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The First Study of Investigation of Clinical Isolates of *Alcaligenes Xylosoxidans* and *Alcaligenes Faecalis* by Phenotypic and Genetic Methods in Iran

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ABSTRACT

Background and Aim: *Alcaligenes ssp*. is a non-fermentative Gram-negative bacillus, which causes nosocomial infections, including urinary tract infections, pneumonia, sepsis, and may be confused with *Pseudomonas aeruginosa*. *Alcaligenes* infections usually are not well identified and due to possible errors and similarities with *Pseudomonas*, their diagnosis with phenotypic tests is not sufficient. In this case, molecular methods seem to be more effective. We aimed to investigate the real presence of clinical isolates of *Alcaligenes xylosoxidans* and *Alcaligenes faecalis* by phenotypical, and genetic methods and their antibiotic susceptibility.

Materials and Methods: From September 2019 to March 2020, we analyzed 36 clinical isolates from a Sina hospital in Hamadan, Iran, which have been identified as *Alcaligenes* in the hospital's microbiology lab, by routine phenotypicall methods. Using the PCR method and tracking *AX* and *77F-r* genes, we identified *A. xylosoxidans* and *A. faecalis* respectively; the antibiotic resistance of each isolate was determined by the disc diffusion method.

Results: Of 36 samples of phenotypically identified *Alcaligenes*, only 13 (36.11%) were confirmed as *A. xylosoxidans* and 3 (8.33%) as *A. faecalis* by PCR test. Among *A. xylosoxidans* isolates, the highest susceptibility (92.3%) was against cephalosporin and the highest resistance (76.92%) was against ciprofloxacin. Among *A. faecalis* isolates, the most susceptibility (100%) was against ceftazidime, piperacillin/tazobactam, imipenem, meropenem, and cefepime, and the most resistance (66.66%) was against gentamicin and ceftriaxone.

Conclusion: Regarding the importance of accurate diagnosis of *Alcaligenes* in combating nosocomial infections, it seems with phenotypic and biochemical tests, there is a possibility of error in their diagnosis; so using the PCR method, each species can be determined more accurately.

Keywords: *Alcaligenes*, Molecular detection, PCR method, Nosocomial infection

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1. Introduction

Alcaligenes genus, is a Gram-negative, aerobic, rod-shaped, non-fermentative bacteria with amphitrichous flagella and rarely non-motile. Additionally, some strains of *Alcaligenes* are capable of anaerobic respiration, but they must be in the presence of nitrate or nitrite; otherwise, their metabolism is respiratory and never fermentative; the genus does not use carbohydrates **(1)**.

Strains of *Alcaligenes* such as *A. faecalis* and *A. xylosoxidans* are found mostly in the intestinal tracts of vertebrates, decaying materials, dairy products, water, and soil **(2-4)**; they can be isolated from the human respiratory **(2, 3)** and gastrointestinal tracts **(5)** and wounds in hospitalized patients with compromised immune systems **(6, 7)**. They are occasionally the cause of opportunistic infections, including nosocomial sepsis **(2, 8)**. *Alcaligenes* sp. is an important hospital pathogen that is morphologically very similar to *Pseudomonas aeruginosa* **(9)** and maybe confused with *Pseudomonas* species **(8)**.

Alcaligenes species causes bacteremia in immune-deficiency patients with situating catheters **(10)**. The

bacterium, along with other infections such as *P. aeruginosa*, has been reported to cause respiratory tract infections in cystic fibrosis patients. At present, the clinical aspects of this bacterium are not very clear in relevant infections **(9, 11)**. Due to high rate of infections caused by this bacterium and its resistance to some common antibiotics, different mortality rates have been observed in patients with infections caused by this bacterium **(12)**.

Routinely phenotypic methods such as dedicated culture media, biochemical tests, and API (Analytical Profile Index) detection kits use to diagnose this bacterium **(4)**. Although phenotypic methods are a precise identification method, molecular methods are more effective and more accurate in identifying non-typical isolates. Already, molecular methods such as PCR, DNA fingerprinting, RAPD-PCR, and PCR assay based on 16S ribosomal DNA, are used to investigate the molecular nature of this bacterium **(11, 13, 14)**.

Antibiotic resistance among pathogenic bacteria especially strains causing nosocomial infections, is particularly important. Also, due to acquisition of antibiotic resistance genes by bacteria over time in different geographical areas and changes in the pattern of bacterial susceptibility to different antibiotics, choosing the right antibiotic for treatment has become a challenge. **(16)** Based on the mentioned statements and due to few published data about the accurate diagnosis of clinical strains of *Alcaligenes* in Iran, in this study as the first time in Iran, we investigated the biochemically and genetically confirmation of the presence of *A. faecalis* and *A. xylosoxidans* in clinical samples and their antimicrobial susceptibility patterns.

