Egyptian Journal of Medical Microbiology Volume 29 / No.1 / January 2020 153-160 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Identification and characterization of a soil isolate of *Chromobacterium violaceum* from Egypt with potential to cause disease

[1]Yasser M. Ibrahim, [1,2]Ahmed M. Abouwarda, [1]Nouran H. Assar*

[1]Department of Microbiology, General Division of Basic Medical Sciences, National Organization for Drug Control and Research (NODCAR), Giza, 12611, Egypt
[2]Department of Biology, Faculty of Science and Arts-Khulais, Jeddah University, Saudi Arabia

Key words:
quorum sensing, Chromobacterium violaceum, violacein, pathogenicity

****Corresponding Author:***
Nouran Hamed Assar
drnouranhamed@hotmail.com
Tel.: 002010008257223

ABSTRACT

Background: *Chromobacterium violaceum (C. violaceum) is a Gram-negative bacterium mainly held to be a harmless saprophyte and only known as a biosensor strain for quorum sensing studies. However, the emergence of C. violaceum as an opportunistic pathogen is evident. In the present study, we aimed to isolate C. violaceum from soil and to test the ability of the isolates to cause infection in a murine model.* ***Methodology:*** *C. violaceum strain YM1 was isolated from soil using rice grains enrichment method. We identified the bacterium based on its biochemical characteristics using API 20NE as well as Vitek2. The identity of the bacterium was further confirmed on the molecular basis by phylogenetic analysis of the 16S rRNA gene sequence (GenBank accession number: MK660325). We then tested the pathogenicity of strain YM1 in a murine infection model.* ***Results****: Identification of strain YM1 revealed that it was closely related to the type strain C. violaceum ATCC 12472. Infectious doses of* 10^4*-*10^8 *cfu of YM1 were able to cause disease in mice when introduced via the intraperitoneal route. Furthermore, we provide experimental data on the antibiotic resistance phenotype of the soil isolate. Strain YM1 was resistant to multiple antibiotics to which the reference strain was susceptible. Fortunately, strain YM1 was found susceptible to fluoroquinolones; ciprofloxacin, levofloxacin, and norfloxacin.* ***Conclusion:*** *Here, we report on the potential clinical significance of soil isolates of C. violaceum that could be pathogenic despite their free-living style. Data presented here suggest that it is necessary to recognize C. violaceum as a suspected pathogen especially in conditions involving skin injury associated with soil.*

INTRODUCTION

Chromobacterium violaceum (*C. violaceum*) is a saprophytic bacterium that is associated with soil and water habitats in tropical and subtropical areas. This bacterium is a Gram-negative, facultative anaerobe, motile, catalase and oxidase positive bacillus. Taxonomically, *C. violaceum* was classified in class Betaproteobacteria, order Neisseriales, family Neisseriaceae[1] and now in the newly proposed family Chromobacteriaceae[2]. The Genus Chromobacterium comprises 11 recognized species. Pigmented strains of *C. violaceum* form smooth violet colonies on agar culture media due to the production of water-insoluble pigment called violacein. This pigment, therefore, allows rapid identification of the bacterium in mixed cultures. It is also considered the main secondary metabolite of *C. violaceum* and exhibits a number of biological activities as well as pharmacological applications[3].

For many years, *C. violaceum* was mainly held to be a harmless saprophyte and only known as a biosensor strain for quorum sensing studies[4]. However, despite the free-living style of *C. violaceum*, it has been found to cause infection in human. The first case of *C. violaceum* infection in man was reported from Malaya by Lesslar in 1927[5]. Since then, more than 150 cases of *C. violaceum* infection were reported worldwide. Due to the escalated number of reported cases and the severity of disease, the bacterium is now considered an emergent pathogen. It has been associated with pneumonia, gastrointestinal infection, meningitis, endocarditis, liver abscesses, and sepsis[6]. Contamination with soil infested with the organism is thought to be the primary source of infection as *C. violaceum* could not be isolated from food and is not part of the normal flora of the human body.

There is a number of publications that reported the isolation of *C. violaceum* from different ecosystems. However, reports about the disease-causing ability of soil isolates of this bacterium are rare especially in

Egypt. Therefore, this study was designed to isolate *C. violaceum* from soil and to test the ability of the isolates to cause infection in a murine model. Antibiotic susceptibility of the soil isolate was also investigated. To the best of our knowledge, this is the first report on the pathogenic nature of *C. violaceum* strains from Egypt.

