chromosomes [5].

Resistance gene can occur on chromosomes, transferable plasmid, transposons gene [6], [7]. Erlier studies have shown that genes for resistance markers do occur on plasmids called R- resistance factor (RF) and they can be transferable[8], and most of them have demonstrated it by plasmid curing experiments alone[9].

In the present study we demonstrate the role of plasmid in distribution of multidrug-resistance, and conjugation in gram negative bacteria.

Materials and methods

Samples collection:

Thirty of bacterial isolates from 45 samples were obtained from urine, stool, sputum, otitis media, collected from outpatients and inpatients(wound and burned patients) in Tikrit Teaching Hospital from September to December (2013), were identified by conventional methods. "It was identified depending on its morphology (colony shape, size, colour, borders, and texture) and then it was examined by the microscope after being stained with Gram's stain. After staining, the biochemical tests were done on each isolate to complete the final identification [10]".

Antibiotic susceptibility test:

All isolates were analyzed for the presence of drug resistance by the method of Bauer *et al.* [11], on Mueller Hinton agar (HiMedia.) by using commercial available paper discs. The antibiotic discs and their concentration used in this study are shown in Table (4).

Extraction of DNA:

Preparation of Solution:

1. Lysis Buffer Solution: It is prepared by: 1- Dissolve 0.242 g of Tris Base, 57 µL of Glacial acetic acid and 1 ml of 0.5 M EDTA solution. 2-To the above solution add 0.02 g of sodium acetate and 0.1 g of SDS. 3-The volume make up to 8 ml by distill water. Adjust the pH to (7.8), the volume complete to 10 ml by distill water. 4-Mix well, dissolve the solution with heating in water bath to assist dissolution.
2. 5 M NaCl Solution: It is prepared by dissolving 2.92 g of Sodium Chloride in 10 ml of distill water.
3. Ethanol 70 %: comprise of 70 ml absolute ethanol and 30 ml of distill water.
4. Ethanol 100 % and Chloroform.

Procedure:

1. 1.5 ml of broth culture was harvested with centrifugation for 5 min at 14,000 rpm.

2. The cell pellet was re-suspended and lysed in 200 µL of lysis buffer (40 mM Tris-acetate pH 7.8, 1 Mm EDTA, 20 mM sodium-acetate, 1 % SDS) by vigorous pipetting.
3. 66 µL of 5M NaCl solution was added (to remove most proteins and cell debris) and mixed well, and then the viscous mixture was centrifuged at 12,000 rpm for 10 min at 4°C.
4. After transferring the clear supernatant into a new eppendorf tube, an equal volume of chloroform was added, Then mix on a rotating mixer for 15 min when a milky solution was completely formed.
5. Following centrifugation at 14,000 rpm for 5 min, The supernatant is then removed to another eppendorf tube and double volume of 100% Ethanol was added.
6. The tubes were inverted 5-6 times gently to precipitated DNA, then centrifuged at 10.000 rpm for 3 minutes.
7. The supernatant from this last step discarded and 1 ml of ethanol (70%) was added, again tubes centrifuged 10.000 rpm for 3 minutes.
8. Repeating Step 7.
9. The supernatant discarded and 100 µL of distil water was added to re-suspended (dissolved) DNA.
10. The stock DNA is kept frozen at (-20°C) until use [12].

Plasmid profile:
Plasmid samples were electrophoresed through 0.8%,1% agarose (Sigma) in TBE buffer at 80V, 180 mA for 1hour and in 60V,90 mA for 100 minute. The gel was stained in 40 µg/l of ethidium bromide solution. Picture was taken by using gel documentation system and estimation of DNA carried out using nanodrop2000 (Thermo scientific company) Spectrophotometer .

Conjugation test:
Conjugation experiments were carried out according to Harley and Prescott [13].by using *E. coli* (4) and *P. aeruginosa* (14) strain as donor and *E. coli* (6) and *P. aeruginosa* (15) as recipient. The presence of plasmid in trans-conjugant was checked through electrophoresis and the transconjugants were also tested for each antibiotic resistance already recorded for the donor strains.

