Antibacterial Activity of Some Essential Plant Oils against Clinical Strain of Corynebacterium Stationis

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Abstract:

  Corynebacterium stationis is a facultative anaerobic gram-positive bacteria. It is an opportunistic pathogen that is one of more than 42 *corynebacterium* species and subspecies found in humans, the majority of which have been linked to opportunistic illness. Human excrement, blood, and sea water are commonly used to isolate it. Mastitis in cattle is caused by the bacteria *c. stationis* and is characterized by inflammation of the mammary gland.. Milk from the infected cows is contaminated with bacteria, making it unsafe for human consumption and causing bacterial diseases in humans. .In this study , we investigated the antimicrobial activity of some essential plant oils against *c. stationis* bacteria isolated from human feces. One bacterial isolate was identified biochemically then characterized by 16s rRNA genotyping and was designated as *Corynebacterium stationis* strain M.S . Antibiotic susceptibility test showed resistance of *c. stationis* M.S to three antibiotic (Erythromycin ,Clindamycin and Azithromycin). By using thirteen Egyptian essential plant oils .we invitro investigated and indicated the efficacy of black seed oil and rosemary oil against *c. stationis* M.S with inhibition zone of 18.00 ±1.3 mm. The Minimum inhibitory (MICs) and Minimum bactericidal concentrations (MBCs) of black seed and rosemary oils against *c. stationis* M.S were found to be 19.5 mg/L and 39 mg/L respectively . Killing time of *c. stationis* M.s upon growing with 200 mg/L of black seed and rosemary were after 6 and 7 hours, respectively.

Key words :   *Corynebacterium Stationis* ,Opportunistic ,Essential Oils.

1.Introduction

  "Club-shaped" Gram-positive, medically relevant species of the genus Corynebacterium and of the "irregular" Gram-positive coryneforms mostly but not exclusively from the suborder Micrococcineae. Those coryneforms were selected because they most closely resemble Corynebacterium species sensu stricto phenotypically and so can be difficult to differentiate from members of that genus. Coryneforms described here are rare, opportunistic human pathogens, and they are Gram-positive bacilli or coccobacilli. Nearly all are catalase positive, and they express a range of pigments and metabolic processes.[1]

  These coryneform bacteria are increasingly being recognized as causing opportunistic disease under specific circumstances, such as in patients who are immunocompromised, have prosthetic devices, or have been in hospitals/nursing homes for long-term periods of time

Corynebacterium stationis is gram-positive, facultative anaerobic organism .It is an opportunistic pathogen that represents one of more than 42 species and subspecies of corynebacterium recovered from humans ,most of which have been associated with opportunistic disease [2] It is usually isolated from human stool, blood and sea water [1] .Mastitis in bovines caused by c. stationis which is characterized by inflammation of the mammary gland and it is a complex and costly disease in dairy herds. The bacterial contamination of milk from the affected cows makes it unhealthy for human consumption and causes human bacterial infections . [3]   Interestingly , c .stationis is resistant to some commonly used antibiotics such as penicillin and clindamycin .[3]

  Despite the prevalence of this organism ,unfortunately, only few papers published dealing with this opportunistic pathogens all over the world .and didn't produce any trustable alternative therapy to deal with these infections .Accordingly, we have designed this study to isolate this bacteria from human clinical specimens and to shed light on the efficacy of using the essential oils of some of the Egyptian native medicinal herbs as a safe alternative treatment.

2. Material and methods

2.1 Sample collection and processing:

  Twenty two stool specimens were collected from private Laboratory at Banha ,Egypt and inserted into nutrient broth tubes as a transport media then transported according to [4] under aseptic conditions to microbiology laboratory ,faculty of science, Benha university ,Egypt where the study was carried out.

2.2. Isolation and cultivation of bacteria

  By using micropipette , 100 μl of nutrient broth containing stool sample was inoculated on trypticase soy agar enriched with 10% human blood . The initial growth of bacteria was obtained after 24 h culture in anaerobic conditions at 37 °C.

