Molecular detection and production of mcrocin from *Escherichia coli* isolated from different clinical samples.

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Abstract:

In this study, nine genes responsible for microcins production were detected, from seventeen clinical isolate of *E.coli* collected from 50 samples by using PCR techniques. catechol microcin was present in all isolates, and microcin H47 are present in 6 isolates ,where microcin M are found in 5 isolates. Also partial purification of microcin by ammonium sulphate and dialysis are conducted in this study, and find the relationship between microcin production and virulence factors of UPEC in patients with UTI. Where microcin consider narrow spectrum antibacterial peptide that inhibit the growth of other bacteria from related species during nutrition deficiency specially in low iron level.

الكشف الجزيئي وإنتاج المايكروسين من الإشريكية القولونية المعزولة من عينات سريرية مختلفة.

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الخلاصة:

في هذه الدراسة، تم الكشف والتحري عن تسع جينات مسؤولة عن إنتاج المايكروسين، من اصل سبعة عشر عزلة سريرية من الايشريشيا القولونية التي تم جمعها من 50 عينة، باستخدام تقنيات بي سي ار،حيث كان الكاتيكول مايكروسين موجودة في جميع العزلات، والمايكروسين اج 47 موجود في 6 عزلات، بينما تم العثور على المايكروسين ام في خمس عزلات فقط. كما تم خلال هذه الدراسة إجراء تنقية جزئية للمايكروسين بواسطة سلفات الأمونيوم والديلزة في هذه الدراسة، وإيجاد العلاقة بين إنتاج المايكروسين وعوامل الضراوة للاشيرشيا القولونية المسببة لامراض المسالك البولية في المرضى الذين يعانون من التهاب المسالك البولية. حيث يعتبر المايكروسين بروتين مضادة للجراثيم ضيق الطيفوالذي يمنع نمو البكتيريا الأخرى من الأنواع ذات الصلة خلال نقص التغذية وخاصةعند انخفاض مستوى الحديد.

Introduction:

Escherichia coli are well known to be one of the most important flora in human intestine (1). Urinary tract infections are among the most common infectious diseases encountered in humans and *Escherichia coli* is their leading etiologic agent (2). Uropathogenic *E. coli* encompasses a group of bacteria possessing a variable virulence gene assortment (2).The bacteria are known to produce multiple true virulence factors and virulence associated factors, among them is the production of bacteriocins.

Escherichia coli can produce various types ofenzymes and proteins and also has the ability to produce antagonistic agent such as bacteriocins .Bacteriocins are protein substances produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s). They are typically considered to be narrow spectrum antibiotics, though these Bacteriocins were first discovered by A.

Gratia in 1925(3*). Escherichia coli* is known to produce two types of bacteriocins, classified by their molecular weight into colicins (25-80 kDa) and microcins (<10 kDa). Microcins are low-molecular-weight proteins secreted by certain bacteria that act by limiting the growth of other bacteria that share the same ecological niche (4). Asensio *et al.* were the first to identify a class of low molecular weight antibiotics, produced by enteric bacteria isolated from the feces of newborn infants, which could inhibit the growth of related microorganisms (5). These antibiotics were able to diffuse through cellophane, and inhibit the growth of an indicator bacterium, and it is soluble in methanol-water (5:1) and thermo stable. The generic name "microcin" was proposed to differentiate these antibiotics from colicins.Microcins are low molecular weight (<10 kDa) ribosomally synthesised peptide antibiotics, produced by diverse members of the *Enterobacteriaceae* and are active against *E. coli* and closely related bacterial species (6).microcins and colicins are similar in many ways, but in contrast to colicins, microcin synthesis is not lethal to the producing strain and is not regulated by the DNA damage inducible SOS system. Further, almost all colicins are plasmid encoded, whereas microcin encoding genes are also found on the chromosome (7).Microcin can be divided according to their size, biochemical properties and mechanism of action of two classes: **Class I**:This class Includes peptides with a molecular weight below 5 kDa (microcin B17, C7, D93, J25). (8). **Class II microcins**: Class II microcins are higher molecular mass peptides between 5-10 kDa. These microcins are further subdivided into two groups, namely class IIa and IIb (7).