Results
The present study included the isolation and identification of gram negative bacterial species that cause different infections and study some virulence factors of isolates in addition to the genetic content of these isolates. These bacteria include the following species: *Pseudomonas aeruginosa* (15), *Escherichia coli* (7), *Klebseilla pneumonia*, *Proteus mirabilis* and *Aeromonas hydrophila* (2), *Serratia marcescens* and *Citrobacte freundii* (1) for each isolate Table (1).
bacterial isolates were obtained from urine, stool, sputum, otitis media, wound and burned patients Table (2).The results of antimicrobial susceptibility and virulence factors of 30 isolates included in the present study are shown in Table (3),(4) and Fig (1). only 17 strains of multiple antibiotic resistant isolates which have many virulence factor were examined for the presence of plasmid. These isolates were found to harbor one to three plasmid bands, Picture (1),(2). The purity of DNA range between 1.93 -1.39 ,Table (5).
Conjugation experiments were attempted on these isolates to determined changes in plasmid content associated with antibiotic pattern. The isolates containing plasmid bands considered as donor strains while isolates free of plasmid bands considered as recipient. It was noted that the trans- conjugants became resistance to most antibiotics used which confirm that all antibiotics resistance determinants has been transferred from donor cell to the recipient cell (Table 6),(picture 3,4).Agarose gel electrophoresis of the transconjugant showed the transferring of plasmid bands form donor to the recipient cell (picture 5).

Table (1): Samples of Grame Negative Bacteria(Numbers &percentages).

Bacterial species	Total number of isolates	(%)
Pseudomonas aeruginosa	15	50
Escherichia coli	7	23.33
Klebseilla pneumoniae	2	6.66
Proteus mirabilis	2	6.66
Aeromonas hydrophila	2	6.66
Serratia marcescens	1	3.33
Citrobacter freundii	1	3.33
Total	30	100

Table (2): Number and percentage of isolates according to the source of bacterial samples.

Source of bacteria.	Isolates	
	number of isolates	%
Otitis media	7	23.3
Wound	7	23.3
Burn	4	13.3
Urine	4	13.3
Sputum	4	13.3
Stool	4	13.3
Total	30	100

Table(3) Virulence factors of bacterial isolates

no.	Bacteria isolate	lipase	Hemolysin production	Gelatinase	Capsule formation	Urease
1	*Psuedomonas aeruginosa* 1	+	β	+	–	-
2	*P. aeruginosa* 2	+	β	+	–	-
3	*P. aeruginosa* 3	+	β	+	–	-
4	*P. aeruginosa* 4	-	β	-	–	-
5	*P. aeruginosa* 5	-	β	-	–	-
6	*P. aeruginosa* 6	-	β	-	–	-
7	*P. aeruginosa* 7	+	β	-	–	-
8	*P. aeruginosa* 8	+	β	-	–	-
9	*P. aeruginosa* 9	+	γ	-	–	-
10	*P. aeruginosa* 10	-	γ	-	–	-
11	*P. aeruginosa* 11	+	β	-	–	-
12	*P. aeruginosa* 12	-	γ	-	–	-
13	*P. aeruginosa* 13	-	γ	-	–	-
14	*P. aeruginosa* 14	+	β	-	–	-
15	*P. aeruginosa* 15	-	γ	-	–	-
16	*E.coli* 1	-	α	-	-	-
17	*E.coli* 2	+	β	-	–	-
18	*E.coli* 3	-	α	-	–	-
19	*E.coli* 4	+	β	-	+	-
20	*E.coli* 5	-	α	-	–	-
21	*E.coli* 6	-	γ	-	–	-
22	*E.coli* 7	+	β	-	-	-
23	*Klebseilla pneumoniae* 1	+	γ	-	+	-
24	*K. pneumoniae* 2	+	γ	-	+	-
25	*Proteus mirabilis* 1	+	γ	+	-	+
26	*P. mirabilis* 2	+	β	-	-	+
27	*A. hydrophila* 1	-	γ	-	-	-
28	*A. hydrophila* 2	-	γ	-	-	-
29	*Serratia marcescens*	-	γ	+	-	-
30	*Citro.freundii*	+	γ	-	-	-

β-hemolysis(complete hemolysis);α-hemolysis(partial hemolysis); γ- hemolysis(Non-hemolysis)

Table (4) Antibiotic resistance of bacterial species .

Antibiotics	Concentration (µg/disc)	Symbol	Bacterial species (No.)						
			Pseudo. aeruginosa (15)	*E. coli* (7)	*K. pneumonia* (2)	*P. mirabilis* (2)	*A. hydrophila* (2)	*S. Marcescens* (1)	*Citr. Freundii* (1)
Amoxicillin	25	AX	8(53.3%)	5(71.4)	1(50)	1(50)	0(0)	1(100)	1(100)
Ampicillin	10	AM	8(53.3%)	7(100)	2(100)	2(100)	1(50)	1(100)	1(100)
Cephalexin	30	CL	7(46.6%)	5(71.4)	1(50)	2(100)	2(100)	0(0)	0(0)
Ciprofloxacin	5	CIP	2(13.3%)	3(42.8)	0(0)	0(0)	2(100)	0(0)	0(0)
Clarithromycin	15	CLR	15(100%)	7(100)	2(100)	2(100)	2(100)	1(100)	1(100)
Erythromycin	15	E	15(100%)	7(100)	2(100)	2(100)	2(100)	1(100)	1(100)
Nitrofurantoin	300	F	15(100%)	5(71.4)	2(100)	2(100)	0(0)	1(100)	1(100)
Rifampin	5	RA	5(33.3%)	7(100)	2(100)	2(100)	2(100)	1(100)	1(100)
Tetracycline	30	TE	15(100%)	5(71.4)	1(50)	1(50)	0(0)	0(0)	1(100)