2.3. Biochemical identification and 16s rRNA gene sequencing

  only 5 out of the 22   collected samples were grown anaerobically, showed bacterial growth on the blood selective media. Biochemical properties identified single type   of anaerobic bacterial isolate. This bacterial isolate were found to be gram-positive bacilli ,non-motile are commonly found in short rods , occur singly, in pairs and in 'V' forms. .The biochemical profile of this anaerobic bacteria was consistent with bacteria formerly known as corynebacterium stationis The partial 16S rRNA gene sequencing was used to confirm the biochemical identification. Universal 16s rRNA   primers   8F   (50   -AG AGTTTGATCCTGGCTCAG-30) and 1492R (50 -

GACGGGCGGTGTGTRC A-30) [5] were used to amplifiy the 16s rRNA of anaerobic bacteria. Sanger sequences were generated at Sigma company Genomics Core Facility (Cairo, Egypt) using an ABI PRISM 3730xl DNA Analyzer (Life Technologies Corporation, CA). Sequences similarity were conducted using the 98 BLASTn search at the NCBI website, phylogenetic and molecular evolutionary analysis were conducted using MEGA software version X   [6]

2.3.1. Isolates accession numbers.

 Corynebacterium stationis M.S was deposited in the Genebank database and were assigned the accession number MW543939.1

2.4. Antibiotic susceptibility test:

Antibiotics susceptibility testing was performed using the disc diffusion method [7] for the following antibiotics (Oxoid, UK); The antibiotics tested in this study include , Amoxicillin (25 μg), Tetracycline(30 μg), Chloramphenicol (30 μg), ,Ciprofloxacin (5 μg), Norfloxacin(10 μg), vancomycin(30 μg),Gentamycin(10 μg),Rifampin(5 μg),Linezolid (30 μg),Ceftazidime (30 μg),Cefotaxime (30 μg),Clindamycin (2 μg),Azithromycin (15 μg),Erythromycin (15 μg) [8]

2.4. Antibacterial activity of some plant oils

 Agar well diffusion method   [9]–[11]   was used to evaluate the antimicrobial activity of the following plant oils; Tea tree, Cinnamon, Rosemary, Cactus, Lavender, Basil, Lemon, Thyme, Parsley, Almond ,sage ,black seed and Lupine. The crude oils were purchased from the commercial markets in Egypt. Nutrient agar plates were inoculated by spreading a 100 μl of the actively grown bacteria. A sterile cork was used to punch holes with a diameter of 6–8 mm and 10 μl of the tested oils (200 mg/L) was introduced into the well. The inoculated agar plates were incubated at 37 C for 24 h. Diameters of the inhibition zones were measured as (mm).

2.5. MICs and MBCs of black seed and rosemary oils

The MIC of black seed and rosemary oils were determined using a broth assay [12] in 96-well microtiter plates (Sigma Aldrich, USA). Fresh cultures of the tested isolates were prepared in nutrient broth, inocula of concentrations 2×10^7 cfu/ml were used. A two-fold dilution series of black seed and rosemary oils were prepared in 1% DMSO to yield final concentrations ranging from 5000 mg/L to 9.7 mg/L. Chloramphenicol was employed as a positive control. After 18 h and 48 h for (*corynebacterium stationis*), the optical density at 600 nm was measured with a microplate reader (680 XR reader, Bio-Rad). Bacterial growth was confirmed by adding 10 μl of a sterile 0.5% aqueous solution of triphenyltetrazolium chloride (TTC, Sigma–Aldrich) and incubating at 36 °C for 30 min. The viable bacterial cells reduced the yellow TTC to

pink/ red 1,3,5-triphenylformazan (TPF). All assays were performed in triplicate. Streaks were taken from the two lowest concentrations of each oil concentration exhibiting invisible growth and were sub-cultured onto Blood agar media. The plates were incubated at 37 °C for 24–48 h, then inspected for bacterial growth in corresponding to both the oils. MBC was taken as the concentration of the oil that did not exhibit any bacterial growth.

2.6. Time-kill kinetics

This experiment was performed as described previously [13] Prior to the experiment, bacteria were incubated on nutrient broth (Oxoid- England-CM0003) for 90 mins at 37 oC to ensure that all the bacteria were in the action in the logarithmic growth phase. The initial bacterial concentration was measured as cfu/ml. the isolate was inoculated into three flasks, one as growth control, one for each type of oil. All flasks were incubated at 37 oC while shaking at 150 rpm. Aliquots were taken at 0, 1, 2, 3, 4,6 and 7 and viable colony counts on blood agar were calculated as cfu/ml.