2.Materials and Methods

Isolates tested

In a descriptive-analytical study from September 2019 to March 2020 at a Sina hospital in Hamadan, Iran, we analyzed all 36 isolates collected from hospitalized patients who were phenotypically identified by the referring clinical microbiology laboratories as *Alcaligenes.* We get the ethical approval letter from the institutional research ethics review committee of Hamadan University of medical sciences. (NO. IR.UMSHA.REC.1396.443).

Phenotypic profiling

The bacteria studied in this research were isolated from blood and urine samples of hospitalized patients in Sina hospital. To phenotypic identification, all isolates were grown on selective media such as SIM, TSI, MR, VP, Citrate, Urea agar, and MacConkey agar (Merck, Germany) and incubated at 37ºc for 24 hours. To confirmation the purity, the samples were examined by Gram staining. Phenotypic characterizations were performed on all isolates, using protocols for Gram-negative, non-fermentative bacteria such as motility tests, oxidative fermentation of glucose and lactose, Bile-esculin, DNase, Nitrate reduction, and Indole reactions. Clinical isolates approved by biochemical experiments were stored at -20 °C. Following the phenotypic and biochemical tests, we used the PCR test to more investigate accurately the detection and confirmation of the phenotypic results.

DNA extraction, PCR amplification, and DNA sequencing

Bacterial genomic DNA for PCR amplification was extracted by the boiling method as previously descrybed **(15)**. The NanoDrop spectrophotometer (Thermo Scientific™ 840274100) was used to determine the amount of extracted DNA. The purity of DNA (OD A260/280) was measured in ng/µl. The quality of the extracted DNA was assessed on 1% Agarose gel.

The PCR was performed in BioRad T100 PCR Thermocycler with *AX* and *77F-r* gene-specific primers, to genetically confirm the phenotypically identified *A. xylosoxidans* and *A. faecalis*, respectively. The specific primers used for the amplification of the *AX* gene were *AX*-F 5́ GCAGGAAAGAAA CGTCGC GGGT 3́ and *AX* -R 5́ ATTTCCATCTTTCTTTCCG 3. The *77F-r* specific primers (*77F-r*-F 5 GGCGGACGGGTGAGTAATA 3 and *77F-r*-R 5 CTGCAGATACCGTCAGCAGT 3) were also used to confirm *A. faecalis* **(16)**. The PCR reaction mixture of 12.5 µl contained 1 µl of DNA template (20 ng), 0.5 µl of each primer (concentration of each primer was 0.5 µM), 6 µl of Taq DNA Polymerase Master Mix RED (amplicon, Denmark) and, 4.5 µl distilled water was used. The first PCR step was performed at 95°C for 3 min and was followed by 30 cycles of denaturation (95 °C for 1 min), annealing (55 °C and 58 °C for 1 min in annealing *AX* and *77F-r*, respectively), and extension (72 °C for 1 min). The last step was performed at 72 °C for 5 min. Positive control (A clinical sample confirmed by sequencing) and negative control (*Pseudomonas aeruginosa* ATCC 27853) were utilized to avoid false-positive results. The amplified PCR products were analyzed using 2% agarose gels and stained with GelRed® nucleic acid stain. An amplified PCR product corresponding to the expected size of *AX* and *77F-r* genes was used for sequencing. Sequences obtained for each of the analyzed genes were assembled and compared to available sequences in Genbank, using the BLAST (Basic Local Alignment Search Tool) algorithm of the NCBI (National Center for Biotechnology Information) **(16)**.

Antimicrobial susceptibility assay

We performed the antimicrobial susceptibility tests for each isolate by the disc diffusion method (Kirby-Bauer). The results were interpreted as either sensitive, intermediate, or resistant according to the Clinical Laboratory Standards Institute (CLSI-2018) susceptibility breakpoints for non-fermenting gram-

negative bacteria **(17)**. Antibiotic discs (*Mast* (*UK*)), used for the tests included: ampicillin (AP10 ug), trimethoprim/sulfamethoxazole (TS25 ug), ciprofloxacin (CIP5 ug), imipenem (IMI10 ug), Gentamicin (GM10 ug), Meropenem (MEM10 ug), Ceftazid-ime-(CAZ30 ug), ceftriaxone(CRO30 ug), piperacillin-/tazobactam (PTZ110 ug), ampicillin/sulbactam (SAM20 ug), cefepime (CPM30 ug).

