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Research Article

ANTIMICROBIAL EFFECT OF SILVER NANOPARTICLES WITH *Kluyvera cryocrescens* AND BIOFILM CULTURES

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Abstract

Kluyvera is recently identified belong to the family Enterobacteriaceae infrequently cause infection in animal and human. The organism has been isolated from different clinical specimens, but it is important to know that *Kluyvera cryocrescens* not clearly demonstrated. In fact, it has been considered as an alternative to saprophytic, opportunistic or pathogenic. In 1981 was the redefinition of this species and case reports of the various clinical infections that occur under different host conditions have been published. In this study, *Kluyvera cryocrescens* was able to produce biofilm which isolated from urine, blood and milk specimens were indicated by Congo red method. Currently, *Kluyvera* sp. showed highly sensitivity rate to all tested antibiotics in Vitek2 AST as well as Sliver nanoparticles effects showed antibacterial activity with inhibition zone revealing sensitivity of *Kluyvera cryocrescens*.

Article History

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Key words: *Kluyvera cryocrescens*, AgNPs, Antimicrobial activity and Biofilm.

1. Introduction

Kluyvera sp. is Gram negative bacteria, less commonly encountered Enterobacteriaceae (Long *et al.,* 2012; Sarria *et al.,* 2001). It is opportunistic pathogen and predominantly colonizes the gastrointestinal, respiratory and urinary tracts and it widely distributed in different environmental sources such as hospital sinks, water, soil, milk, and cows but it rarely causes infection (Mahmood *et al.,* 2020). Little cases were reported as adult bacteremia (Yoshino *et al.,* 2016). *Kluyvera cryocrescens* was reported as a second species commonly isolated from environment and rarely isolated from clinical specimens (Farmer *et al.,* 1981). This genus shared the most properties of member Enterobacteriaceae in growing on MacConkey agar, produce acid and gas from fermentation of D-glucose as shown in the Tab2. All previous studied mentioned that *Kluyvera* sp. had peritrichous instead of polar flagella (Farmer *et al.,* 1981; Sarria *et al.,* 2001).

Kluyvera cryocrescens reported as a virulent pathogens because of it resistance activity to the ampicillin and 1st and 2nd generation of Cephalosporins (Carter and Evans, 2005). Recently isolation of this species occurs frequently with clinical specimens in Iraq. This research focused on the ability of antimicrobial effect against bacteria and compared it to the silver nanoparticles effect.

2. Materials and Methods

Samples were presented to the Al-Qasim Green University (Microbiology Lab) for

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investigation mentioned in Table - 1. Specimens were cultured on different type of media (Nutrient agar, MacConkey agar and Blood agar) by Streaking method for single isolated colony. After 24 hours (incubation period), growth was noticeable on all types of media. Gram staining was done by using Standard method used. Fresh culturing was done for all samples and used for identification by Biomerieux Vitek 2 System and results appeared in Table – 2. Samples were 94 % probability *Kluyvera cryocrescens*.

Table – 1: Samples Information Details

Source	Number	Negative	*Kluyvera cryocrescens*
Blood (Human age 35 years)	21	18	3
Milk cows	64	52	12
Urine (Human age 43 years)	12	5	7

Table – 2 : Biochemical details by Biomerieux Vitek 2 System

APPA	-	ADO	-	PyrA	-	IARL	-	dVEL	+	BGAL	+
H2S	-	BNGA	-	AGLTp	-	dGLU	+	GGT	-	OFF	+
BGLU	+	Dmal	+	dMAN	+	dMNE	+	BXYL	-	BALap	-
ProA	+	LIP	-	PLE	+	TyrA	+	UPE	-	dSOR	-
SAC	-	Dtag	-	dTRE	+	GIT	+	MNT	+	sKG	+
ILATK	+	AGLU	-	SUCT	+	NAGA	-	AGAL	+	PHOS	-
GLYA	-	ODC	+	LDC	-	IHISa	-	CMT	+	BGUR	-
O129R	+	GGAA	-	IMLTa	-	ELLM	+	ILATa	-		

