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MOLECULAR AND BIOCHEMICAL IDENTIFICATION OF *ACHROMOBACTER XYLOSOXIDANS* ISOLATED FROM CYSTIC FIBROSIS PATIENTS IN AL–DIWANIYA CITY

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ABSTRACT

Achromobacter xylosoxidans is an emerging human pathogen that has been increasingly recovered from cystic fibrosis (CF) patients. This is the first report to investigate the prevalence of *A. xylosoxidans* in CF patients in Iraq, particularly in Al- Diwaniya city. In order to evaluate the frequency of this pathogen, 270 sputum samples were collected from patients suffering from CF attending Al-Diwaniya teaching hospital during the period January 2015 to January 2016. A total of 12 isolates (5.42%) of *A. xylosoxidans* were recovered from the sputum specimens and identified based on biochemical tests and confirmed using Vitek2 Compact system. Isolates were further verified using 16Sr DNA gene based analysis and DNA sequencing. The activity of antimicrobial agents against the isolated and identified *A. xylosoxidans* isolates was determined using standard disk-diffusion and broth dilution methods. The isolates of *A. xylosoxidans* were (100%) resistant to Cephalothin and Nitrofurantoin, (91.66 %) to Ampicillin and Kanamycin, (83.33%) to Tobramycin and Tetracycline, and Meropenem, (75 %) to Chloramphenicol, (66.66%) to Imipenem, and (40.66%) to Gentamicin. Isolates showed (100%) susceptibility to Pipracillin and (83.33%) to Ciprofloxacin and PolymyxinB. Findings of this study can help to understand the epidemiology and clinical implications of *A. xylosoxidans* and to improve clinical management attempts in CF patients.

KEYWORDS: Cystic fibrosis, *Achromobacter xylosoxidans*, prevalence, antibiotic-resistance.

INTRODUCTION

Cystic fibrosis (CF) is a genetic disorder that results from affecting a gene called the cystic fibrosis transmembrane conductance regulator gene (CFTR). The product of the CFTR gene regulates and facilitates the transportation of electrolytes across cell membranes, so that patients suffering from cystic fibrosis have a high concentration of salt in their sweat.[1] Defective CFTR protein affects the function of the pancreas and changes the consistency of mucosal secretions. The last of these impacts most likely plays a vital role in the defective resistance of CF patients to many pathogens.[1] The main cause of morbidity and mortality in patients with cystic fibrosis (CF) is the respiratory infections with several bacterial species including *Pseudomonas aeruginosa* and other Gram-negative bacteria, such as *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia* and non tuberculous mycobacteria.[2]

A. xylosoxidans is a Gram-negative, aerobic, oxidase and catalase positive bacillus. It is frequently misidentified as *Pseudomonas aeruginosa*[3,4], so the prevalence of *A. xylosoxidans* is underestimated in CF patients.[4] *A. xylosoxidans* is an opportunistic human pathogen able to cause a range of infections, such as pneumonia, meningitis, bacteremia, and peritonitis.[5-7] Moreover, it is able to persistent infection of the respiratory tract of people with cystic fibrosis (CF).[8,9] It colonizes the respiratory tract of children and of patients with CF, which is associated with an exacerbation of pulmonary symptoms.[9] Clinical *A. xylosoxidans* isolates usually show multiple drug resistance for aminoglycosides, cephalosporins (except ceftazidime), and aztreonam.[10] Acquired resistance in *A. xylosoxidans* especially for β-lactams has been also characterized, and it is frequently encoded by genes carried on mobile genetic elements (MGEs), such as integrons. Accurate identification of *A.xylosoxidans* is critical to clearly understand the epidemiology and clinical implications of this emerging pathogen and to enhance clinical management efforts in CF patients. Therefore, the objectives of this study are to: (1) Isolate and identificate *A. xylosoxidans* from CF people in Al-Diwaniya city using molecular and biochemical approaches (2) Evaluate the prevalence of *A. xylosoxidans* in CF patients. (3) Investigate the sensitivity of *A. xylosoxidans* isolated from CF patients during the study period to the common used antimicrobial agents.