3.Results

Patient Characteristics

Out of 36 samples collected in this study, 19 (52.8%) samples belonged to women, and 17samples (47.2%) were from men. The patients 'age was between 27 and 95 years, with an average age of 58.2.The patients 'age was between 27 and 95 years, with an average age of 58.2.

Genetic Confirmation of *Alcaligenes* species

The PCR results revealed that only 16 (44.44%) samples were *Alcaligenes* species, which included 13 (36.11%) samples as *A. xylosoxidans* using the *AX*-specific primers and 3 (8.33%) samples as *A. faecalis* using the *77F-r*-specific primers. A 163 bp and a 391 bp PCR fragment were seen following the electrophoresis, corresponding well with the expected size of a part of *AX* and *77F-r* genes, respectively (Fig. 1 and 2).

We compared the *AX* and *77F-r* gene sequences to the whole genome sequences of the genus of *Alcaligenes* present in the NCBI database. The alignment results demonstrate a 100% identity with the 16S rRNA genes of the *A. xylosoxidans* and *A. faecalis* bacteria. This compliance confirmed the specificity of the PCR products as a positive control.

Antimicrobial Susceptibility

The in vitro susceptibility of 36 *Alcaligenes* isolates to 11 antimicrobial agents is summarized in Table 1. The most susceptibility (80.55%) among *Alcaligenes* species was to Cefepime, followed by imipenem, piperacillin-tazobactam, and ceftazidime with a 75% rate. Also, the most resistance (92.3 %) was seen against Cefepime antibiotic in 13 *A. xylosoxidans* isolates followed by ciprofloxacin (76.92%) and meropenem (38.46%) (Table 2).

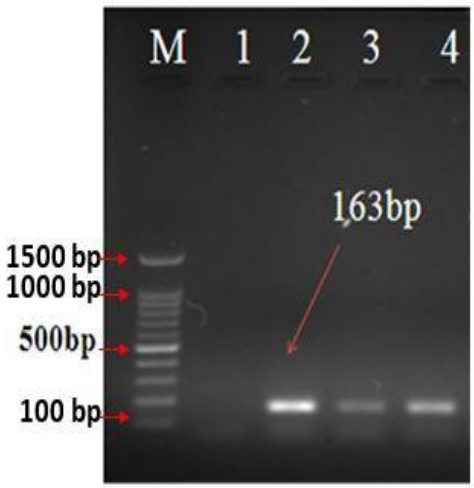

Figure 1. Agarose gel electrophoresis (2% agarose) of PCR amplified products using species-specific PCR primer sets (163 bp) in *A. xylosoxidans* strains. Lane M, 100 bp DNA ladder, lane 1: negative control, lane 2: positive control, lane 3, 4: *A. xylosoxidans* strains.

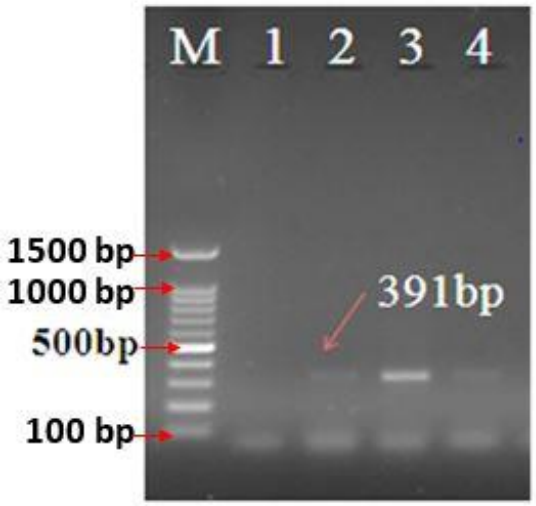

Figure 2. Agarose gel electrophoresis (2% agarose) of PCR amplified products using species-specific PCR primer sets (391 bp) in A. *faecalis* strains. Lane M, 100 bp DNA ladder, lane 1: negative control, lane 2: positive control, lane 3, 4: *A. faecalis* strains.