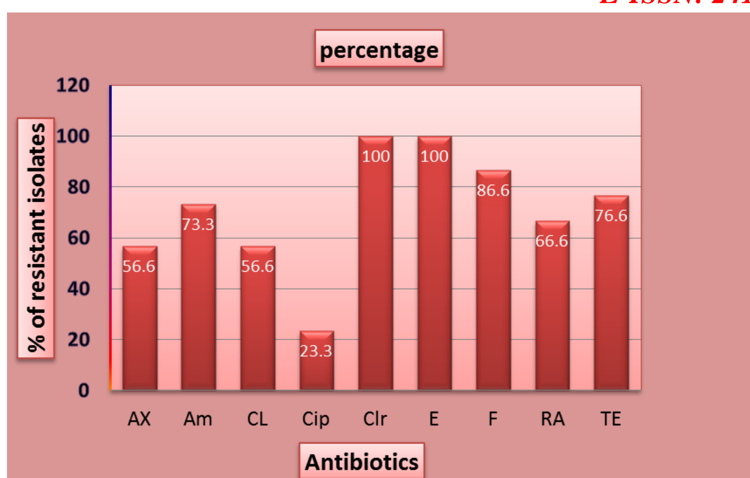


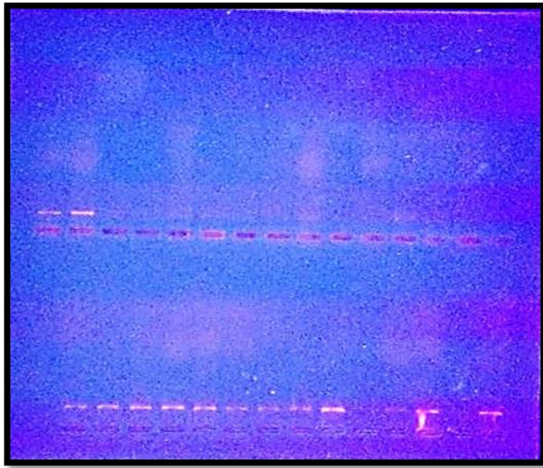
Figure 1: Antibiotic resistance of bacterial isolates.
AX=Amoxicillin,AM= Ampicillin,CL= Cephalexin,CIP= Ciprofloxacin, CLR= Clarithromycin,E= Erythromycin,F= Nitrofurantoin,RA= Rifampin,TE= Tetracycline

Table(5):Estimation of DNA spectrophotometry.

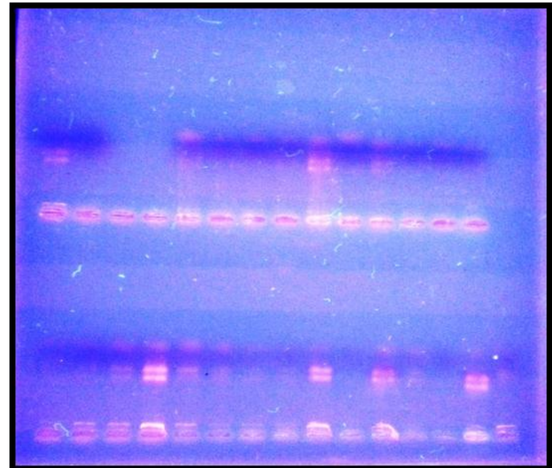
N0.	Bacterial isolates	purity	Nucleic acid concentration in ng\ µl
	Blank	1.16	0.5
1	*A. hydrophila* 1	1.60	99.2
2	*P. mirabilis* 1	1.86	38.8
3	*K.pneumoniae* 1	1.93	62.5
4	*E.coli* 4	1.85	92.2
5	*E.coli* 7	1.70	76.3
6	*E.coli* 2	1.64	35.4
7	*E.coli* 6	1.84	64.7
8	*S. marcescens*	1.70	25.2
9	*P. aeruginosa* 9	1.92	86.0
10	*P. aeruginosa* 10	1.40	20.6
11	*P. aeruginosa* 11	1.75	35.8
12	*P. aeruginosa* 12	1.90	57.0
13	*P. aeruginosa* 13	1.92	60.7
14	*P. aeruginosa* 14	1.78	65.4
15	*C.freundii*	1.50	65.8
16	*P. aeruginosa* 2	1.60	88.0
17	*P. aeruginosa* 15	1.64	38.2
	Blank(after conjugation)	0.85	0.7
1	*E.coli* 4+ *E.coli* 6	1.41	16.2
2	*P.aeruginosa* (14)+ *P.aeruginosa* (15)	1.91	65.2
3	*E.coli* 4+ *E.coli* 6	1.74	25.2
4	*P.aeruginosa* (14)+ *P.aeruginosa* (15)	1.39	15.3

