RESEARCH
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EXTRA CELLULAR BIOFILM FORMATION OF CLINICAL PATHOGEN KLEBSIELLA PNEUMONIAE AND THEIR INHIBITORY ACTIVITY WITH ANTIMICROBIAL SECONDARY METABOLITES

Running title: Extra cellular biofilm formation of clinical pathogen Klebsiella pneumoniae and their inhibitory activity with antimicrobial secondary metabolites
Type of study: Original study

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Abstract

Introduction: *K.pneumoniae* can survive in the hospital environment and colonize the bowel, respiratory and bacterial meningitis. Humans are known to be the primary reservoir. This is a nosocomial pathogen. In recent years, the bacterium is reported to have developed new virulence genes making them resistant to a major class of antibiotics. One of the major sources for isolation and identification of therapeutics against bacterial infection are microbial secondary metabolites. Those that are obtained from Actinobacteria have antifungal, antibacterial, antioxidant, antitumor and antiviral activities. *Nocardia sp* is one such bacteria.

Materials and Method: The isolation of the actinomycetes was done from the marine soil. The isolation and culture of the bacteria was done in Bennett's culture medium and methanolic extract was prepared. The strains of Klebsiella pneumoniae were obtained from pus cells of infected patients. It was grown in Nutrient agar and MacConkey agar was used as a differential medium. 0.1% of Acridine orange dye was used to stain the biofilm of *K.pneumoniae*. Number of viable cells was observed at regular intervals of time under the microscope such as 24 hours, 48 hours and 72 hours.

Results: Secondary metabolite of Nocardiopsis was able to effectively inhibit the growth of the biofilm which was stained with Acridine Orange at 25µg/dL. The number of viable cells was heavily decreased after 72 hours of incubation.

Conclusion: From the results above, it can be concluded that the secondary metabolite of Nocardiopsis was able to inhibit the biofilm growth of *K.pneumoniae*.

Keywords: anti-microbial, biofilm formation, Klebsiella, Nocardiopsis, secondary metabolites

Introduction:

Klebsiella pneumoniae bacteria is a gram-negative bacteria that is most commonly found in the pus cells of wounds, GI and urinary tract infections, septicemia and bacterial meningitis (1). This organism can even survive in the hospital environment and colonize the bowel, respiratory and bacterial meningitis (2). Humans are known to be the primary reservoir. Transmission of infection is mainly through hospital patients and staff. The most common cause for the outbreak in neonatal intensive care units is also because of *K.pneumoniae*. (3) This is not only considered as a leading cause for community infections (4) but also accounts for 11.8% of all nosocomial bacterial infections around the world. Patients with septicemia and with alcohol dependence have shown mortality rates from 50% to 100%.

The mechanism through which Klebsiella pneumoniae shows its virulence is by the presence of lipopolysaccharides present in the gram negative cell wall which acts as the endotoxin. Siderophores released by the bacterium is a compound with low molecular weight which serves as an iron-chelating substance through which virulence is achieved. Several antimicrobial agents have been researched and employed to fight against these notorious bacteria. However, uncontrolled and frequent usage of anti-bacterial agents proved to be ineffective as the *K.pneumoniae* strains quickly developed multi-drug resistance (5).

In recent years, the bacterium is reported to have developed new virulence genes making them resistant to a major class of antibiotics (6). Because of this, WHO has declared it as a "priority pathogen". Biofilms are the major virulence factors of K.pneumoniae and we are yet to understand the complex mechanism behind its strong formation. Some of the antimicrobials in which the bacterium can grow are fluoroquinolones, carbapenems and aminoglycosides (7). Carbapenem resistance of the bacteria is related to a change in the outer cell membrane, increased production of Extended-spectrum beta lactamase enzyme in organisms and an upregulation in efflux pumps. (8)

One of the major sources for isolation and identification of therapeutics against bacterial infection are microbial secondary metabolites. Those that are obtained from Actinobacteria are being arduously researched because of their antifungal, antibacterial, antioxidant, antitumor and antiviral activities. *Nocardia sp* is one such bacteria which is known for its pathogenicity (9). It is a gram positive, partially acid-fast bacilli which is filamentous in nature. It is known to cause Nocardiosis which is of two forms: pulmonary and cutaneous. Approximately 500-1000 new cases of nocardiosis are reported yearly in the United States. The mortality rate in immunosuppressed patients is around 85% and in patients with disseminated infection, it was observed to be in the range of 7-44% (10). However, recent studies have shown that these microorganisms have the ability to produce bioactive secondary metabolites, and have distinct metabolic pathways. These secondary metabolites also show anticancer and immunosuppressive activities (11).