METHODOLOGY

Isolation of *C. violaceum* from soil:

We attempted to isolate *C. violaceum* using culture media suggested for the enumeration of these bacteria such as Bennet's agar[7] and Ryall & Moss agar[8], as well as common media such as Lauria Bertani (LB) agar and Nutrient agar (HiMedia). A simple old method of isolation of *C. violaceum* developed by Corpe[9] was also used. Soil samples were collected from agricultural lands around the applied research centre of medicinal plants, Giza, Egypt. Serial 10-fold dilutions of each soil suspension were made in sterile distilled water and spread onto the appropriate agar plate. For Corpe method, five grams of each soil sample were spread in sterile 15-cm glass petri dishes, soaked with sterile distilled water and sprinkled with precooked rice grains. Plates were incubated at 25 ºC for 5 days.

Phenotypic characterization of isolates:

Isolates were preliminarily identified by their cultural, morphological and biochemical characteristics compared to those of the type strain *C. violaceum* ATCC 12472[10]. Motility was detected using a semi-solid agar medium[11]. Catalase production was assessed using 3% hydrogen peroxide. Oxidase activity was tested using oxidase discs (HiMedia) for cultures grown on Muller Hinton agar (MHA) for 24h to prevent obscuring the reaction by the pigment. Pigmentation of cultures grown on MHA was not observed before 48h of incubation (this study). Cyanid production, indole production, growth at different temperatures and NaCl concentrations, anaerobic growth, utilization of carbohydrates were carried out according to standard protocols[12, 13]. Haemolysis assay was performed on LB agar supplemented with 5% sheep blood[14].

Identification of the selected isolate:

The selected isolate YM1 was identified using API 20NE strips (bioMérieux) according to the manufacturer's instructions. The identification was also confirmed by Vitek-2 system (bioMérieux) using colorimetric GN cards. Bacterial suspension was prepared using a Densicheck system (bioMérieux) and the strain was tested according to the manufacturer's instructions. The Vitek-2 system software analyzed the data and the result was automatically reported[15].

16S ribosomal RNA gene amplification and sequencing:

Genomic DNA was prepared according to a published protocol[16]. Primer pair YM1-F (5'-GCGGTTGTGCAAGTCTGATG-3') and YM1-R (5'-CCTCCTTGCGGTTAGCCTAC-3') was used to amplify an 874-bp fragment of the 16S rRNA gene by PCR (SensoQuest GmbH, Germany). The PCR reaction (100 µl) was set up as follows; 5 µl (~500 ng) genomic DNA, 1 µM of each primer (Sigma), 10 µl of thermopol buffer, 2 µl of $MgSO_4$, 2 units of Vent® polymerase and 50 µM of dNTPs (Promega). The reaction volume was made up to 100 µl using dH_2O. The PCR reactions were heated to 95°C for 5 min and run through 35 cycles of (95°C for 30s, 60 °C for 30s, 72 °C for 1 min) and then heated to 72 °C for 5 min.

Purified PCR product was sent to Sigma-Scientific Co. for sequencing. DNA Sequence was carried out based on the dideoxy method developed by Sanger *et al*[17]. Sequence homology searches were performed using BLAST[18] and the NCBI (https://www.ncbi.nlm.nih.gov/). Phylogenetic analysis was inferred by Tree Viewer 1.17.5 software using the neighbour-joining method[19] calculated by Kimura's two-parameter model[20].

Nucleotide sequence accession number:

The 16S rRNA partial gene sequence of the soil isolate (*Chromobacterium violaceum* strain YM1) was deposited in the GenBank Data Library under accession number (MK660325).

Mouse infection model and *in vivo* experiment:

Female BALB/c mice were housed at the faculty of science and arts, Khulais, University of Jeddah according to the standard laboratory animal care advisory committee guidelines. To prepare the standard inocula of bacteria, Cells from 900 µl of glycerol stock were collected by centrifugation at 13,000 ×g for 3 min. Pellets were resuspended in 900 µl of sterile phosphate buffered saline (PBS) and diluted as necessary to give different infectious doses (10^2-10^8 cfu). The infectious doses were confirmed by plating out serial dilutions on LB agar medium. A group of five mice was used for each infectious dose as well as an uninfected control group. Mice were injected with 200 µl of bacterial suspension containing the appropriate infectious dose of either strain YM1 or 12472 into their peritoneal cavities. Mice were observed daily for two weeks to evaluate survival[21]. When showing signs of lethargy or upon becoming moribund, mice were humanely euthanized and were considered to reach the endpoint of the experiment[22].