Table(6):Bacterial Conjugation

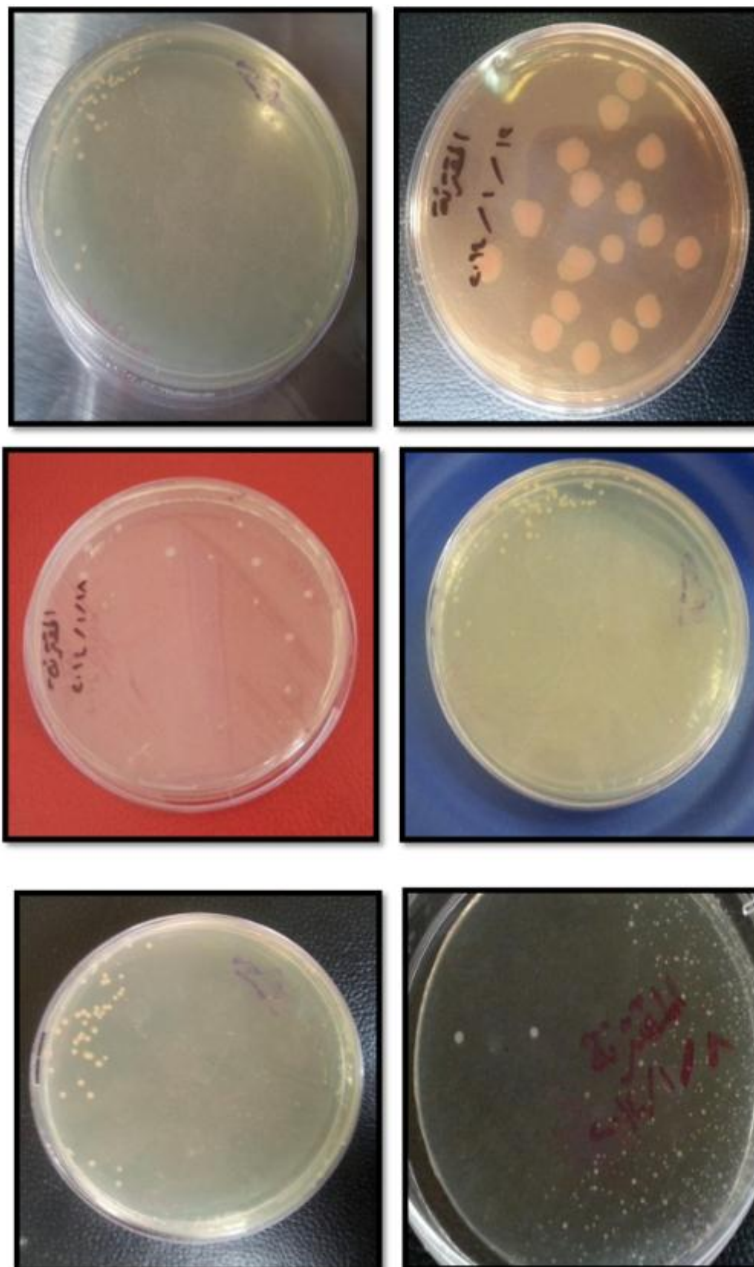
Donor isolates		Recipient isolates		Conjugation
Bacterial isolates	Resistant to antibiotics	Bacterial isolates	Resistant to antibiotics	Resistant to antibiotics
E.coli (4)	Ax^S,Cip^R	*E.coli* (6)	Ax^R,Cip^S	Ax^R,Cip^R
P.aeruginosa (14)	Ax^S ,Cl^R	*P.aeruginosa* (15)	Ax^R ,Cl^S	Ax^R ,Cl^R
P.aeruginosa (14)	Te^R,Cip^S	*E.coli* (4)	Te^S,Cip^R	-



Picture (1) :Agarose gel electrophoresis of bacterial isolates (after 1 Hour of Electrophoresis , agarose 0.8%).