Catechol microcin: These types of microcins use catecholate siderophore receptors (catecholate siderophor receptors are FepA, Cir, Fiu and iroN) for recognition/translocation across the outer membrane (9).When microcins enter the target cells through the pathway employed the siderophores of the catechol type so these modified microcins in range of catechol group (10).

Aims of study:-

1- Isolation of *Escherichia coli* from urine samples.
2- Molecular screening of producer by using PCR techniques to detect the genes.
3- Production of microcins by traditional methods.
4- Partial fractionation of microcins by using $(NH_4)_2SO_4$.
5-Assay for detection of microcin by using standared strain of *Escherichia coli*.

Materials and Methods:

Bacteriocins production: Pathogenic strains of *Escherichia coli* can produce bacteriocins in vitro by this procedure:

1-Prepare 400ml from M63 broth as in Karen procedure, and inoculate with pathogenic strain of *E.coli*. At 37 °C for 24 h.

2- Prepare other 400 ml from M63 broth and sterile it by autoclave, and keep it sterile in refrigerator as control.

3-Filterate both broths by Millipore filter in a sterile flask.

4-Prepare dialysis bag by maceration it with sufficient amount from sterile M63 broth and phosphate buffer to keep the same pH.

5-Fill the dialysis bag with filtrate that extract from broth (this broth contain pathogenic strain of *E.coli* producer to bacteriocins specially microcins).

6-Closed both ends of dialysis bag by strong threat.

7- Put the dialysis bag in 500 ml beaker and the beaker was filled with sterile M63 broth and put in refrigerator for 4 days.

8- After 4-days, we take two samples from two solutions to assay the activity for both solutions.

Extraction of microcins by ammonium sulfate fractionation:

1-Filtrate both solution1 and solution2 above by Millipore filter .
2-Prepare phosphate buffer and adjusted the pH to 7.0.
3- Prepare 20% ammonium sulfate salt and added to 50 ml of solution2 (adding 5.7 mg of $(NH_4)_2SO_4$ to 50 ml of solution2 as in the table).
4-Centerfuge this solution for 45 min with cooling in 10000 rpm speed to obtain the precipitate (this ppt must contain salt-protein complex).(take 2 ml from this solution And keep it to assay its activity).
5-Increase the conc. of solution from 20%to 40% by added 7mg from ammonium sulfate to previous solution
(As in the table) and centrifuge this solution For 30 min at 10000rpm speed (take 2 ml from this solution To assay its activity).
6-Filterate this solution and prepare dialysis bag by putting it in buffer solution For 1 hr.
7- Fill dialysis bag by solutions that contain 40% from ammonium sulfate and closed both ends of bag by strong threat and put in a 500 ml beaker that fill with buffer solution And keep it in refrigerator for 2-days.
8- After 2-days, we assay the activity of both solutions (solution in side bag and solution outside bag).

Assay for microcins activity:

Activity of microcins can be detected from other activity of bacteriocins by the following procedure:
1-Prepare M9 solid media and inoculate with standard strain *E.coli* (MM294).
2-Take about (2.5cmX5cm) pieces of dialysis membrane (sterile this membrane with 95% alcohol and washing with sterile D.W, then left to dry).
3- Put this membrane above the inoculate plate and fixed it on the surface of plate by small pin in all directions.
4-Take about 5-colonies from different pathogenic strain *E.coli* samples, and put each colonies in different places of dialysis membrane.
5-Incubate this plate at 37ºC for 24 h.

Detection of some virulence gene markers by PCR:

The primers and PCR conditions used to amplify genes encoding virulence factors with PCR are listed in table (1)
The primers includes 1)*mchB***,** 2)*mchC, 3)mcmi ,4)mciA ,5)mccF ,6)J25, 7)mcmIA ,8)mcmA and 9) mceA*).genes. as well as the primer specific for the int. Each 25µl of PCR reaction contained 2.5µl of each upstream and downstream primer, 2.5µl of free nuclease water, 5µl of DNA extraction and 12.5µl of master mix. The PCR amplification product were visualized by electrophoresis on 1% agarose gels for 45min at 70volts. The size of the amplicons were determined by comparison to the 100 bp allelic ladder (bioneer, USA).