METHODS AND MATERIALS

Patients: Sputum samples were collected from 270 (130 male and 140 female aged between 1-50 years) patients suffering from CF attending Al-Diwaniya teaching hospital during the

period January 2015 to January 2016. CF was diagnosed based on standard methods with the help of a specialist.[11]

Samples processing

Samples were treated as mentioned in[11] by mixing with equal volume of 1% dithiothreitol then incubating at 37°C for 30 minutes. All samples were examined microscopically and plated on several agar media, including MaCconkey agar and blood agar then incubated at 37°C for 24 h-72 h.

Identification

Bacterial isolates were identified by Gram stain, standard techniques including biochemical tests for the identification of non- *P. aeruginosa*, non lactose fermenting Gram negative bacteria[12], and Gram-negative identification card automated Vitek2 Compact system (Biomeriux). Isolates that identified as *A. xylosoxidans* were further confirmed using 16Sr DNA gene based analysis.

DNA Preparation

From an overnight growing plate, one colony was picked and heated at 95 °C for 15 minutes in 20 µl of lysis buffer (0.25%(vol/vol) sodium dodecyl sulfate and 0.05 M NaOH). 180 µl of sterile distilled water were mixed with the lyis buffer after lysis, and DNA samples were stored at -20 °C.[13]

Determination of 16Sr DNA gene

Using the primers (AX-F1, sequence 5′-3′ GCAGGAAAGAAACGTCGCGGGT, nucleotide positions 427–448 and AX-B1, sequence 5′-3′ ATTTCA CATCTTTCTTTCCG, nucleotide positions 576–595) , respectively, the 16S rDNA gene (163 bp) was amplified. PCR reactions were performed in a total volume of 50 µl using Thermo Scientific™ Phusion High-Fidelity DNA Polymerase (Fisher scientific) with initial heating at 94°C for 1 min, denaturation at 94°C for 1 min(35 cycles), annealing at 56°C for 45 seconds, and extension at 72°C for 1 min. The last extension step was performed at 72°C for 10 minutes. Positive control (using DNA of a reference strain of *A. xylosoxidans* obtained from the Central Public Health Laboratory) and negative control (without DNA) PCRs were utilized to avoid false positive results. The amplified PCR products were analyzed using 2% agarose gels and stained with ethidium bromide. Bands corresponding to the expected size of the 16S rDNA gene then extracted from the gels and purified using the Gene JET^{TM}Gel Extraction Kit (Thermo

scientific). Double strand sequencing was utilized using BigDye v1.1 Terminator chemistry to confirm the sequence of the 16S rDNA gene.

Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) values of Meropenem ,Ampicillin, Imipenem ,Cephalothin, PolymyxinB, Gentamicin, Kanamycin, Tobramycin, Amikacin, Tetracycline, Nitrofurantoin, Chloramphenicol, Ciprofloxacin, and Pipracillin for the identified *A. xylosoxidans* isolates were performed by using standard disk-diffusion and broth dilution methods according to Clinical and Laboratory Standards Institute (CLSI).[14]

RESULTS

A total of 270 sputum samples were obtained from patients suffering from CF and attending Al-Diwaniya teaching hospital during the period January 2015 to January 2016. Table (1) summarizes the total numbers and percentages of bacterial species isolated and identified during the study period. The predominant bacterial species isolated from CF patients was *P. aeruginosa* with a percentage (73.30%) then *Burkholderia cenocepacia* with a percentage (16.74). A total of 12 isolates (5.42%) of *A. xylosoxidans* were recovered from the sputum specimens and identified based on biochemical reactions and characteristics listed in table (2). The identification of *A. xylosoxidans* isolates was further verified using Gram-negative identification card automated Vitek2 Compact system (Biomeriux). PCR analysis of each of the 12 isolates for the expression of the 16Sr DNA gene (Figure 1) was in agreement with the results of biochemical identification. Further verification of the 16Sr DNA gene was performed by sequencing the bands corresponding to the expected size of the gene.