3. Results and Discussion

3.1. Isolation, biochemical characterization and 16S rRNA typing of *corynebacterium stationis* .

 The collected samples were grown anaerobically, 5 out of the 22 stool samples showed bacterial growth on the blood selective media. Biochemical properties identified one type of bacterial species . This species was found to be non-motile, gram-positive bacilli , are found in short rods , occur singly, in pairs and in club shapes . The biochemical profile of this anaerobic bacteria was consistent with formerly known as *corynebacterium stationis.*

BlASTn alignments and phylogenetic tree analysis (Fig 1) of the assembled 16s rRNA gene sequences showed highest similarity with the previously partially sequenced 16S rRNA of as *corynebacterium stationis* on the NCBI website. Bacteria were designated as *corynebacterium stationis* Strain M.S .

3.2. Antibiotic susceptibility testing

 Qualitative results from the antibiograms (Table1), showed the resistance of antibiotic to *Corynebacterium stationis* M.S was 21.4 %

All of *C. stationis* isolates were resistant to three antibiotics (Clindamycin , Erythromycin and Azithromycin) this is in contrast to previous study showed no resistance to all antibiotics tested . [14]

The evolution of multidrug-resistant microorganisms is accelerating at an alarming rate. Antibiotics may be acquired straight from drugstores, which is one of the main reasons. [15]–[17] According to the Centers for Disease Control and Prevention, at least 2 million people in the United States get infected with antibiotic-resistant bacteria every year, with at least 23,000 of them dying.

3.3. Antibacterial activity of some plant oils against the isolated bacteria :

corynebacterium stationis M.S showed resistance to 11 out of 13 selected essential plant oils. Rosemary and Black seed and were the only two oils that were effective against this tested bacteria . Black seed oil was more effective than rosemary against *corynebacterium stationis* M.S with inhibition zone of 18.00 ±1.3 mm . Unfortunaely there was no previous published paper to compare with our results.

Figure (1) : Molecular phylogenetic analyses of corynebacterium stationis strain M.S by Maximum Likelihood Model of MEGA 10.0 package
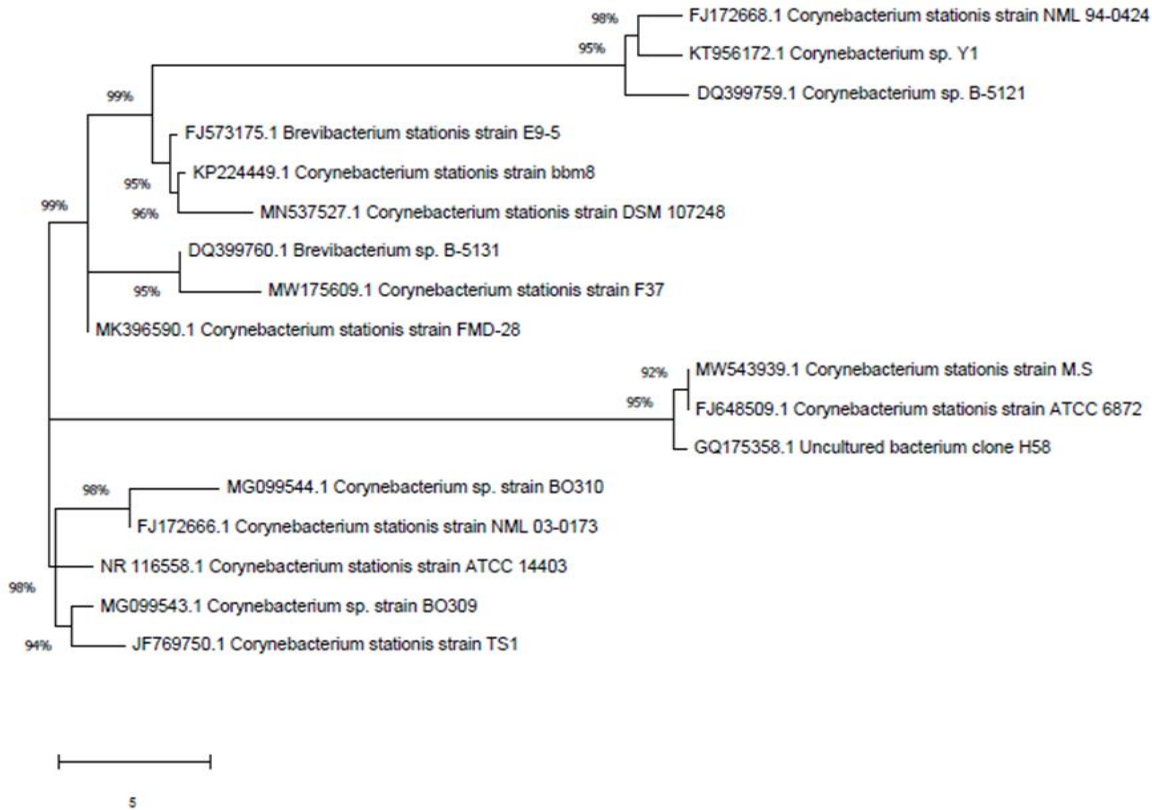

