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RESEARCH ARTICLE

Antibiotic Resistance and Molecular Characteristics of Upcoming Pathogenic *Kocuria* spp. Isolated from Iraq and Egypt

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ABSTRACT

Kocuria spp. isolates were collected from different clinical samples from Medical City Educational Laboratories/ Baghdad and Ain Shams Specialized Hospital, Cairo. Identification was carried out by VITEK2 automated compact system. Antibiotic susceptibility, biofilm formation, and detection of macrolides resistance genes *erm*A, *erm*B, *erm*C, *msr*A & *mef*, integron *intI*1 and *intI*2 were also investigated. Results indicated that 60 isolates were collected from the two countries, 42 isolates from Iraq, and 18 isolates from Egypt. The bacterial isolates were identified by their morphological and biochemical characteristics. They were susceptible to pipracillin/Tazobactam and vancomycin, resistant to erythromycin, azithromycin and ofloxacin. *Kocuria* spp. had different potential to form biofilm, *Kocuria kristinae* were strong biofilm producers, while *Kocuria rhizophila* varied between non-biofilm producers in isolates from Iraq and weak producers in isolates from Egypt. The detection of macrolide resistance genes indicated that the *mef* gene was present in 7.14% of isolates from Iraq, while the *erm*A gene was detected in 6.66% of isolates from Egypt. Integron detection showed that *intI*1 was present in isolates from both Iraq and Egypt, whereas *intI*2 was detected in Iraq isolates only. To the best of our knowledge, this is the first comparative study conducted to investigate the presence of *Kocuria* spp. in Iraq and Egypt. The study highlights the importance of *Kocuria* spp. which is considered an opportunistic pathogens, however, it is responsible for different human infections.

Keywords: Antibiotics susceptibility, Biofilm formation, *Kocuria* spp, Macrolides resistance genes, multidrug resistance bacteria

Introduction

Kocuria spp. are aerobic Gram-positive cocci arranged in pairs, short chains, and tetrads. They produce irregular clusters, and on the agar surface, the bacterial colonies appear as pink, orange, yellow or cream according to different species and strain.[1] *Kocuria* spp. belongs to the class Actinobacteria, order Actinomycetales family Micrococcaceae. This bacterium was first recognized by Microslav kocur, a Slovakian case of Urinary Tract Infection (UTI) in 1974.[2,3] These bacteria are non-capsulated, non-spore-forming non-motile, and non haemolytic. They are obligate aerobes, coagulase negative and catalase positive.[4] *Kocuria* spp. are capable of producing various enzymes. They display attractive enzymatic activities in food like the production of catalase and proteases, which play a role in fermented and

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unfermented meat production and others. Further, these bacteria have the ability to have beneficial interactions with other microorganisms, which leads to increased production of enzymes and volatile compounds in foods.[5,6]

Generally, these bacteria are considered non-pathogenic commensals that colonize the skin, mucosa, and oropharynx, as well as environmental organisms inhabiting the animals, plants, soil and air.[7,8] Recently, there has been a rise in the occurrence of infections caused by *Kocuria* spp. causing both superficial infections and invasive infections in immunocompromised patients. The most common infections associated with *Kocuria* are urinary tract infection, endocarditis, brain abscess, peritonitis, cancer and renal failure in dialysis patients.[9,10] This study aimed to highlight the occurrence and importance of *Kocuria* species in different clinical samples as an upcoming pathogen by the detection of biofilm formation and macrolides resistance genes (*erm*A, *erm*B, *erm*C, *msr*A, and *mef*).

Materials and methods

Collection and identification of bacterial isolates

A total of 60 clinical isolates were collected from Iraq and Egypt, 42 isolates from Medical City Educational Laboratories/Baghdad, and 18 isolates from Ain Shams Specialized Hospital in Egypt. *Kocuria* spp. were isolated from various samples such as urine, blood, and wound swabs. All isolates were cultured on nutrient, blood, and MacConkey agar, then incubated for 24–72 h at 37˚C. Species identification was performed by the Vitek2 ID GPC system (bioMérieux, France), using Gram-positive identification cards (GP cards).[11] All samples were subjected to further confirmatory biochemical tests.[12] Susceptibility testing was performed by modified Kirby-Bauer disc diffusion technique,[13] and minimum inhibitory concentration (MIC) by micro broth dilution method.[14]

Qualitative assessment of biofilm production

Assessment of biofilm production was carried out by the Congo Red Agar method (CRA). This method was described by Freeman and colleagues.[15]

Detection of antibiotic resistance genes

Genomic DNA was extracted from bacterial cells using an ABIO pure extraction kit (USA), according to the manufacturer's instructions. PCR was performed in a total volume of 50 μl. Each reaction mixture contained the following: 25 μl of master mix (Thermo Scientific), 3 μl of 20 ng DNA template and 2 μl of 10 μM of each primer (*erm*A, *erm*B, *erm*C, *msr*A, *mef*, *intI*1 and *intI*2)[16–18] all primers are presented in Table 1.