Results and discussion:The results as shown in table 2 and Fig 1,2,3

The microcin specific gene, most frequent were those encoding microcin M (35%), followed by microcin H47 (29%), microcin V (17%), microcins B17, C7 and L (3%) and microcin J25 (1%).(7). Almost all bacteria require iron (11),so that a number of microcins are induced when iron is limiting and that many employ receptors for iron acquisition (12), Thus, it has been hypothesized that microcins may be involved in competition for iron (13). In bacteriocin-producing strains, about

42% produced one type of bacteriocin, 41% produced two, 16% produced three, and one strain was found to produce four different bacteriocins(14). Budič *et al*.found an association of microcins with virulence factors, in A number of urovirulence factors (adhesins, cytotoxins, siderophores, etc.) have been identified, which appear more frequently in uropathogenic *E. coli* than in commensal or enteropathogenic strains (7). Microcins H47 was strongly associated with toxins (hlyA,cnf1, usp,), the siderophore(fyuA) , adhesins (sfa , papC , and papG) as well as tcpC ,(15) . Azpiroz, *et al*. who found strong association with various virulence factors among strains producing microcin H47 (16). Microcin H47 located on chromosome by clustered of genes, Enterobactin provides by the all which is converted into salmochelins, which is employed in microcins synthesis and secreted by type 1 apparatus , so, microcins H47 composed of fore moieties, peptide and salmochlin. Peptide for deleterious activity and salmochelin confer the uptake properties of molecules. So, it's up taker through catechol pathway.

Iron may be up taken and mediated by salmochelin. Mcc E492 Chromosom located differs from H47 in its final mode of action, when H47 acts on the proton channel of ATP synthetase but E492 act on ionophore and carriers post translation modification consisting of the linkage of salmochelin to peptide.

In microcin production and purification,the result shown in the table (3)

The result showed that microcin is first produced at high level because its production is simultaneous and along with bacteriocins production, then, it was separated by using cellophane membrane which prevents the crossing of bacteriocins above 15 kD, but allow the passing of microcins which are below 15 kD, however, the activity enhanced with progression of purification steps. According to the result of this study, It was observed that fractionation by Ammonium sulphate may increase the amount of microcins activity against standard strain of *E.coli*.

The result showed that the maximum effect of broth in *E.coli*(MM492) strain culture appear in supernatant before and after filteration with Millipore filter (no significant effect of filteration by Millipore filter because of this filteration allows passage most type of bacteriocins due to large pore size of filter and the range of most bacteriocins size located under range size of this type of filter), so this maximum effect due to all types of bacteriocins that found in broth(microcins, colicins, and others),so gave the synergism effects of bacteriocins with each other's.

In the second step to separate microcin by cellophane membrane Tab.3, was used directly on culture media , it was observed that the activity of bacteriocins is reduced after cellophane membrane using.this mean that this activity is restricted to microcin because of the cellophane of dialysis bag that allows passage only molecules with molecular size less than 15kDa (microcins only) and exclude the rest types of bacteriocins (specially colicins) from passage (because of molecular size of other bacteriocins larger than 75 kDa.(17). However, the dialysis bag is used to ensure the presence of only the micricin. In third steps, dialysis bag only that used to partial purification of microcins, its used to eliminate the solution from the salt ,and the activity is increased and this means that microcin only found , so that bag allowed microcins to passage through dialysis membrane according to its molecular size. In forth step of microcins purification in Tab.3, the ammonium sulphate salt was added at final saturation from (20-40)% and this step increase the precipitation of microcin inside the dialysis bag. The precipitation itself has an activity of microcins, when the latter is dissolved by the phosphate buffer (pH7.0). The activity of microcins remains intact and then subjected for dialysis to remove the salts.

The ammonium sulphate used because of its being safe, don’t interfere with products and easily to dissolve, so, this salt is useful in salting out (its effect based on the electrolyte-nonelectrolyte

interaction. It is used as method of separating proteins based on the principle that proteins are less soluble at high salt concentrations. The salt concentration needed for the protein to precipitate out of the solution differs from protein to protein. This process is also used to concentrate dilute solutions of proteins). Dialysis can be used to remove the salt if needed (18). The activity of microcin is highly observed on bacteria and increased due to decreasing in the amount of proteins.The production of microcins and purification of it, are very important steps, and this product may be useful in treatment of *E.coli* infection particularly in UTI, Where microcins was produced normally in urine as the one of the most important products that be synthesized by UPEC extracelluarly. Briefly is the first study in Hilla Province and according to molecular investigation for 9 types of microcins it was proposed that these microcins should be studded at biotechnology level to show their effect and activities separately because some isolates was found to possess more than microcin type in their genome ,this may reflect that these isolate can use multiple means for production these microcins, how other ,production of them may be judged by several factores,the secretion process that its required for each microcin, the site of infection and the environmental factors that may determine which of them that could be produced by bacteria isolates.