There are limited studies on the inhibitory activity of actinobacteria, especially Nocardiopsis against the biofilm of *K.pneumoniae.* The aim of the present study is to assess the extracellular biofilm formation of the pathogen *Klebsiella pneumoniae* and their inhibition by using the antimicrobial secondary metabolites of *Nocardia species*.

Materials and Methods:

The current study was done in the department of Forensic odontology of Saveetha Dental College, Chennai, Tamil Nadu, India for a duration of 3 months. The isolation of the actinomycetes was done from the marine soil. The morphological characteristics of the actinobacteria was observed under the microscope using the Nonomura key. Chemotaxonomical analysis was performed to determine the similarities and differences between organisms using their biochemical compositions. The isolation and culture of the bacteria was done in Bennett's culture medium. A single colony of Nocardiopsis was transferred from the agar plate into a sterile flask containing a suitable liquid seed medium broth based on ISP 2. The flask was incubated in a shaking incubator at room temperature for about 2 days to obtain a dense and actively growing seed culture. The seed culture was inoculated into Erlenmeyer flasks containing a larger volume of the production medium. The production medium was optimized to stimulate secondary metabolite production. The cultures were incubated on a rotary shaker at the appropriate temperature for 1 week. After fermentation, the culture broth was harvested by filtering it. This was done to separate the bacterial biomass from the liquid medium which contained the secondary metabolites. The liquid culture medium was transferred to a separating funnel and extracted from the secondary metabolites using methanol. The extraction process was repeated several times to maximize the recovery of the metabolites. The crude extract was obtained using a rotary evaporator. 25µg/dL of the extract was added to the biofilm to notice the inhibitory effect.

The strains of Klebsiella pneumoniae were obtained from pus cells of infected patients. It was grown in Nutrient agar and MacConkey agar was used as a differential medium. The culture was allowed to grow and form biofilm for 3 days. After the formation of biofilm, the culture was washed with PBS (phosphate-buffered saline) to remove any non-adherent cells and debris. 0.1% of Acridine orange dye was used to stain the biofilm and check for the number of viable cells. Fluorescent microscopy was used to observe the number of viable cells. It was then treated with the secondary metabolite extract of Nocardiopsis. The number of viable cells was observed at regular intervals of time under the microscope such as 24 hours, 48 hours and 72 hours. Confocal images were taken after 72 hours to view the biofilm for the number of dead cells.

Results:

The isolation of the actinomycete Nocardiopsis was done using Bennett's medium and the morphology, chemotaxology, morphological identification and micromorphology was observed.

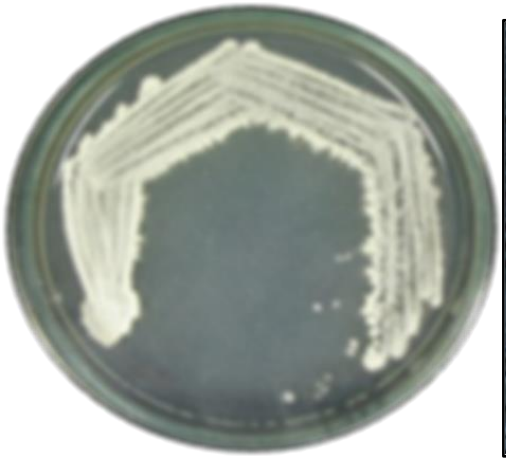

Fig 1 (a): Isolation of Nocardiopsis species

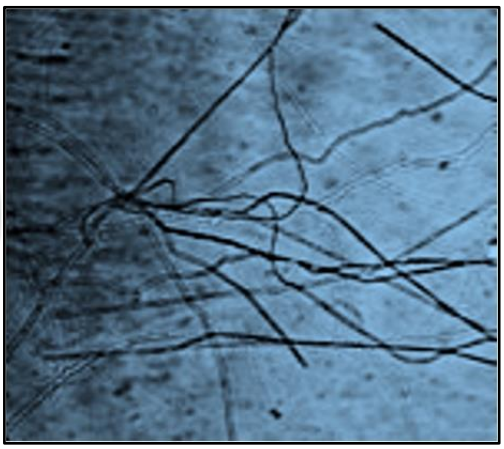

Fig 2 (b): Long spore chain of Nocardiopsis

Table 1: Biochemical Characteristics

CELL WALL AMINO ACID	
LL-DAP	–
DL-DAP	+
GLYCINE	–
ALANINE	–
CELL WALL SUGAR	
ARABINOSE	–
GALACTOSE	+
XYLOSE	–
MADUROSE	–
RIBOSE	+
INFERENCE	III/N.C
SPORE MORPHOLOGY	Long chain
INDEX	Nocardiopsis sp

Table 2: Morphological Character studied (as per Nonomura key)

Color of aerial mycelium	–
Melanoid pigment	–
Reverse side pigment	–
soluble pigment	+
Sporechain	Long Chain

Tabe 3: Carbon source assimilation

Arabinose	+
xylose	+
Inositol	±
Mannitol	±
Rhamnose	+
Sucrose	–
Raffinose	±

After the tests conducted, it can be confirmed that the actinomycete that was used was *Nocardiopsis* species.