PCR protocol was as follows: initial denaturation at 94˚C for 5 min, then 35 cycles of 40s at 94˚C, 40s at 55˚C, 40s at 72˚C and a final step of 10 min at 72˚C. PCR products were analyzed by 1% agarose gel electrophoresis using TAE buffer, and agarose gel was examined under UV transillumination. A Qiagen extraction kit was used to purify the PCR products before DNA sequencing.

Statistical analysis

The Statistical Analysis System-SAS (2018) program was used to detect the effect of different factors in study parameters. Chi-square test was used to compare significance between percentages ($P \leq 0.05$ and 0.01 probability).[19]

Results and discussion

Isolation of Kocuria species

The samples were isolated from various clinical sources, including wounds, urine, and blood. Out of 42 isolates collected from Iraq, 22 isolates (52.38%) were males, and 20 isolates (47.61%) were females. This result failed to reach the level of statistical significance (P-values .0.705). On the other hand, from Egypt, 14 isolates (77.77%) were from males and 4 isolates (22.22%) from females. This result showed highly significance in statistical analysis (P-values 0.0003).

Identification of clinical isolates

Kocuria spp. were isolated and diagnosed depending on the VITEK2 compact system and confirmed by biochemical tests as four species. *Kocuria kristinae* and *Kocuria rhizophila* appeared in yellow colonies, while *Kocuria rosea* had pink to orange colonies and *Kocuria varians* had white colonies on nutrient agar. Microscopic examination showed that *Kocuria* is Gram-positive cocci in pairs, tetrads, and clusters as shown in Fig. 1. The isolates were identified by the Vitek2 ID GPC system. The isolates from Iraq were identified as 26 isolates (61.90%) *Kocuria kristinae*, 14 isolates (33.33%) *Kocuria rosea*, one isolate (2.38%) of *Kocuria rhizophila* and one isolate of *Kocuria varians*. The isolates from Egypt were identified as 8 isolates (44.44%) *Kocuria kristinae*, 9 isolates (50%) *Kocuria rosea* and one isolate (5.55%) *Kocuria rhizophila*, with a P-value (0.0001).

Table 1. Primers used in the PCR assay for detection of resistance genes in *Kocuria* spp.

Gene	Primer 5′–3′	annealing temperature (°C)	Product size bp
*erm*A	F5′-TAT CTT ATC GTT GAG AAG GGA TT-3′ R5′-CTA CAC TTG GCT TAG GAT GAA A-3′	55	139
*erm*B	F5′-CTA TCT GAT TGT TGA AGA AGG ATT-3′ R5′-GTT TAC TCT TGG TTT AGG ATG AAA-3′	55	142
*erm*C	F5′-CTT GTT GAT CAC GAT AAT TTC C-3′ R5′-ATC TTT TAG CAA ACC CGT ATT C-3′	55	190
*msr*A	F5′-TCC AAT CAT AGC ACA AAA TC-3′ R5′-AAT TCC CTC TAT TTG GTG GT-3′	55	163
mef	F5′-AGTATCATTAATCACTAGTGC-3′ R5′-TTCTTCTGGTACAAAAGTGG-3′	55	348
*intI*1	F5′-GGCTTCGTGATGCCTGCTT-3′ R5′-CATTCCTGGCCGTGGTTCT-3′	57	148
*intI*2	F5′-GTTATTTTATTGCTGGGATTAGG-3′ R5′-TTTTACGCTGCTGTATGGTGC-3′	56	166