Table 1. In vitro susceptibility profile of 36 Alcaligenes species isolated from hospitalized patients to 11 Antimicrobial agent

Antibiotic	No. of isolates (%)		
	Susceptible	Intermediate	Resistant
Gentamicin	12 (33.33)	3 (8.33)	21 (58.33)
Ceftazidime	27 (75.00)	1 (2.77)	8 (22.22)
Ceftriaxone	11 (30.55)	14 (38.88)	11 (30.55)

Antibiotic	No. of isolates (%)		
	Susceptible	Intermediate	Resistant
Piperacillin/Tazobactam	27 (75.00)	5 (13.88)	4 (11.11)
Ampicillin	9 (25.00)	6 (16.66)	21 (58.33)
Trimethoprim/sulfamethoxazole	21 (58.33)	3 (8.33)	12 (33.33)
Meropenem	21 (58.33)	1 (2.77)	14 (38.88)
Ampicillin/Sulbactam	17 (47.22)	3 (8.33)	16 (44.44)
Imipenem	27 (75.00)	7 (19.44)	2 (5.55)
Ciprofloxacin	8 (22.22)	2 (5.55)	26 (72.22)
Cefepime	29 (80.55)	1 (2.77)	6 (16.66)

Table 2. In vitro susceptibility profile of 13 A. xylosoxidans and 3 A. faecalis strains isolated from hospitalized patients to 11 Antimicrobial agent

Antibiotics	No. of isolates (%)					
	Susceptible		Intermediate Susceptibility		Resistant	
	A. xylosoxidans	*A. faecalis*	*A. xylosoxidans*	*A. faecalis*	*A. xylosoxidans*	*A. faecalis*
Gentamicin	4 (30.76)	1 (33.33)	8 (61.53)	0	1 (7.70)	2 (66.66)
Ceftazidime	11 (84.61)	3 (100)	0	0	2 (15.38)	0
Ceftriaxone	5 (38.46)	1 (33.33)	5 (38.46)	0	3 (23.07)	2 (66.66)
Piperacillin/Tazobactam	10 (76.92)	3 (100)	2 (15.38)	0	1 (7.70)	0
Ampicillin	7 (53.84)	0	3 (23.07)	2 (66.66)	3 (23.07)	1 (33.33)
Trimethoprim/sulfamethoxazole	11 (84.61)	2 (66.66)	1 (7.70)	0	1 (7.70)	1 (33.33)
Meropenem	8 (61.53)	3 (100)	0	0	5 (38.46)	0
Ampicillin/Sulbactam	9 (69.23)	2 (66.66)	1 (7.70)	0	3 (23.07)	1 (33.33)
Imipenem	11 (84.61)	3 (100)	0	0	2 (15.38)	0
Ciprofloxacin	2 (15.38)	1 (33.33)	1 (7.70)	1 (33.33)	10 (76.92)	1 (33.33)
Cefepime	12 (92.30)	3 (100)	0	0	1 (7.70)	0

In three samples that were identified as *A. faecalis* isolates, the complete susceptibility (100%) to ceftazidime, piperacillin/tazobactam, imipenem, and cefepime were observed, and 66.66% of isolates were resistant to Gentamicin and Ceftriaxone.

4.Discussion

Nosocomial infections or healthcare-associated infections are important issues in patients under medical care (30). *A. xylosoxidans* and *A. faecalis* bacteria are the most common *Alcaligenes* species in the prevalence and spread of nosocomial infections **(6, 18)**. Most infections are transmitted from contaminated hospital equipment to immunocompromised hosts **(5)**. Sometimes, it is inevitable misidentification of Gram-negative non-fermenters cultured from clinical isolates due to the diversity of colony morphology and biochemical reactivity **(19)**. In the case of *Alcaligenes* spp. regarding the similar morphology of colonies and some unique results of phenotypic and biochemical tests such as being gram-negative and rod-shaped, aerobic and oxidase and catalase-positive and motility in case of phenotypic identification, clinical strains of *Alcaligenes* sometimes misidentified by *P. aeruginosa* **(9)**.

Regarding this fact that for bacteria whose phenotypic diagnosis may be misdiagnosed with similar cases, it is better to use more accurate methods such as molecular methods. In the present study, 20 of 36 samples (56%) which were initially considered as *Alcaligenes* using standard phenotypic identification in the microbiology laboratory, were not confirmed genotypically as *A. xylosoxidans*. This is