Biofilm detection

To detect biofilm - forming bacteria by Congo red agar method according to (Feynman, 2012) by prepared a Congo red stain as stock solution, autoclaved at 121 °C for 20 minutes. Then, Brain Heart Infusion Broth with Agar and 5 % Sucrose were added at 55 °C (Hassan *et al*., 2011). The bacterial strains were inoculated and incubated at 37 °C for 24 to 48 hrs. Then read the result as following: if the bacteria formed black colonies with a dry crystalline consistency that was mean it biofilm producer isolates while if it formed red colonies that was mean the non-biofilm producer isolates (Kaiser *et al*., 2013).

Antibiotics susceptibility tests

VITEK 2 AST system to determine the Minimum Inhibitory Concentration (MIC) to many tested antibiotics. All the following steps were done according to the manufacturer's instructions as VITEK 2 AST system supplemented with antimicrobial susceptibility testing cards Enterobacteriaceae contains more than 15 antibiotics (Table - 3). The results read also digitally on monitor connected to VITEK2 system apparatus.

Silver nanoparticles synthesis

Twenty drops of 0.1 M $AgNO_3$ was added dropwise (1 drop per sec.) to 50 ml of 0.001 M $NaBH_4$ in beaker (250 ml) on magnetic stirrer at 400 for 30 min in dark condition and in room temperature, then the change in color was noted. The reaction mixture was stirred vigorously on a Magnetic stirrer (Murgueitio *et al.*, 2016; Rashid *et al.*, 2013) according to equation below:

$AgNO_3 + NaBH_4 \rightarrow Ag + H_2 + B_2H_6 + NaNO_3$

Table - 3: Antibiotics provide by VITEK AST card for Enterobacteriaceae with MIC breakpoints according to M100 (10)

Antibiotic	MIC Breakpoints (µg/mL)			Antibiotic	MIC Breakpoints (µg/mL)		
	S	I	R		S	I	R
Ticarcillin	≥8	16	≤32	Cefepime	≥2	-	≤16
Ticarcillin/ Clavulanic acid	≥16/2	32/2-64/2	≤128/2	Trimethoprim /Sulfamethoxazole	≥2/38	-	≤4/76
piperacillin	≥16	32-64	≤128	Gentamicin	≥4	8	≤16
Piperacillin-tazobactam	≥16/4	32/4-64/4	≤128/4	Tobramycin	≥4	8	≤16
Aztroenam	≥4	8	≤16	Ciprofloxacin	≥1	2	≤4
Meropenem	≥1	2	≤4	Amikacin	≥16	32	≤64
Ceftazidime	≥4	8	≤16	Minocycline	≥4	8	**≤16**
Imipenem	≥1	2	≤4				

+ = Deduced drug * = AES modified ** = User modified

Optimize Silver nanoparticles characterization

The silver nanoparticles were characterized by UV Spectrophotometer and Size analyzer (Gomaa, 2017). All these analyses were carried out at pharmacy and Science College, Kufa and Veterinary College of Al-Qasim Green University, Iraq.

UV–visible spectrophotometer analysis

The Surface Plasmon Resonance of silver nanoparticles was measured by UV–visible spectrophotometer at wave length ranging from 300 - 500 nm. By sampling 1 ml of AgNPs solution to different wave length were measured every ten degree at resolution of 1 nm (Karthik and Radha, 2012).

Size analyzer

Laser diffraction particle size analyzers, which measure light scattering and assume an index of refraction to calculate the particle size distribution (Levoguer, 2013). Silver nanoparticles sample was examination in size analyzer after incubated in Sonicator water bath at 35 °C for 30 minutes. Emulsion diluted sample with deionized water were put in grove of apparatus and the size were measured during 5 minutes by using Laser beam scattering in beta sizer apparatus. The results were monitoring on computer's screen.