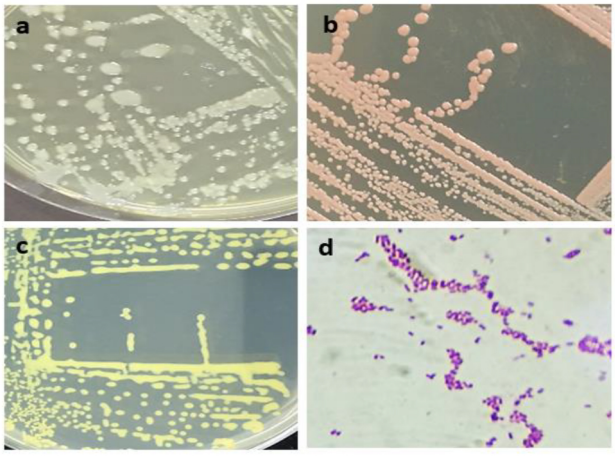

Fig. 1. Culture characteristics of *Kocuria* spp. a- *Kocuria varians*, b- *Kocuria rosea* and c- *Kocuria kristinae* & *Kocuria rhizophila*, appearance on nutrient agar after 24h of aerobic incubation (smooth, opaque, round to convex colonies, pale cream to orange and yellow pigmented colonies). d- Gram's stain of *Kocuria kristinae*, showing deeply stained positive cocci arranged in pairs, short chains, tetrads and clusters.

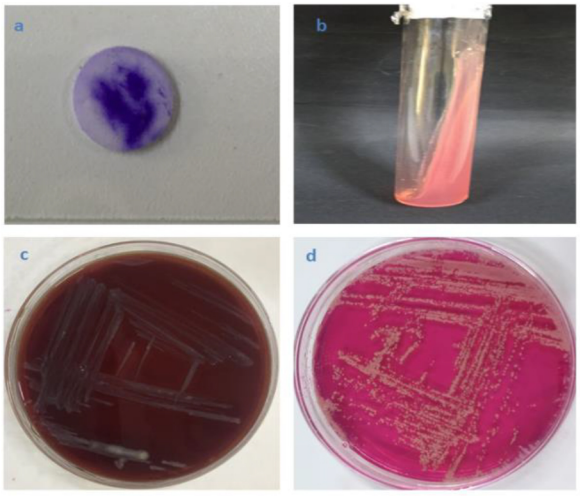

Fig. 2. Biochemical tests and virulence enzymes. a- Oxidase positive/*Kocuria kristinae* & *Kocuria rosea*, b- Urease positive/*Kocuria varians*, c- Non-haemolytic bacteria on blood agar/all *Kocuria* species, d- Non-fermented mannitol bacteria (pale-cream growth)/all *Kocuria* species.

Detection of some virulent enzymes

In the current study, all bacterial isolates were positive for catalase test, while all isolates were negative for coagulase test. On the other hand, oxidase test showed strong positive for *Kocuria kristinae* isolates and weak positive for *Kocuria rosea*, both *Kocuria rhizophila* and *Kocuria varians* showed negative results for the oxidase test. Further, all the isolates were negative for urease except for *Kocuria varians* which were positive. Moreover, all isolated bacteria showed non-hemolytic colonies on blood agar Fig. 2.

Production of biofilm

Biofilm production demonstrated that the isolates have different potential to form biofilm according to different species under the same conditions in both countries, Table 2 and Fig. 3.

Antibiotic susceptibility and MIC of Kocuria spp.

All *Kocuria* species were susceptible to vancomycin, ertapenem, imipenem/cilastatin, meropenem, cefepime, ceftriaxone, amikacin, linezolid, teicoplanin and pipracillin/tazobactam, while they were resistant to amoxicillin, ciprofloxacin, ofloxacin, levofloxacin, clindamycin, erythromycin, azithromycin and gentamycin. The values of Minimum Inhibitory Concentration (MIC) were determined according to Clinical and Laboratory Standards Institute guidelines (2020) as shown in Table 3.

Table 2. Biofilm production from bacterial isolates in Iraq and Egypt.

	Iraq					Egypt				
Name of bacteria	Total number	Strong	Moderate	Weak	Non	Total number	Strong	Moderate	Weak	Non
K. kristinae	26 61.90%	19 73.07%	4 15.40%	3 11.53%	0	8 44.44%	5 62.50%	3 37.50%	0	0
K. rosea	14 33.33%	5 35.70%	9 64.30%	0	0	9 50%	4 44.44%	5 55.55%	0	0
K. rhizophila	1 2.38%	0	0	0	1 100%	1 5.55%	0	0	1 100%	0
K. varians	1 2.38%	0	1 100%	0	0	-	-	-	-	-
P-value	0.0001 **	0.0001 **	0.0001 **	0.039 *	0.109 NS	0.0001 **	0.0077 **	0.0051 **	0.109 NS	1.00 NS

*(P ≤ 0.05), **(P ≤ 0.01). **(P ≤ 0.01).