Table (1) Numbers and percentages of Gram negative bacteria isolated from CF patients during the study period

Bacterial species	Number of isolates	Percentage%
Pseudomonas aeruginosa	162	73.30
Achromobacter xylosoxidans	12	5.42
Burkholderia cepacia	5	2.26
Burkholderia cenocepacia	37	16.74
Burkholderia stabilis	3	1.35
Burkholderia gladioli	2	0.90
Total	221	

Table (3) shows the antibiotic sensitivity of the isolated *A. xylosoxidans* for each of the antimicrobial agent used in the current study. The 12 isolates of *A. xylosoxidans* identified in

this study were multidrug resistant, showing (100%) resistant to Cephalothin (MIC>64 µg/ml) and Nitrofurantoin(MIC>250 µg/ml), (91.66 %) to Ampicillin (MIC>32 µg/ml) and Kanamycin (MIC≥ 320 µg/ml), (83.33%) to Tobramycin (MIC>256 µg/ml), Tetracycline(MIC≥ 64 µg/ml), and Meropenem (MIC>8 µg/ml), (75 %) to Chloramphenicol (MIC≤ 6 µg/ml), (66.66%) to Imipenem (MIC>8 µg/ml), and (40.66%) to Gentamicin (MIC≥16 µg/ml). Isolates showed (100%) susceptibility to Pipracillin (MIC<4 µg/ml) and (83.33%) to Ciprofloxacin (MIC≥ 4 µg/ml) and PolymyxinB (MIC>1 µg/ml).

Table (2) Biochemical Characteristics used to identify *A.xylosoxidans*

Characteristic	No.of isolates positive	% Positive
Motility	12	100
Oxidase	12	100
Catalase	12	100
Urease	0	0
Indole	0	0
Citrate, Simmons	11	91.66
Acetamide	5	41.66
Hemolysis	0	0
Growth on MaCconkey agar	12	100
Growth on Cetrimide agar	11	91.66
NaCl 0.5% growth	12	100
NaCl 1.5% growth	11	91.66
NO^-_2 reduction	12	100
NO^-_3reduction	11	91.66
N_2/ N_2 denitrification	9	75
Glucose	10	83.33
Arabinose	0	0
Mannitol	0	0
D-Gluconate	11	91.66
L-Arginine dihydrolase	0	0
Malate	12	100
phenylacetate	11	91.66

Table (3) Antimicrobial susceptibility of *A.xylosoxidans*

Antibiotic	No. of sensitive isolates	%	No. of resistant isolates	%
Ampicillin	1	8.33	11	91.66
Cephalothin	0	0	12	100
PolymyxinB	10	83.33	2	16.66
Gentamicin	1	8.33	11	40.66
Kanamycin	1	8.33	11	91.66
Tobramycin	2	16.66	10	83.33
Amikacin	0	0	12	100
Tetracycline	2	16.66	10	83.33

Nitrofurantoin	0	0	12	100
Chloramphenicol	3	25	9	75
Ciprofloxacin	10	83.33	2	16.66
Meropenem	2	16.66	10	83.33
Pipracillin	12	100	0	0
Imipenem	4	33.33	8	66.66

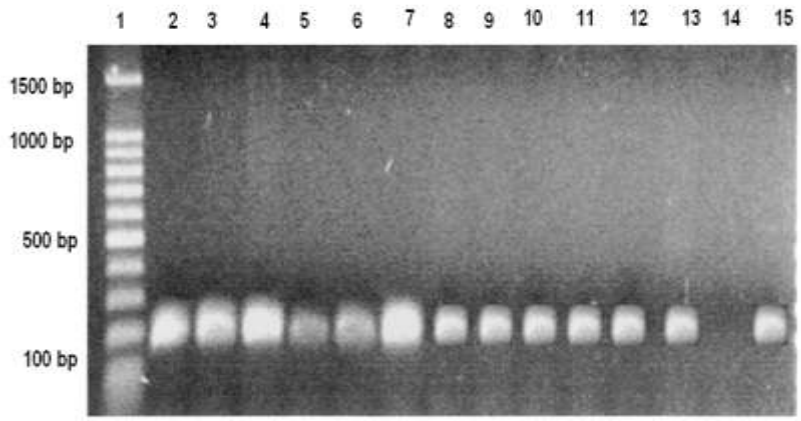

Figure (1). PCR analysis of the 16Sr DNA gene of *A.xylosoxidans* identified in the current study. Lanes are: (1) Molecular marker, (2-13): amplified 16Sr DNA gene of the 12 *A.xylosoxidans* isolates, (14) negative control, (15) positive control. PCR was done for 35 cycles, and products were analyzed using 2% agarose gels. Gels were stained with ethidium bromide and imaged using a Bio-Rad ChemiDoc MP. 16S rDNA gene (163 bp).