Antimicrobial activity assay of AgNPs

The antimicrobial activity of AgNPs were evaluated by agar well diffusion method by using Muller Hinton plate inoculated with tested biofilm-forming bacteria at inoculum 1.5×10^8 CFU/ml by streaking method and waited 10 minutes to dry then made Well by Cork borer in the center of inoculated plate and fill the well with 100 µl of filtered AgNPs, incubated at 37 °C to 24 hours at dark condition. After that, the inhibition

zone diameter was measured by ruler and compared to the nearest whole millimeter.

3. Results and Discussion

Kluyvera sp. is uncommon Gram negative bacteria produce biofilm in clinical specimen, there is no study about *Kluyvera* biofilm formation. In our study, *Kluyvera* sp. produce biofilm which isolated from urine, blood and milk specimens were indicated by Congo red method (Figure - 1). In the Figure - 1, the black color colony indicate biofilm formation of bacteria due to stain exopolysaccharide matrix producing during biofilm process by Congo red stain (Bose *et al*., 2009).

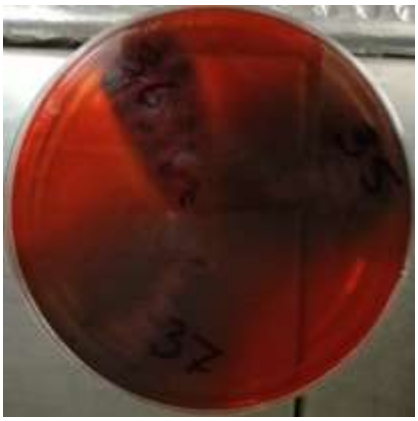


Figure - 1: Congo red plate indicates biofilm formation *Kluyvera* sp.

Table - 4: Vitek AST results of biofilm forming *Kluyvera* sp.

Antibiotic	MIC	Interpretation	Antibiotic	MIC	Interpretation
Ticarcillin	64	I	Cefepime	≤1	S
Ticarcillin/ Clavulanic acid	≤8	S	Trimethoprim /Sulfamethoxazole	≤20	S
Piperacillin	≤4	S	Gentamicin	≤1	S
Piperacillin-Tazobactam	≤4	S	Tobramycin	≤1	S
Aztroenam	≤1	S	Ciprofloxacin	≤0.25	S
Meropenem	≤0.25	S	Amikacin	≤2	S
Ceftazidime	≤4	S	Minocycline	≤1	S
Imipenem	≤0,25	S			

Antibiotics sensitivity test

At usual state, the biofilm-forming bacteria showed antibiotic resistant phenomenon but in current study, *Kluyvera* sp. showed highly sensitivity rate to all tested antibiotics in Vitek AST (Table - 2) in contrast to biofilm-forming bacteria (Hasson *et al.*, 2018) and that may be related to uncommon pathogenicity and newer species of this bacteria lead to didn't developing resistance mechanisms against ordinary antibiotics. Some research reported that *Kluyvera* susceptible to all experimental antibiotics (Stock, 2005). In our research the reaction of *Kluyvera* to the Trimethoprim/Sulfamethoxazole, and

Ceftazidime were sensitive result shown in Tab2 whereas, previous studies mentioned resistant of *Kluyvera* to the same antibiotics which could reveal that difference in the virulence factor of *Kluyvera cryoscense* (Thaller *et al.*,1988; Carter and Evans, 2005).

Silver nanoparticles synthesis

Color change indicator

During AgNPs synthesis, the color of mixture solution of $AgNO_3$ and $NaBH_4$ converted from colorless to dark brown color which indicate AgNPs production and that related to excitation of AgNPs Surface Plasmon vibration (Figure - 2) (Kvitek *et al*., 2008).

UV Spectrophotometer analysis

The optical density of AgNPs production were revealed beak at 390 nm (Figure - 3). The absorption degree of AgNPs depend on Plasmon resonance rate which represent the ratio of silver ion to silver zero valent (Gomaa, 2017).