Susceptibility of *C. violaceum* YM1 to different antibiotics:

Susceptibility tests were carried out by disc diffusion method according to the guidelines of the CLSI[23]. MHA medium was poured in petri dishes and allowed to solidify before the test bacteria were streaked onto the surface of the agar. Antibiotic discs were placed on the surface of the agar and the plates were left in a refrigerator for 2 hours to allow the diffusion of

antibiotics. Plates were then incubated at 28 ºC for 24 hours. The inhibition zone (if any) around each antibiotic disc was measured. Interpretation of results was according to CLSI[23].

Statistical analysis:

Statistical analyses were performed using Student's *t*-test. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Recovery of *C. violaceum*:

Attempts to isolate the bacterium using common culture media were unsatisfactory. Pigmented bacteria suspected to be *C. violaceum* was only isolated using rice grains enriched soils. We could observe the violet-coloured growth of isolates on rice grains after 3 days of incubation at 25 ºC. Fourteen isolates were recovered from different soil samples and were assigned YM1 through YM14.

Characterization of isolates:

All 14 isolates were Gram-negative round-ended bacilli. Table 1 summarizes the results of phenotypic and physiological characterization of all isolates. Five isolates; YM2, 5, 6, 7, and 14 were non-motile, unable to produce cyanide, oxidase negative and grew at 4ºC but not at 37ºC. These features characterize the Genus Iodobacter rather than Chromobacterium. The other nine isolates were motile, six of which; YM3, 4, 8, 9, 12, and 13 were again unable to produce cyanide, grew at 4ºC but not at 37ºC or anaerobically suggesting that they were probably related to the Genus Janthinobacterium. With two of the remaining three isolates; YM10 and YM11 oxidase negative, only isolate YM1 showed the perfect match of the characteristics of the Genus Chromobacterium. Isolate YM1 was therefore selected for further studies.

Table 1. Description of phenotypic and physiological characteristics of violet-pigmented soil isolates

Characteristic	ATCC 12472	YM1	YM2	YM3	YM4	YM5	YM6	YM7	YM8	YM9	YM10	YM11	YM12	YM13	YM14
Motility	+	+	-	+	+	-	-	-	+	+	+	+	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	-	+	+	-	-	-	+	+	-	-	+	+	-
Cyanide production	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Haemolysis	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+
Growth at															
4 ºC	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+
37 ºC	+	+	-	-	-	-	+	-	-	-	+	-	-	-	+
2% NaCl	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
4% NaCl	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	-	-	+	+	+	-	-	+	+	-	-	+
Growth on citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Acid from															
Glucose	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+
Inositol	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Maltose	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-

Biochemical and molecular identification of isolate YM1:

Isolate YM1 was identified to the species level by API 20NE strip. The isolate gave a numerical profile of 5150555 which was 99.9% identified as *C. violaceum* (T value was 0.91) with no test results against this identification. Identification was also confirmed by Vitek-2 system using GN card. Furthermore, we analyzed the sequence of the 874-bp fragment from position 578 to 1451 of the 16S rRNA gene. BLASTN results and phylogenetic analysis revealed that isolate YM1 shared identical 16S rRNA gene sequence with the type strain *C. violaceum* ATCC 12472 as well as *C. violaceum* NBRC 12614 and *C. violaceum* JCM 1249. For other species of the Genus, similarity ranged from 96.58 to 99.31% (Figure 1).