DISCUSSION

A. xylosoxidans is progressively cultured in sputum samples obtained from cystic fibrosis (CF) patients, and it is capable of chronic infections in these patients.

The prevalence of *A. xylosoxidans* infection or colonisation is most likely underestimated.[15] To date, few published data about the prevalence of *A. xylosoxidans* in CF patients exist. Al-Araji[16] isolated this microorganism from different body sites, including chest, burn, urine, ear discharge, peritoneal fluid , and throat in patients from Baghdad city. This is the first study to investigate the prevalence of *A. xylosoxidans* in CF patients in Iraq, particularly in Al- Diwaniya city. Among 270 patients, 12 (median age of 20 years (range 11–27 years) have at least one positive culture of *A. xylosoxidans* recovered from the sputum samples. The prevalence of *A. xylosoxidans* in the current study(5.42%) is similar to[11] which was (6.9 %). High prevalence of *A. xylosoxidans* has been reported in[17] (17.5%) and[15] (17.9%). In the current study, 5 patients (41.66%) infected by *A. xylosoxidans* were co – colonized by *P.*

aeruginosa. It is proposed that *A. xylosoxidans* tends to establish itself in lungs already infected by *P. aeruginosa*.[18] The results also showed that one patient (8.33%) infected by *A. xylosoxidans* was co– colonized by *B. cepacia*, while six patients (50%) were not co–colonized by any other pathogen indicating the ability of *A. xylosoxidans* to infect lungs that are not previously colonized.[11] It is known that because of the variety of colony morphology and biochemical reactivity, misidentification of Gram-negative non-fermenters cultured from CF sputum may arise.[15] Misidentification of 11% of *A. xylosoxidans* strains was reported in.[19] In order to help understanding of the epidemiology of emerging pathogens, precise identification of multiply antibiotic-resistant gram-negative bacilli isolated from CF patients is vital. Accurate identification is also critical for treatment and for the institution of infection control standards if patient-to-patient transmission is observed.[19] Therefore, biochemical and molecular approaches were used in this study for accurate identification of *A. xylosoxidans* in CF patients.

Research suggested that the high prevalence of colonisation is not due to cross-contamination, but it is because of the high rate of antimicrobial resistance.[17] *A. xylosoxidans* and other non-fermentative Gram-negative bacteria show increasing drug resistance as a result of the increase use of antibiotics associated to acute pulmonary exacerbations in CF patients caused by *P. aeruginosa* infection.[11] The current data support this evidence because of frequent previous colonization with *P. aeruginosa* in some patients. Both disk-diffusion and broth dilution methods were used to determine the sensitivity of *A. xylosoxidans* to abtibiotics because some researches referred to some problems regarding the use of the disk-diffusion method, such as unclear inhibition zone.[11] There was no difference in the results obtained from the two methods in the present study. *A. xylosoxidans* isolates recovered from CF patients in this study exhibited multiresistance profile previously described for this species.[20] The identified *A. xylosoxidans* isolates were resistant to multiple antibiotics including imipenem and meropenem recommended to treat CF patients infected with this bacterium. With the appearance of antimicrobial resistance of new pathogens, the scenario has changed regarding the availability and susceptibility of antimicrobial drugs.[11] *A. xylosoxidans* isolates showed high sensitivity to Pipracillin, Ciprofloxacin and PolymyxinB, so we suggest using combination of these antibiotics for more efficient effects to treat CF patients infected with *A. xylosoxidans*.

CONCLUSION

The results of this study can help to facilitate further studies towards understanding the pathogenic role of *A. xylosoxidans* and can help to improve clinical management and infection control efforts in CF patients.

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