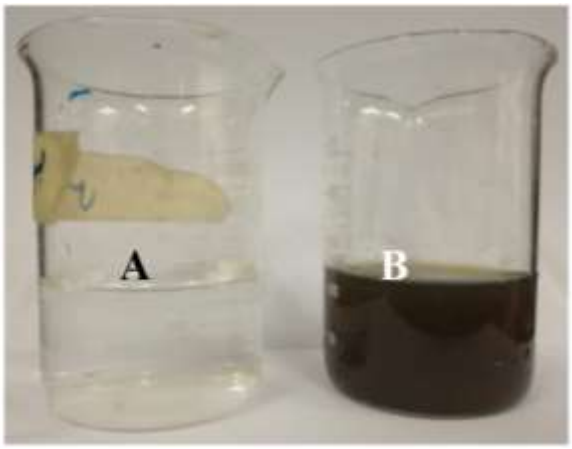


Figure - 2: Color change indicator AgNPs formation, A= Colorless $AgNO_3$ and $NaBH_4$ solutions, B = Dark brown AgNPs

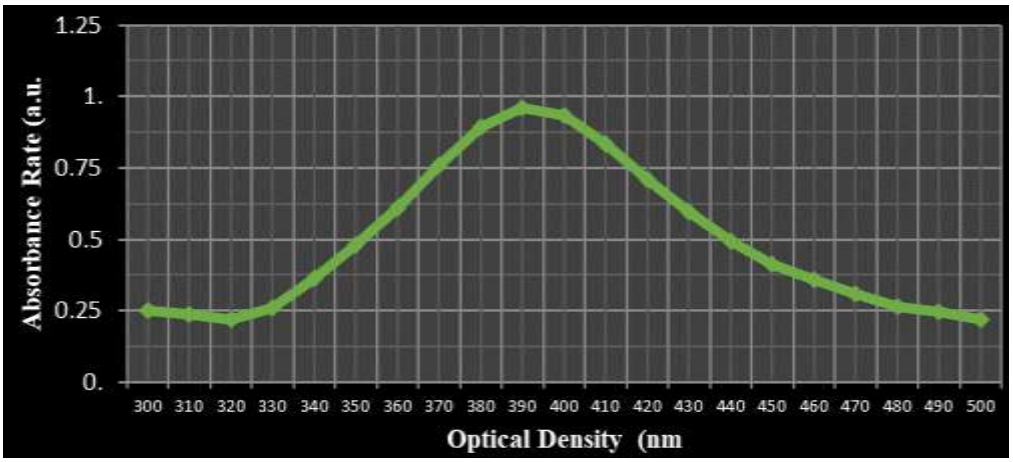
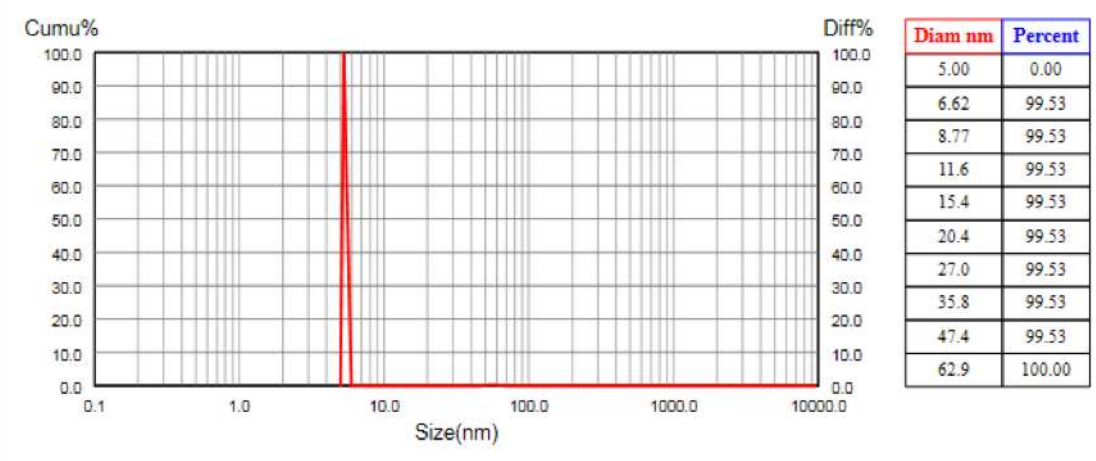