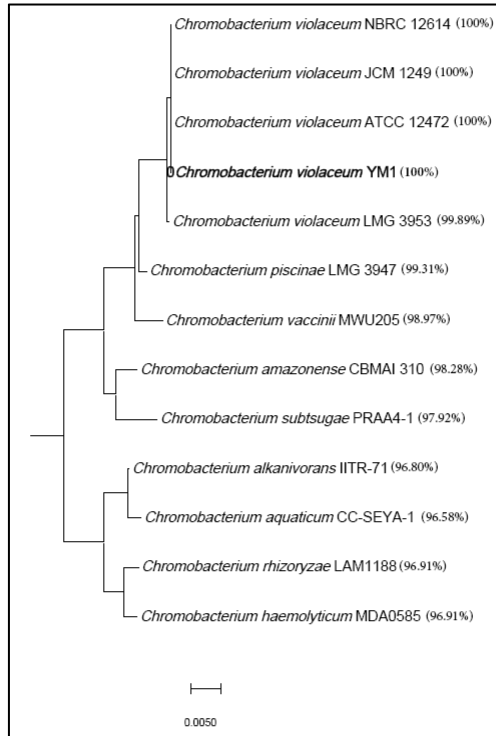

Fig. 1: Phylogenetic tree based on 16S rRNA gene sequence showing *C. violaceum* YM1 strain and other *Chromobacterium spp*. The tree was inferred using the neighbour-joining method calculated by Kimura's two-parameter model.

Pathogenicity of *C. violaceum* YM1:

Virulence of *C. violaceum* in animal models of infection has been reported before[24, 25]. Here, we sought to characterize the virulence of isolate YM1. All mice infected with 10^8 or 10^7 cfu/mouse started to show symptoms of illness at 24h post infection, and were dead by 48h or 72h, respectively (Figure 2A). At 5 or 7 days post infection, 80% of mice infected with 10^6 or 10^5 cfu/mouse died, whereas a dose of 10^4 was able to kill 60% of the tested animals 8 days after infection. On the other hand, mice infected with 10^3 or 10^2 cfu/mouse survived the course of infection similar to the control uninfected group (Figure 2A). We also investigated the virulence of the type strain ATCC 12472 in the same murine model. It was able to cause infection in mice at higher infectious doses (10^4-10^8 cfu) but not at lower ones (10^2 or 10^3 cfu) similar to the soil isolate YM1 (Figure 2B). A dose of 10^3 cfu resulted in death of 20% of mice. However, this was not statistically significant compared to the control uninfected group.

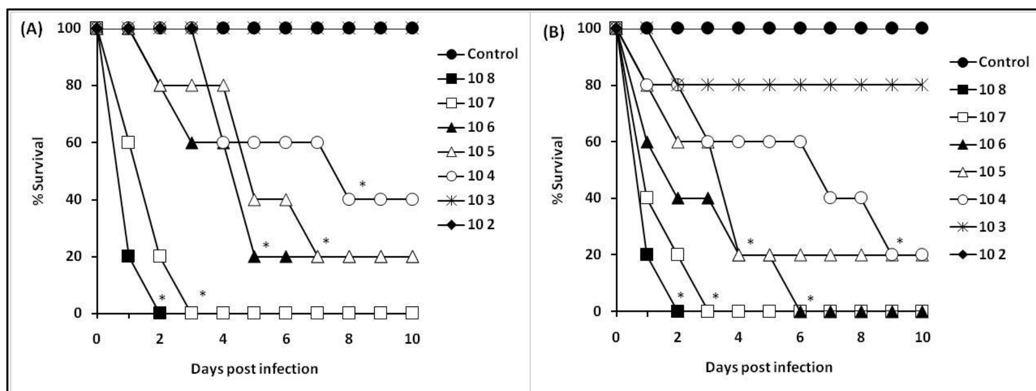

Figure 2: Survival of BALB/c mice infected with either the soil isolate *C. violaceum* strain YM1 in (A) or the type strain *C. violaceum* ATCC 12472 in (B). Mice were infected intranperitoneally with the indicated infectious dose and were observed daily for two weeks to evaluate survival. *, $P < 0.05$ for shorter survival times for infected mice than for the control uninfected group.

Antimicrobial susceptibility of isolate YM1:

We tested the susceptibility of our isolate to 32 antibiotics by disc diffusion method with the type strain ATCC 12472 used as a reference. Similar to the reference strain, isolate YM1 was sensitive to Amikacin, Ciprofloxacin, Doxycycline, Erythromycin, Gentamicin, Imipenem, Levofloxacin, Nitrofurantoin, Norfloxacin, Piperacillin, Piperacillin-Tazobactam, Tigecycline, Tobramycin, and Trimethoprim/ Sulphamethazole (table 2). However, it was found resistant to Azithromycin, Chloramphenicol, Clarithromycin, Fusidic acid, and linezolid to which the reference strain was susceptible. Of the tested antibiotics, fluoroquinolones; Ciprofloxacin, Levofloxacin, and Norfloxacin exhibited the highest activities against both YM1 and ATCC 12472.