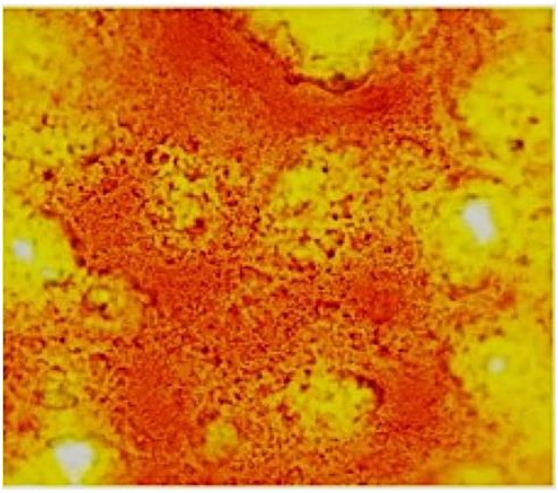

Fig2(a): Biofilm formation of Klebsiella pneumoniae

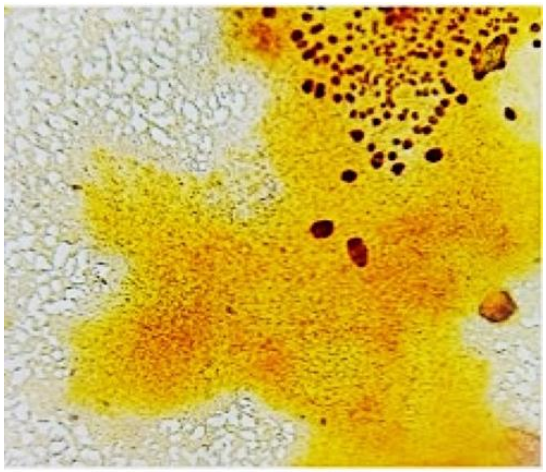

Fig2(b): Biofilm after treatment with actinobacteria secondary metabolite-24 hours

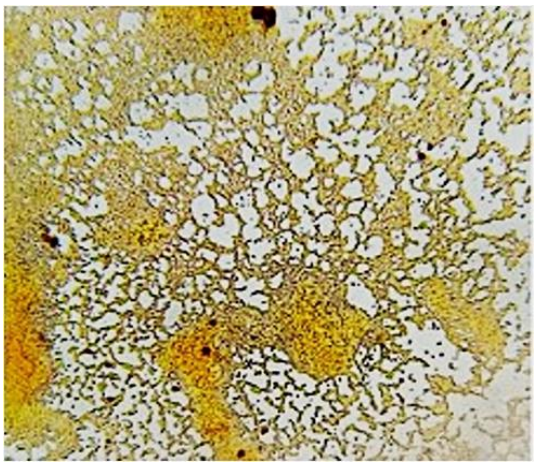

Fig2(c): Biofilmafter treatment with actinobacteria secondary metabolite - 48 hours

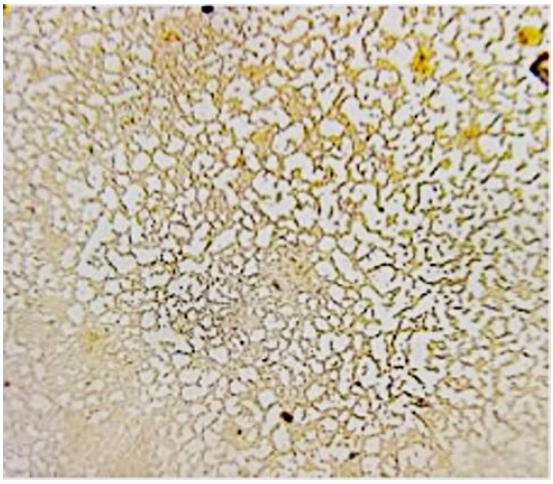

Fig2(d): Biofilm after treatment with actinobacteria secondary metabolite -72 hours

From the images above, it is clear that the secondary metabolite of Nocardiopsis was able to effectively inhibit the growth of the biofilm which was stained with Acridine Orange. The number of viable cells was heavily decreased after 72 hours of incubation as seen in Fig 2.