Picture (2) : Gel Electrophoresis of Plasmid DNA content of bacterial isolates (after 1.5 Hour of Electrophoresis , agarose 1%) .



Picture (3) Congugated isolations of Bacteria

1

A B

Picture (4): Conjugation between *E.coli* 4(A) Ax^S,Cip^R + *E.coli* 6 (B) Ax^R,Cip^S

Picture (5): Conjugation between *P. aeruginosa* (14)+ *P. aeruginosa* (15)

Picture (6) : Agarose gel electrophoresis of transconjugants isolates.

Discussion

There are many bacterial virulence factors occurance commonly on Plasmids (extrachromosomal mobil genetic elements) that transferred by a primary mechanism for exchange of genetic information between bacteria which carrying the genes that code for many bacterial virulence factors,this results was similar to [14] and dissimilar to [15] because the large groups of genes that associated with pathogenicity are located on the bacterial chromosome and termed with Pathogenicity Island(PAI) that containing more virulence genes in the pathogenic members and they typically have a different guanine plus cytosine(G+C) content than the bacterial genome[16].

The high resistance pattern for antibiotics might be due to the mechanisms of resistance which involve the alteration of the target to which antimicrobial agents bind [17] or alteration in membrane permeability, enzymes and metabolic pathways which are usually caused by the acquisition of R_plasmids [18]. In addition the relatively nonspecific efflux pumps that can pump many different drugs could explain the resistance of these isolates [19]. Antibiotics resistance genes are often located in transposons which have the ability to transpose from one DNA molecule to another[20] are thought to have played a major role in the development of antibiotics Plasmids [21], promoting the movement of the genes responsible for drug resistance between different plasmids, or from chromosome to plasmid of a naturally resistant organism [22].

The genes of Antibiotic Resistance are transferred by Plasmids (genetic elements) were transferred with conjugation which encoded by the tra genes that carried by self transmissible plasmids. [23].

The genes encoding conjucative _transfer functions were associated with extra chromosomal replicon like antibiotics resistance and virulence factors called conjugative Plasmids [24, 25, 26, 27].

Most antibiotics Resistance were result of Plasmids by encoded enzymes that degrade or modify antibiotics[28] and occur at a high frequency called (R_ Plasmids, R_ factors) carry two set of genes [29],one set encodes the enzymes that modify antibiotics and the other encodes the proteins that mediated conjugation ,the main process by which resistance genes are transferred from one bacterium to another[30]. But this results dissimilar to [31] the genes which encoding to virulence factors are by a portion of chromosome.

The result of conjugation between *E.coli* and *P.aeruginosa* is similar to [32] based on ability of transfer of prime plasmids between organisms like *E.coli* and *P.aeruginosa* , it was correlated with [33].

The Conjugation involves transfer of DNA between adjacent Bacterium that is located on Plasmids which containing F genes, the genetic transfer requires contact between the two bacteria, then allows bacteria to increase their genetic diversity, and requires a set of F (Fertility) genes ,it was correlated with [34]. but discorrelated with [35]. When transfer of DNA from the genome of bacteria can occur if the F set of genes is integrated in the bacterial chromosome. This process was discovered in the strains of *E.coli* ,that exhibit a higher than usual tendency to transfer genomic DNA are called as a Higher Frequency of Recombination (HFR) strains [36] .

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دراسة بكتريولوجية ووراثية لبعض البكتريا السالبة لصبغة كرام والمعزولة من أخماج مختلفة

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الملخص

عزلت 30 عينة جرثومية من اصل 45 عينه لأصابات مختلفة وشخصت مجهرياً وبأستخدام الفحوصات الكيموحيوية .أختبرت قابلية هذه العزلات الجرثومية على مقاومة المضادات الحيوية الأتية: (amoxicillin, tetracycline, cephalexin, nitrofurantoin, ciprofloxacin, clarithromycin, erythromycin, ampicillin, rifampin)

ولتعيين مواقع المورثات المشفرة لمقاومة المضادات الحيوية فقد أستخدم الأقتران الجرثومي لعدد من العزلات المحتوية على البلازميدات, اذ بينت نتائج الاقتران الجرثومي ان المورثات المقاومة للمضادات الحيوية في العزلات (**P. aeruginosa** (14)**, *E.coli*** (4)) موجودة على الـ DNAالبلازميدي لها وليست على الكروموسوم, اما العزلات الأخرى فلم نستطع الحصول على مستعمرات جرثومية مقترنة مما يدل على عدم امتلاك الدنا البلازميدي لها صفة الانتقال الذاتي.