Table 2: Antibiotic susceptibility of *C. violaceum* strain YM1 compared to that of the type strain ATCC 12472

Antibiotic	Disc content (µg)	Inhibition zone, mm (interpretation)[a]	
		C. violaceum YM1	*C. violaceum* 12472
Amikacin	30	32 (S)	22 (S)
Amoxicillin/Clavulanic acid	30	6 (R)	6 (R)
Ampicillin/Sulbactam	20	6 (R)	6 (R)
Azithromycin	15	6 (R)	36 (S)
Cefepime	30	6 (R)	6 (R)
Cefoperazone	75	6 (R)	6 (R)
Cefotaxime	30	6 (R)	6 (R)
Ceftazidime	30	6 (R)	6 (R)
Ceftriaxone	30	6 (R)	6 (R)
Cefuroxime	30	6 (R)	6 (R)
Chloramphenicol	30	6 (R)	26 (S)
Ciprofloxacin	5	40 (S)	50 (S)
Clarithromycin	15	15 (R)	25 (S)
Clindamycin	2	6 (R)	6 (R)
Doxycycline	30	30 (S)	51 (S)
Erythromycin	15	23 (S)	40 (S)
Fosfomycin	200	9 (R)	9 (R)
Fusidic acid	10	6 (R)	20 (S)
Gentamicin	10	23 (S)	27 (S)
Imipenem	10	39 (S)	36 (S)
Levofloxacin	5	35 (S)	50 (S)
Linezolid	30	6 (R)	28 (S)
Meropenem	10	6 (R)	6 (R)
Nitrofurantoin	300	30 (S)	38 (S)
Norfloxacin	10	33 (S)	53 (S)
Oxacillin	5	6 (R)	6 (R)
Piperacillin	100	25 (S)	21 (S)
Piperacillin-Tazobactam	110	35 (S)	29 (S)
Teichoplanin	30	6 (R)	9 (R)
Tigecycline	15	21(S)	36 (S)
Tobramycin	10	22 (S)	23 (S)
Trimethoprim/Sulphamethazole	25	21 (S)	20 (S)

[a], based on the CLSI for non-enterobacteriaceae [23]

DISCUSSION

It is known that *C. violaceum* constitutes a minor component of the total soil microflora. Failure to recover *C. violaceum* using common culture media in this study could, therefore, be attributed to the overgrowth of the large number of other bacteria that coexist with it. Analysis of the genome revealed the existence of genes necessary for the adaptability of this bacterium to a wide range of environmental conditions such as UV radiation[26]. Proteomic analysis has also identified numerous proteins associated with alternative pathways for energy generation, cell motility, and secondary metabolism[27] that could help the organism

cope with different environmental stresses. We were able to isolate *C. violaceum* only when using rice grains-enrichment soils[9]. We identified the bacterium to the Genus level using conventional methods. API 20NE and Vitek2 were used to identify the organism to the species level. We focused only on the single isolate that shared identical physiological characteristics with the type strain *C. violaceum* ATCC 12472. Other isolates showed physiological and phenotypical similarities to the Genera Janthinobacterium and Iodobacter, species of which were formerly grouped in the Genus Chromobacterium as violet pigment producing bacteria[28]. We also confirmed the identification using a molecular approach. Analysis of the 16S rRNA sequence and phylogenetic tree confirmed that strain YM1 isolated in this study is closely related to the type strain *C. violaceum* ATCC 12472. It was important to use multiple methods for identification because misidentification of *Burkholderia pseudomallei* as *C. violaceum* has been reported[29].