Table (1): Antibiotic susceptibility pattern of bacterial isolate against fourteen antibiotics.

Bacteria	AZM	E	CIP	NOR	CAZ		CTX	DA	LZD	C	RA	VA	AX	TEC	GEN
Corynebacterium stationis M.s	R	R	S	S	I		S	R	S	S	S	S	S	S	S

*Denotes for Resistant (R), Intermediate (I) and Susceptible (S).
Amoxicillin (25 µg), Tetracycline(30 µg), Chloramphenicol (30 µg), ,Ciprofloxacin (5 µg), Norfloxacin (10 µg), vancomycin(30 µg),Gentamycin(10 µg),Rifampin(5 µg),Linezolid (30 µg),Ceftazidime (30 µg),Cefotaxime (30 µg),Clindamycin (2 µg),Azithromycin (15 µg),Erythromycin (15 µg)

Table(2): Antibacterial activity of essential palnt oils against *corynebacterium stationis* M.S. Each value is the mean of three readings (mm) ± standard deviation(SD).

Bacteria	Mean diameter of Inhibition zone(mm) /original zone diameter 5mm												
	Sage	Black seed	Lupine	Almond	Parsley	Thyme	Lemon	Basil	Lavender	Cactus	Rosemary	cinnamon	Tea tree
Corynebacterium stationis M.S	R	18.00 ±1.3	R	R	R	R	R	R	R	R	15.30±0.46	R	R

* Denotes for Resistant or no inhibition (R)

Table (3):   Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of the black seed and Rosemary oils against the isolated bacteria.

Bacteria oil	Black seed oil			Rosemary oil
	MIC	MBC	MIC	MBC
Corynebacterium stationis M.S	19.5	19.5	39	39

Table (4): killer time of *corynebacterium stationis* M.S

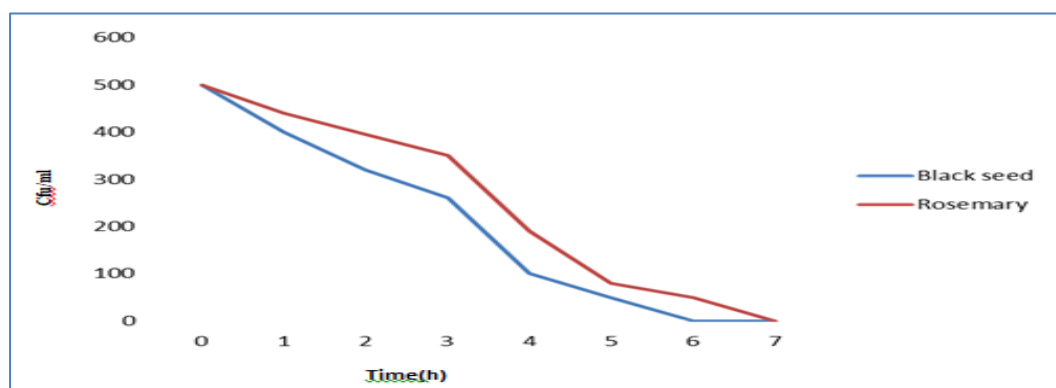|	*Corynebacterium stationis* M.S	
Time	Black seed	Rosemary
0	500	500
1	400	440
2	320	395
3	260	350
4	100	190
5	50	80
٦	٠	50
٧	٠	٠

Fig. (3) Time-kill experiment of corynebacterium stationis M.S Relative viable count of this bacterial isolate was measured for 6 hours and calculated as cfu/ml against black seed oil , rosemary oil .Prior to the experiment, this bacterial isolate was in the action of the logarithmic phase. Aliquots were taken at 0, 1, 2, 3, 4, 5, 6 and 7 and viable colony counts on blood agar were calculated as cfu/ml

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