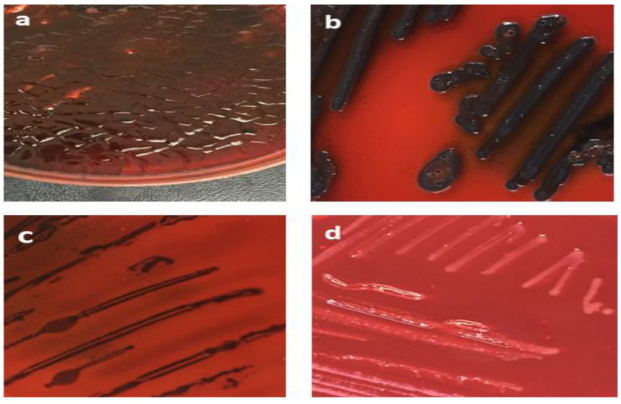

Fig. 3. Biofilm detection by Congo red agar method, showing, a- strong biofilm formation (black crystalline colonies of biofilm producers), b- moderate biofilm formation (dark colonies with the absence of dry crystalline colonies of biofilm producers), c- Weak biofilm formation (weak slime colonies of biofilm producers) and d- Non-biofilm producer (smooth, pinkish-red colonies).

Detection of macrolides resistance genes and Integrons

Macrolides resistance genes *erm*A, *erm*B, *erm*C, *msr*A and *mef* were detected using PCR. The results revealed that *mef* gene was detected in Iraq isolates only, while *erm*A was detected in Egyptian isolates only. In isolates from Iraq, *erm*B gene was detected in 3 isolates (*K. kristinae* and *K. rosea*), and *mef* gene was detected in 3 isolates (*K. kristinae* and *K. rhizophila*) in 7.14%; *erm*C gene was demonstrated in 2 isolates (*K. kristinae* and *K. rosea*). In addition, *msr*A gene was detected in 2 isolates (*K. kristinae* and *K. varians*) in 4.76% in all Iraq isolates.

On the other hand, samples from Egypt revealed that *erm*A and *msr*A genes were detected in 4 isolates (22.22%) (*K. kristinae* and *K. rosea*), *erm*B gene was detected in 5 isolates (27.77%) (*K. kristinae, K. rosea* and *K. rhizophila*), while *erm*C gene was detected only in 2 isolates (11.11%) (*K. kristinae* and *K. rosea*) in 11.11%.

Integrons can carry, spread, and have significant routes of distribution of the antibiotic-resistance genes among bacteria via horizontal transfer. In this study, *int*1 and *int*2 were detected in 31.66 and 6.66% respectively. Detection of integron 1, 2 revealed that *intI1* gene was present in 11 isolates (26.19%) (*K. kristinae, K. rosea* and *K. varians*) and 8 isolates (44.44%) (*K. kristinae, K. rosea* and *K. rhizophila*) from Iraq and Egypt respectively. While *intI*2 was present in 4 isolates (9.52%) (*K. kristinae* and *K. rosea*) in Iraq isolates only and was not detected in isolates from Egypt, Fig. 4.

Discussion

There are few studies about *Kocuria* spp. in Iraq and Egypt, and sufficient importance has not been given to the diseases caused by these bacteria. Therefore, the clinical importance of *Kocuria* spp must be highlighted.

Kocuria spp. has been known to be a part of normal microbiota and is considered a commensal microbe on human skin, mucous membranes and oropharynx.[7] However, five species can cause opportunistic infection in immunocompromised patients. These species are *Kocuria kristinae*, *Kocuria rosea*, *Kocuria varians*, *Kocuria rhizophila* and *Kocuria marina*.[20] Therefore, these bacteria should be considered as a true pathogen contributing to clinical sepsis, and real treatment should be provided to all susceptible patients. Moreover, some studies have recorded that *Kocuria kristinae* functions as commensal of humans, animals and environment to be contaminants of wounds.[21] Previous studies on *Kocuria* spp. isolates from Iraq,[22–24] and Egypt[25] illustrated that *Kocuria* spp. cause symptomatic bacteremia in recurrent UTI patients, and behave as an apportunistic

Table 3. The Minimum Inhibitory Concentration (MIC) of *Kocuria* spp. clinical isolates.