Despite being rare, human infection with *C. violaceum* does occur. The infection is characterized by rapid progress to sepsis[30]. Skin injury in association with exposure to contaminated soil or water is believed to be the main factor predisposing to infection. A study of 106 patients revealed that 68% had skin lesions during the clinical course, 82% had sepsis at presentation with *C. violaceum* as the single causative agent in blood culture of 58% of them[30]. In support of this belief, Madi and co-workers have reported a case of *C. violaceum* septicemia with multiple liver abscesses for a patient who had a history of trauma to her leg while working in a farm[31]. In the present study, we tested the ability of strain YM1 to cause infection in a mice model to find out whether *C. violaceum* could be pathogenic directly from soil. Pathogenicity of *C. violaceum* in mice was reported for strain 6357, which was isolated from human[32] and for strain CVN, which is a spontaneous nalidixic acid-resistant strain of the type strain 12472[21] as well as for strain 12472[24, 25]. Virulence of soil isolates of *C. violaceum* in mice was not tested before. The results of our study revealed that the soil isolate of *C. violaceum* YM1 was able to cause infection in BALB/c mice when introduced via the intraperitoneal route. Reported virulence of the type strain 12472[24, 25] was also confirmed here except for lower infectious doses (10^2 and 10^3 cfu/mouse) contrary to a published data[25]. This could be due to difference in the animal models used.

Knowledge of the pathogenesis of *C. violaceum* infection is far from complete. The release of the genome sequence of *C. violaceum* ATCC 12472[10] has enabled the study of the mechanisms of virulence of this bacterium. One of the most important virulence factors is the type III secretion system (T3SS), a needle-like structure composed of multiple proteins which is believed to enable bacteria inject virulence-associated effectors into host cells[33]. *C. violaceum* encodes two T3SSs whose genes are clustered in Chromobacterium pathogenicity islands 1, 1a, and 2 (Cpi-1, Cp-1a and Cpi-2)[34]. Cpi-1/-1a-encoded T3SS was found to be a major virulence determinant that causes fatal infection by the induction of cell death in hepatocytes of mice[21]. Although the role of Cpi-2 is still unclear, it could be required for survival of *C. violaceum* within macrophages similar to the role of the corresponding Cpi-2 in Salmonella[35]. Two-component systems involved in *C. violaceum* pathogenicity were also identified. Quorum-sensing system was found to be responsible for pathogenicity in a nematode model[36]. A more recent study has proposed that the *C. violaceum* OhrA/OhrR system is required for bacterial defense against organic hydroperoxides (OHPs) and to modulate virulence in the host[24]. Deletion in *ohrR* gene resulted in the downregulation of hemolysin, chitinase, and collagenase, which are known virulence factors[24]. Other virulence factors include type VI pilli involved in cell adhesion and motility, lipopolysaccharides and peptidoglycan that trigger host immune response resulting in septic shock[34], and secreted virulence determinants including collagenase[37].

Diseases caused by *C. violaceum* proved to be fatal in many cases. Therefore, early diagnosis based on bacterial culture and susceptibility data could be life-saving. However, data on the antimicrobial susceptibility of *C.violaceum* strains is limited because their isolation is rare. We aimed to investigate the antimicrobial susceptibility profile of YM1. Results suggested a multidrug resistance phenotype of YM1 compared to that of the type strain. The phenomenon of intrinsic resistance in *C. violaceum* was described based on genes associated with drug resistance identified in the genome of the type strain ATCC 12472. Open reading frames linked with different mechanisms of antibiotic resistance such as beta-lactam and multidrug resistance were reported[38]. These genes are expected to provide a survival advantage of *C. violaceum* over competing microorganisms. Results of the present study also revealed that fluoroquinolone; ciprofloxacin, Levofloxacin, and Norfloxacin showed the highest activity against YM1 as well as against the reference strain. This is in agreement with a reported data suggesting that sensitivity of *C. violaceum* strains to Quinilones was between 94 and 100% of the tested strains[30]. Moreover, ciprofloxacin was reported as an effective treatment for *C. violaceum* infection[39, 40].

CONCLUSION

We show that a soil isolate identified as *C. violaceum* is pathogenic in an animal model of infection and provide data on its antibiotic resistance phenotype as well as effective antibiotics that could be considered for treatment of infection caused by this bacterium. Data

presented here as well as previous data on the unique adaptability of this bacterium to extreme conditions suggest that this organism could be emerging as a "professional" pathogen.