Reference:

1. Eckburg, PB., Bik, EM, Bernstein, CN., Purdom, E., Dethlefsen, L., Sargent, M., Gill, SR., Nelson, KE., Relman, DA. Science 308 (5728):1635–1638. (2005).
2. Marı´a, F., Azpiroz, Magela, L. *Antimicrobial Agents and Chemotherapy*, 51(7): 2412–2419. (2007).
3. Gratia, JP. *The Genetics Society of America*. 156 (2): 471–476. (2000).
4. Corsini, G., Karahanian, E., Tello, M., Fernandez, K., Rivero, D., Saavedra, JM. *FEMS Microbiol Lett*. ; 312(2):119-125. (2010).
5. Asensio, C., J. C. Perez-Diaz, M., Martinez, C., Baquero, F. *Biochem. Biophys. Res. Commun.* 67:7-14. (1976).
6. Zschüttig, A., Zimmermann, K., Blom, J., Goesmann, A., Pöhlmann, C., *et al*. *PLoS ONE* .7(3): e33351. (2012).
7. Budič, M., Rijavec, M., Petkovšek, Ž., Žgur-Bertok, D. *PLoS ONE*. 6(12): e28769. (2011).
8. Pons, AM., Delalande, F., Duarte, M., Benoit, S., Lanneluc, I., Sablé, S., Van dorsselaer, A., Cottenceau, G. *Antimicrob Agents Chemother*. 48(2):505-513. (2004).
9. Gaëlle, V., Destoumieux-Garzón,D., Peduzzi,J. *1st ed.Springer New York Dordrecht Heidelberg London* .309-332. (2011).
10. Trujillo, M., Rodríguez, E., Laviña, M. *Antimicrob Agents Chemother*.45(11):3128-31. (2001).
11. Andrews, S.C., Robinson, A.K., Rodrı´guez-Quin˜ones, F. *FEMS. Microbiology. Reviews* 27: 215–237. (2003)
12. Duquesne, S., Destoumieux-Garzon, D., Peduzzi, J., Rebuffat, S. *Nat Prod Rep* 24: 708-734. (2007).
13. Gordon, DM., O'Brien, CL. 152: 3239–3244. (2006).
14. David, M., Gordon and Claire L. O'Brien. *Microbiology*. 152, 3239–3244.(2006).
15. Rijavec, M., Muller-Premru, M., Zakotnik, B., Zgur-Bertok, D. *Med Microbiol* .57: 1329–1334. (2008).
16. Azpiroz, MF,. Poey, ME., Laviña, M. *Microb Pathog* .47: 274-280.(2009).
17. Cascales, E., Buchanan, SK., Duché, D., Kleanthous, C., Lloubès, R., Postle, K., Riley, M., Slatin, S., Cavard, D. *Microbiol. Mol. Biol. Rev*. 71(1):158-229. (2007).
18. Berg, Jeremy, M.*Biochemistry*. Sixth Ed. New York: W.H. Freeman. 68-69, 78. (2007).

Table 1 Primers sequences and PCR condition using to detect virulence gene:

Genes	Primer sequence (5´- 3´)	Size (bp)	PCR conditions	Reference
mcmIA F	CCATAAAAAAAATGAATATCACCC	695	94ºC 4min 1x 94ºC 2min 60ºC 1min 30x 72ºC 1min 72ºC 7min 1x	(2)
mcmIA R	CTAACTTAAAGCGTTACATAGG			
Mcmi F	CCATAAAAAAAATGAATATCACCC	415	94ºC 4min 1x 94ºC 2min 60ºC 1min 30x 72ºC 1min 72ºC 7min 1x	(2)
Mcmi R	GCAATTAATTCTGCCTGCCC			
mcmA F	TAACTTCCACTCCCCGCA	278	94ºC4min 1x 94ºC 2min 60ºC1min 30x 72ºC 1min 72ºC 8min 1x	(2)
mcmA R	ATGAGAAAACTATCTGAAAATGAAAT			
mchB F	ATGCGAGAAATAACAGAATCACAG	228	94ºC 4min 1x 94ºC 2min 60ºC 1min 30x 72ºC 1min 72ºC 8min 1x	(2)
mchB R	TTAGCTACCGCCACCAGCAGAAG			
mchC F	AGTGGTATTCAGCGTAATGG	551	94ºC 4min 1x 94ºC 2min 58ºC 1min 30x 72ºC 1min 72ºC 8min 1x	(2)
mchC R	GTCAATCTCAGGAAACGTGT			