		MIC (μg)							
Country	Bacterial isolates	MEM	IPM/CS	AK	VA	LNZ	CRO	CPM	TZP
Iraq	10 isolates 8 (*K. kristinae*) 2 (*K. rosea*)	32	None	16	64	None	None	None	64
	20 isolates 15 (*K. kristinae*) 5 (*K. rosea*)	64	64	None	4	4	64	None	None
	12 isolates 1 (*K. varians*) 1 (*K. rhizophila*) 6 (*K. kristinae*) 4 (*K. rosea*)	None	None	32	128	None	16	64	4
Egypt	8 isolates 4 (*K. kristinae*) 4 (*K. rosea*)	16	16	16	64	64	128	16	64
	10 isolates 1 (*K. rhizophila*) 5 (*K. kristinae*) 4 (*K. rosea*)	32	None	16	None	4	None	4	None

IPM/CS = Imipenem /Cilastatin (10 μg); MEM = Meropenem (10 μg); AK = Amikacin (30 μg); CPM = Cefepime (30 μg); CRO = Ceftriaxone (30 μg); TZP = Piperacillin/Tazobacam (20 μg); VA = Vancomycin (30 μg); LNZ = Linezolid (30 μg).

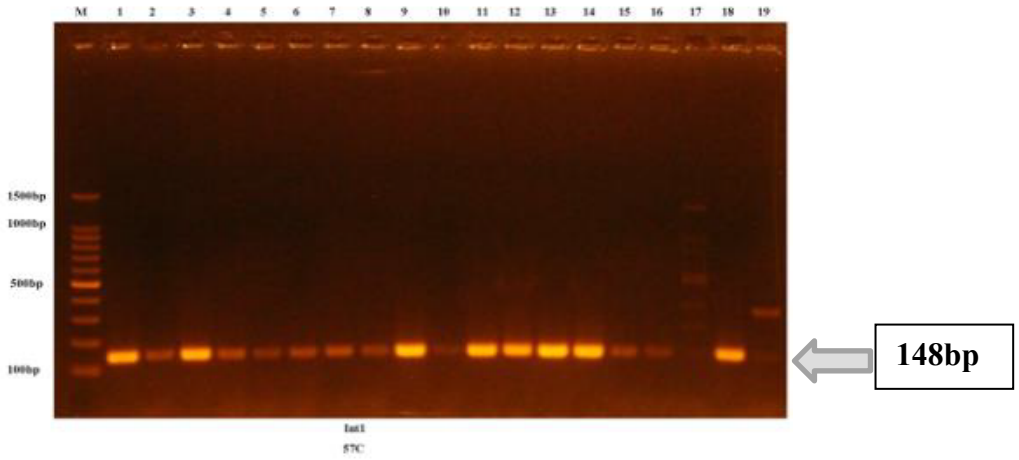

Fig. 4. PCR amplification of *int*I1 gene (148bp).

pathogen in patients with tonsillitis. Another study in Egypt demonstrated by Maany *et al.*,[26] showed dental plaque polysaccharides which produced by *Kocuria rosea*.

In the current study, *Kocuria* spp. have pigmented colonies on nutrient agar due to the production of carotinoides the pigments shades from red to yellow. Recently, the production of carotinoids from bacteria has become of considerable interest for industrial applications.[27]

The current study showed variability in the formation of biofilm between strong moderate, weak and non-biofilm producers. A high production of biofilm was observed in the *Kocuria kristinae*. M any studies demonstrated considerable adhesion and biofilm formation in *Kocuria kristinae* strains.[28] While isolates that are weak to non-biofilm producers could be other environmental conditions such as temperature, pH, CO_2 levels, osmolality, nutrients and factors derived from the biotic environment that affect the biofilm production.

In this study, all bacterial isolates appeared susceptible to vancomycin, meropenem, ertapenem, imipinem/cilastatin, amikacin, cefepime, cefitriaxone, linezolid, teicoplanin and pipracillin/tazobactam, but resistant to amoxicillin, ofloxacin, ciprofloxacin, lev-

ofloxacin, erythromycin, azithromycin, clindamycin and gentamycin. These results are congruent with other studies,[5,29] respectively.