Authors' contributions:

- Yasser M. Ibrahim, Ahmed M. Abouwarda and Nouran H. Assar conceived and designed the study.
- Yasser M. Ibrahim, Ahmed M. Abouwarda and Nouran H. Assar carried out the experiments.
- Yasser M. Ibrahim, Ahmed M. Abouwarda and Nouran H. Assar analyzed the data.
- Yasser M. Ibrahim wrote the manuscript.
- Ahmed M. Abouwarda and Nouran H. Assar critically reviewed the manuscript.

Acknowledgment:

- We appreciate the financial support from the National Organization for Drug Control and Research.

Conflicts of interest:

- The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

REFERENCES

1. Dewhirst FE, Paster BJ, Bright PL. Chromobacterium, Eikenella, Kingella, Neisseria, Simonsiella, and Vitreoscilla Species Comprise a Major Branch of the Beta Group Proteobacteria by 16S Ribosomal Ribonucleic Acid Sequence Comparison: Transfer of Eikenella and Simonsiella to the Family Neisseriaceae (emend.). Int J Syst Evol Microbiol 1989; 39:258-266.
2. Adeolu M, Gupta RS. Phylogenomics and molecular signatures for the order Neisseriales: proposal for division of the order Neisseriales into the emended family Neisseriaceae and Chromobacteriaceae fam. nov. Antonie Van Leeuwenhoek 2013; 104:1-24.
3. Kothari V, Sharma S, Padia D. Recent research advances on *Chromobacterium violaceum.* Asian Pac J Trop Med 2017; 10:744-752.
4. McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, et al. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology 1997; 143:3703-11.
5. Sneath PH, Whelan JP, Bhagwan Singh R, Edwards D. Fatal infection by *Chromobacterium violaceum.* Lancet 1953; 265:276-7.
6. Batista JH, da Silva Neto JF. *Chromobacterium violaceum* Pathogenicity: Updates and Insights from Genome Sequencing of Novel Chromobacterium Species. Front Microbiol 2017; 8:2213.
7. Keeble JR, Cross T. An Improved Medium for the Enumeration of Chromobacterium in Soil and Water. J Appl Bacteriol 1977; 43:325-327.
8. Ryall C, Moss MO. Selective Media for the Enumeration of Chromobacterium spp. in Soil and Water. J Appl Bacteriol 1975; 38:53-59.
9. Corpe WA. A study of the wide spread distribution of Chromobacterium species in soil by a simple technique. J Bacteriol 1951; 62:515-517.
10. Vasconcelos A, Almeida D, Almeida F. The complete genome sequence of *Chromobacterium violaceum* reveals remarkable and exploitable bacterial adaptability. Proc Natl Acad Sci USA 2003; 100:11660-11665.
11. Tittsler RP, Sandholzer LA . The Use of Semi-solid Agar for the Detection of Bacterial Motility. J Bacteriol 1936; 31:575-580.
12. Gillis M, Logan N. Chromobacterium. in: FR W. B. Whitman, P. Kampfer, M. Trujillo, J. Chun, P. DeVos, B. Hedlund and S. Dedysh (ed) John Wiley & Sons 2015.
13. Reddy CA. Methods for general and molecular microbiology. 3rd ed. Washington, DC: ASM Press 2007
14. Young C-C, Arun AB, Lai W-A, Chen W-M, Chao J-H, Shen F-T, et al. *Chromobacterium aquaticum* sp. nov., isolated from spring water samples. Int J Syst Evol Microbiol 2008; 58:877-880.
15. Wallet F, Loiez C, Renaux E, Lemaitre N, Courcol RJ. Performances of VITEK 2 Colorimetric Cards for Identification of Gram-Positive and Gram-Negative Bacteria. J Clin Microbiol 2005; 43:4402-4406.
16. Neumann B, Pospiech A, Schairer HU. Rapid isolation of genomic DNA from Gram-negative bacteria. Trends in Genetics 1992; 8:332-333.
17. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74:5463-7.
18. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990; 215:403-10.

19. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-25.
20. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980; 16:111-120.
21. Miki T, Iguchi M, Akiba K, Hosono M, Sobue T, Danbara H, et al. Chromobacterium pathogenicity island 1 type III secretion system is a major virulence determinant for Chromobacterium violaceum-induced cell death in hepatocytes. Mol Microbiol 2010; 77:855-72.
22. Toth LA. The moribund state as an experimental endpoint. Contemp Top Lab Anim Sci 1997; 36:44-8.
23. CLSI. Performance standards for antimicrobial susceptibility testing. 27th ed. CLSI supplement M100 ed. Wayne, PA: Clinical and Laboratory Standards Institute 2017
24. Previato-Mello M, Meireles DA, Netto LES da Silva Neto JF. Global Transcriptional Response to Organic Hydroperoxide and the Role of OhrR in the Control of Virulence Traits in *Chromobacterium violaceum.* Infect Immun 2017; 85:e00017-17.
25. Maltez VI, Tubbs AL, Cook KD, Aachoui Y, Falcone EL, Holland SM, et al. Inflammasomes Coordinate Pyroptosis and Natural Killer Cell Cytotoxicity to Clear Infection by a Ubiquitous Environmental Bacterium. Immunity 2015; 43:987-97.
26. Hungria M, Nicolas MF, Guimaraes CT, Jardim SN, Gomes EA, Vasconcelos AT. Tolerance to stress and environmental adaptability of *Chromobacterium violaceum.* Genet Mol Res 2004; 3(1):102-16.
27. Castro D, Cordeiro IB, Taquita P, Eberlin MN, Garcia JS, Souza GHMF, et al. Proteomic analysis of *Chromobacterium violaceum* and its adaptability to stress. BMC microbiology 2015; 15:272.
28. Buchanan RE. Studies in the Nomenclature and Classification of the Bacteria: V. Subgroups and Genera of the Bacteriaceae. J Bacteriol 1918; 3:27-61.
29. Amornchai P, Chierakul W, Wuthiekanun V, Mahakhunkijcharoen Y, Phetsouvanh R, Currie BJ, et al. Accuracy of *Burkholderia pseudomallei* Identification Using the API 20NE System and a Latex Agglutination Test. J Clin Microbiol 2007; 45:3774-3776.
30. Yang CH, Li YH. *Chromobacterium violaceum* infection: a clinical review of an important but neglected infection. J Chin Med Assoc 2011; 74(10):435-41.
31. Madi DR, Vidyalakshmi K, Ramapuram J, Shetty AK. Successful Treatment of *Chromobacterium violaceum* Sepsis in a South Indian Adult. Am J Trop Med Hyg 2015; 93(5):1066-1067.
32. Segal BH, Ding L, Holland SM. Phagocyte NADPH oxidase, but not inducible nitric oxide synthase, is essential for early control of *Burkholderia cepacia* and *chromobacterium violaceum* infection in mice. Infect Immun 2003; 71(1):205-10.
33. Galan JE, Wolf-Watz H. Protein delivery into eukaryotic cells by type III secretion machines. Nature 2006; 444:567-73.
34. Brito CF, Carvalho CB, Santos F, Gazzinelli RT, Oliveira SC, Azevedo V, et al. *Chromobacterium violaceum* genome: molecular mechanisms associated with pathogenicity. Genet Mol Res 2004; 3(1):148-61.
35. Hensel M. Salmonella pathogenicity island 2. Mol Microbiol 2000; 36:1015-23.
36. Swem LR, Swem DL, O'Loughlin CT, Gatmaitan R, Zhao B, Ulrich SM, et al. A quorum-sensing antagonist targets both membrane-bound and cytoplasmic receptors and controls bacterial pathogenicity. Mol Cell 2009; 35:143-53.
37. Castro-Gomes T, Cardoso MS, DaRocha WD, Laibida LA, Nascimento AM, Zuccherato LW, et al. Identification of secreted virulence factors of *Chromobacterium violaceum.* J Microbiol 2014; 52:350-3.
38. Fantinatti-Garboggini F, Almeida R, Portillo Vdo A, Barbosa TA, Trevilato PB, Neto CE, et al. Drug resistance in *Chromobacterium violaceum.* Genet Mol Res 2004; 3(1):134-47.
39. Olalekan A, Itua F, Mutiu B, Egwuatu T, Akinloye O Iwalokun B. Case Report on Pleural Empyema Thoracis and Urinary Tract Infection Caused by *Chromobacterium violaceum* from Lagos, Nigeria. Case Reports in Medicine 2019; Article ID 5321484:5.
40. Darmawan G, Kusumawardhani RNY, Alisjahbana B, Fadjari TH. *Chromobacterium violaceum*: The Deadly Sepsis. Acta Med Indones 2018; 50(1):80-81.