Figure - 3: UV-Vis spectrophotometer analysis of silver nanoparticles synthesis



Diam nm	Percent
5.00	0.00
6.62	99.53
8.77	99.53
11.6	99.53
15.4	99.53
20.4	99.53
27.0	99.53
35.8	99.53
47.4	99.53
62.9	100.00

Figure - 4: Size distribution analysis of synthetic AgNPs particle size was approximately 5 nm

Figure - 5: Zone of inhibition of biofilm formation *Kluyvera* sp. growth as a result antibacterial activity of synthetic AgNPs

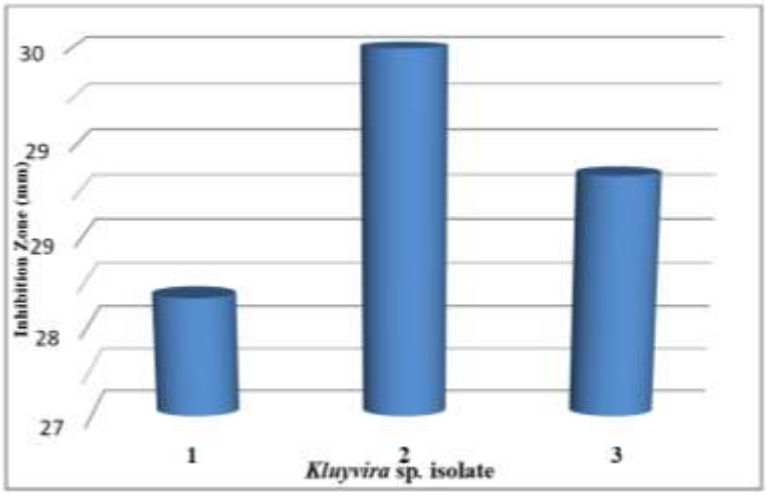


Figure - 6: Zone of inhibition of biofilm formation *Kluyvera* sp. growth isolated from (1 = Urine, 2 = Blood and 3 = Milk) as a result of antibacterial activity of synthetic AgNPs

Size analyzer test

The size of synthetic AgNPs was determined by dynamic light scattering by nano laser particle size analyzer. The figure below showed the size of AgNPs at 5 nm. The size of synthetic nanoparticles is very important and effective because the antibacterial activity of nanoparticles depend upon its size, the small particles more effective as antibacterial than large one (Kvitek *et al*., 2008).

The antibacterial activity of synthetic AgNPs

The antibacterial activity of AgNPs was evaluated by agar well diffusion method. The results revealed that inhibition zone at average 29 nm (Figure - 4 and Figure - 5) to all isolates.

The antibacterial activity of AgNPs against biofilm forming bacteria studies is limited except littles studies (Morones *et al*., 2005; Marini *et al*., 2007; Kvitek *et al*., 2008; Abady, 2021;) and no study about its effect against biofilm forming *Kluyvera* sp. The inhibition zone of biofilm formation *Kluyvera* against silver nanoparticles at 29 mm were highest in contrast to other closely studies (Morones *et al*., 2005) and that explanation to *Kluyvera* inability to antibiotics resistant so it seem to be more susceptible to AgNPs antimicrobial activity. The antibacterial activity of AgNPs suppose related to many mechanisms but it still unknown, AgNPs may interact with the bacterial cell membrane lead to disturb the permeability and functions of respiration (Kvitek *et al*., 2008) and may penetrate the bacteria cell (Morones *et al*., 2005). Many researchers also proposed that Ag^{+} ions interact with the thiol groups in bacteria proteins, affecting the replication of DNA (Marini *et al*., 2007).

5. Conclusion

Kluyvera sp. showed highly sensitivity rate to all tested antibiotics in Vitek2 AST as well as sliver nano particles effects showed antibacterial activity with inhibition zone revealing sensitivity of *Kluyvera*. This research point to some differences in the antimicrobial effect between *Kluyvera* sp. for the Iraqi patients isolates.

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