Tab.2: result of presence gene in *E.coli* isolates

Primer name	*Sample name*																	*No. of positive*	*% of gene*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*		
1)mchC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	17	100
2)mchB	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	7	41.1
3)mcmi	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	17.6
4)mcmIA	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	1	5.8
5)mcmA	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	1	5.8

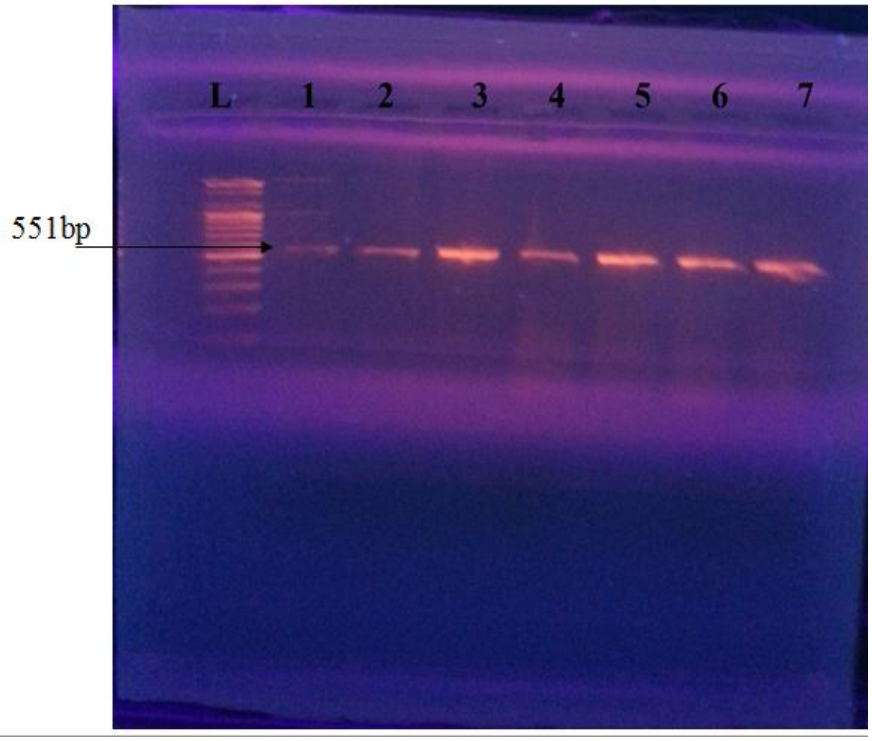

Figure 1 : Gel electrophoresis of PCR product of *mchC* (Catechol microcin).(17) *E.coli* isolates with positive result for *mchC*.