There are limited data cited throughout the literature concerning antibiotic resistance mechanisms in *Kocuria* spp. As well as, there are no specific guidelines yet to perform antibiotic susceptibility testing for *Kocuria* enforced many studies, including ours. Therefore, we relied on studies that considered *Kocuria* spp. as *Staphylococcus*.[30] For this reason, all results mentioned above were performed according to the Clinical and Laboratory Standards Institute guidelines for *Staphylococcus*.[31]

In our study, macrolides resistance genes were detected in *Kocuria* spp. Genes such as *erm*A, *erm*B and *erm*C have been shown to confer resistance to macrolides by target site alteration of the ribosome. The *mef* & *msr*A genes, encode macrolides efflux and may be all these genes encode the efflux pump and 23SrRna. The later have been detected in this study in a low number of bacterial isolates. This was coincident with Guyomard, *et al.*[32] Although the *Kocuria* spp. were resistant to macrolides, the number of genes detected was low. This could be due to intrinsic resistance, another mechanism of resistance, or to the limitation of PCR primers used in the detection of the resistance genes, which was used in macrolides resistance in staphylococci.[16] Integrons are transposons (like genetic elements), which are considered a reservoir for antibiotic resistance genes to be disseminated in the environment and have excessive ability for chromosomal integration in bacteria.[33] Our results showed that there was a strong relationship between the prevalence of *intI*1 gene and antimicrobial resistant, and with the capacity of biofilm formation, this result agree with.[34]

Conclusion

Kocuria spp. behave as opportunistic pathogens, responsible for different infections in different ages, and capable of forming biofilms and multi-drug resistant bacteria. The resistance of *Kocuria* to antibiotics further increases its clinical importance. Hopefully, the antimicrobial susceptibility patterns presented in this work might provide a hint for treatment until the CLSI guidelines are formed.

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Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for republication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Medical City Educational Laboratories, Baghdad.

Authors' contribution statement

M.B. Performed data curation and analysis and writing; M.K. study design, data analysis, editing, and revision; I. M. study design, editing, and revision; E.H. revision; E. M. revision; S. T. Study design, data analysis, revision and editing.

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المقاومة للمضادات الحياتية والخصائص الجزيئية لبكتريا كوكوريا الممرضة البازغة حديثا والمعزولة من العراق ومصر

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الخلاصة

تم جمع المعزولات البكتيرية من عينات سريرية مختلفة من مختبرات مدينة الطب التعليمية في بغداد ومستشفى عين شمس التخصصي في القاهرة. وقد تم تعريف هذه المعزولات بواسطة نظام VITEK2 الآلي المدمج. كما تم فحص الحساسية للمضادات الحيوية، وتكوين الأغشية الحيوية، والتقصي عن جينات مقاومة الماكروليدات *ermA*، و*ermB*، و*ermC*، و*mefA & msr*، و*intI1* integron، و*intI2*. حيث تم جمع 60 عزلة من البلدين (42عزلة من العراق، و18 عزلة من مصر). تم تشخيص العزلات البكتيرية من خلال صفاتها المورفولوجية والكيميائية الحيوية. كانت العزلات حساسة للبيبراسيللين/تازوباكتام والفانكومايسين، ومقاومة للإريثرومايسين والأزيثروميسين والأوفلوكساسين. وقد تباينت قدرة بكتريا الكوكوريا على تكوين الأغشية الحيوية، حيث كانت *Kocuria kristinae* منتجة قوية للأغشية الحيوية، بينما تراوحت *Kocuria rhizophila* بين غيرمنتجة في عزلات العراق ومنتجة ضعيفة في عزلات مصر، وأظهرالكشف عن جينات مقاومة الماكروليدات أن جين *mef* كان موجودا في العزلات من العراق بنسبة 7.14% فقط بينما وجد جين *ermA* في العزلات من مصر بنسبة 6.66% فقط، كما ثبت بالكشف عن integron أن *intI1* كان موجودا في العزلات من العراق ومصر بينما تم اكتشاف *intI2* في العزلات من العراق فقط. على حد علمنا، هذه هي أول دراسة مقارنة أجريت للتحقيق في وجود *Kocuria* spp. في العراق ومصر. تسلط الدراسة الضوء على أهمية *Kocuria* spp. والتي تعتبر من مسببات الأمراض الانتهازية، حيث أنها مسؤولة عن حالات العدوى البشرية المختلفة.

الكلمات المفتاحية: حساسية المضادات، تكوين الأغشية الحية، كوكوريا، الجينات المقاومة للمايكروليدات، البكتريا المتعددة المقاومة .