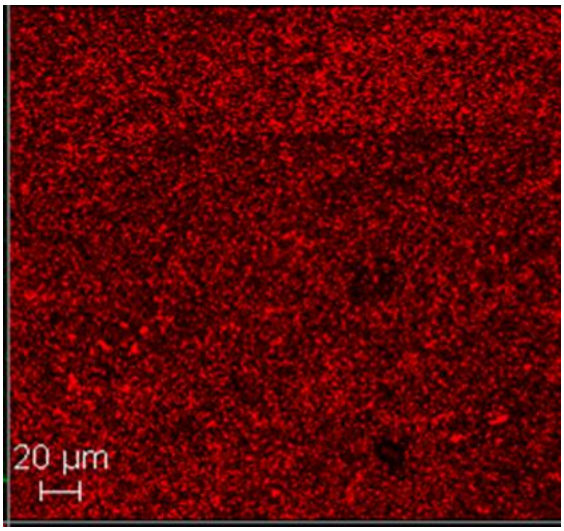

Fig 3: Confocal image of dead cells after treatment

The number of dead cells in the biofilm after 72 hours post treatment with the secondary metabolite was observed in the confocal image.

Table 4: Identification Of Pathogen

GRAM STAIN	--
SHAPE	**Rod**
MOTILITY	--
INDOLE	--
MR	+
VP	--
CITRATE	+
TSI	+
OXIDASE	--
CATALASE	+
UREASE	+
LACTOSE	+
MALTOSE	--
SUCROSE	--
XYLOSE	+
STARCH	+
INOSITOL	--
GENUS	**Klebsiella**

The pathogen was confirmed to be *Klebsiella pneumoniae* by assessing the characteristics given in table 4. This was done as a confirmatory test.

Discussion

The morphological and biochemical characteristics of the organism from which the secondary metabolite was derived, were observed. The cell wall of the organism was found to be DL-DAP positive, and was found to have galactose and ribose which implies that it was a gram negative bacteria. These characteristics of the cell wall and the spore morphology was considered and finally was identified as Nocardiopsis species. (Table 1).

Further, carbon source assimilation was tested (Table 3) as a confirmatory test for Nocardiopsis species. For the identification of pathogen, shape motility and other features were analyzed. The organism was found to be non motile, rod-shaped and was known to contain citrate, catalase urease, lactose, xylose and starch. From the above results, it was confirmed that the pathogen is a Klebsiella species.

The pathogen, Nocardia,which is the actinobacterial agent used, is known to have produced many bioactive secondary metabolites. Some of the metabolites are Nargenicin A1, Nocardicin A1, Tubelactomicin A, Brasilcaridin A. These are versatile in their activity and have shown excellent antimicrobial. Antibacterial, immunosuppressive activities. Because of their extensive properties, several studies are being conducted to unravel the properties and the genomic sequence of the species.

A study was conducted on the anti-microbial activity of Nocardiopsis against pathogenic bacteria like E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhirium and Shigella soni. They also saw the activity of the actinomycete against fungi like Aspergillus niger and Aspergillus fumigatus (12). The results of the study were such that Nocardiopsis possessed a broad-spectrum antimicrobial activity which was similar to the result we got.

In a study conducted by Al-Mathkhury HJ et al, the bacilli *L. fermentum* and the supernatant of *L.acidophilus* was not able to inhibit the planktonic cells of Klebsiella pneumoniae (13). But the results of that study showed that the acidic supernatant exhibited the inhibitory effect. The drugs Gentamicin and Amikacin showed very less to no inhibitory activity and left live cells, the supernatant was able to completely inhibit the biofilm. Ahmed A et al, in their study used chlorhexidine conjugated gold nanoparticles to inhibit the biofilm growth of *K.pneumoniae*. The Au-CHX was able to stop the growth of the bacterial biofilm and also showed antibacterial characteristics against clinical isolates of the pathogen. The mechanism of the nanoparticle is

such that it prevents the early colonization of the bacteria to inhibit the biofilm growth (14).

Another study by Rajivgandhi G et al stated that the antibacterial activity of biosynthesized zinc nanoparticles with actinomycetes was effective against the biofilm of *K.pneumoniae* (15). The highest zone of inhibition was seen at 24mm when 250 µg/mL of the actinomycete inculcated with zinc nanoparticles was used. It also showed an improved drug delivery. The author also states that ZnO nanoparticles are biologically compatible compounds and can be used as dental fillers with added antimicrobial activity. The results of the current study agree with the above mentioned studies.

Conclusion

From the results of the study, the secondary metabolites derived from the actinobacteria Nocardiopsis was capable of inhibiting the biofilm growth of the nosocomial bacteria *Klebsiella pneumoniae*.

Conflict of interest

There was no conflict of interest between the authors of the study.

Source of funding

The source of funding for the present study was provided by Zenith Food solutions Pvt, Ltd. Chennai.

Ethical clearance number

Since this is an in vitro study, the ethical clearance is not applicable.

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