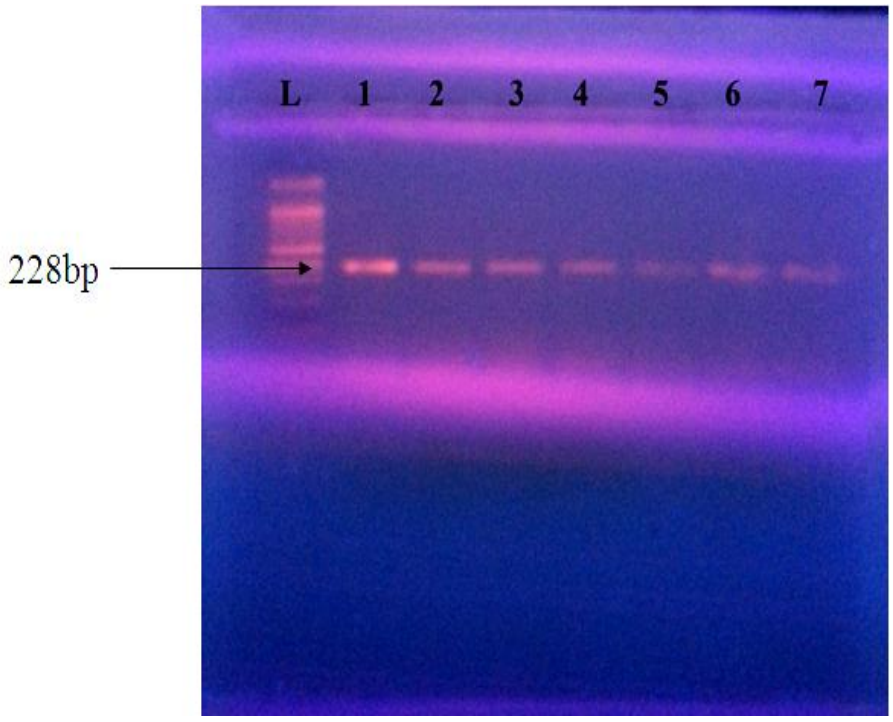

Figure 2 : Gel electrophoresis of PCR product of *mchB* (H47).(7) *E.coli* isolates with positive result for *mchB.*

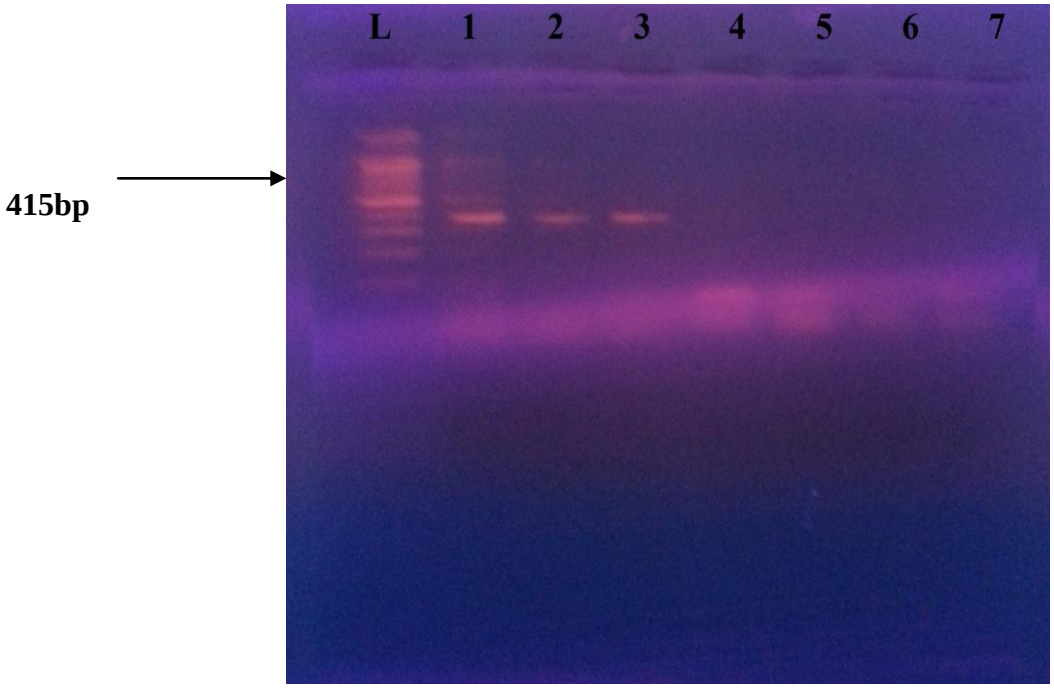

Figure 3): Gel electrophoresis of PCR product of *mcmi* .(3) *E.coli* isolates with positive result for *mcmi*.

Tab.3: Effect of different technique in microcins purification on bacterial growth

Steps	Technique	Diameter of inhibition zone in mm	Dilution factor	Total activity U/ml
1	Total bacteriocin	18	1/1	18
2	(Cellophane) microcins	16	1/1	16
3	Crude product(dialysis without salt)	13	1/2	26
4	Crude 20-40 % And dialysis	10	1/10	100