incomparable with the findings of Saiman *et al.* **(4)** who was reported 12 (11%) misidentification of *A. xylosoxidans* strains, these were found to be *P. aeruginosa* (n=10), *Stenotrophomonas maltophilia* (n=1), and *Burkholderia cepacia* (n=1) .As described in this study it could be assumable that in our study some of the phenotypically confirmed isolates of *Alcaligenes*in may belong to other similar bacteria such as the *Pseudomonas* family. Also we assumed that in some unequipped microbiological laboratories, due to lack of enough material, instruments, or time, or having not enough specific mediums or DNA techniques, it could be possible that the prevalence of true infections would be ignored. **(9)**. Maybe this is one of the reasons for the low rate of published data about clinical strains of *Alcaligenes* reports, especially in Iran. For example in one study in Nigeria, out of 100 urine samples isolated by only culture and phenotypic diagnosis, just 7 (19.4%) species of *A. xylosoxidans* species were identified **(19)**. In another study, in order to identify Non-fermentative Gram-negative bacilli by using the differentiation test included: growth on Mac Conkey, TSI growth characteristics, oxidative fermentation of glucose and lactose, oxidize and motility tests, DNase, Nitrate reduction, Bile-esculin, and Indole reactions, of 6952 clinical specimens that were studied, 19 (39.5%) *A. faecalis* were isolated **(20)**. Also, Kumhar *et al.* **(21)** isolated 4.9% *A. faecalis* from blood samples of hospitalized patients. In another study, Aisenberg *et al.* **(12)** reported low prevalence (< 2%) of *A. faecalis.* The result of these studies prevalence also confirms that the prevalence of this bacterium is not the same in different hospitals and geographical areas and identifying the genus and species of these bacteria is important in controlling their antibiotic resistance of them. However, based on our searches, there was no phenotypic and genetic study about clinical strains of *Alcaligenes* in Iran.

For accurate detection of pathogenic bacteria, molecular methods, such as the 16S rRNA gene sequencing, DNA fingerprinting techniques, and an arbitrary primed PCR typing method provide new possibilities for detecting bacterial isolates **(11, 22, 23)**. Here as the first, we report the identification of gram-negative species related to *Alcaligenes* using the PCR method with gene- specific primers in Iran. Based on PCR results, the prevalence of *A. xylosoxidans* in our clinical samples was 36.11%. In one study, Liu *et al.* **(14)** developed a PCR assay based on a 16S rRNA sequence in their analysis, which of 149 isolates, 47 (31.54%) *A. xylosoxidans* recovered from cystic fibrosis (CF) patients which were near our results.

According to the fact that *Alcaligenes spp.* which cause bacteremia and other nosocomial infections, carries both intrinsic and acquired mechanisms of resistance, the treatment of patients with bacteremia usually encounter multidrug resistance **(24)**. But studies on the pattern of *Alcaligenes*' resistance to antibiotics are limited and further studies are needed. This study, different from previous studies that were limited to phenotypic diagnosis, in addition to accurate diagnosis of *Alcaligenes* species using PCR, was tried to determine the antibiotic resistance of each species separately. Our results also showed that the highest susceptibility (92.30%) in *A. xylosoxidans* was to Cefepime, followed by Ceftazidime, Trimethoprim /sulfamethoxazole, and Imipenem with 84.61% frequency. The isolates of *A. faecalis* were completely (100%) susceptible to Ceftazidime, Piperacillin/Tazobactam, Imipenem and Cefepime. According to Saiman *et al.* (2001), the highest sensitivity of *Alcaligenes* was to imipenem, meropenem, and piperacillin-tazobactam, with 59%, 51%, and 55% frequency, respectively. The pattern of antibiotic susceptibility was similar to the present study, although the susceptibility rate was lower than that of in present study **(4)**. In another study, Aisenberg *et al.* (2004) reported that *A. xylosoxidans* isolates showed the highest sensitivity (94%) to Trimethoprim/ sulfamethoxazole antibiotic. The isolates of *A. xylosoxidans* were resistant to Cefoxitin (98%) and Amikacin (90 %) **(12)**.

It should be noted that some of our limitations are including investigating the infectious predisposing factors, quantitative antibiotic susceptibility tests, determination of changes in antibiotic resistance pattern in the coming years, consequences of infection caused by this bacteria, and comparison of its clinical significance with other pathogens of nosocomial infection, which seems to be necessary to be investigated in future studies.

Regarding the high rate of *Alcaligenes* infections in nosocomial infections around the world, **(24)** and also this fact that the most appropriate treatment for nosocomial infections is choosing one/some antibiotics with high efficacy and effectiveness, it should be considered that in lake of accurate diagnosis of pathogenic bacteria and their antibiotic resistance, frequent and continuous use of antibiotics due to overdose, improper treatment and misdiagnosis, will increase the proliferation, and prevalence of resistant bacterial strains **(25-27)**.

5. Conclusion

Alcaligenes species are a known cause of nosocomial infections with increasing prevalence in the world in recent years; but unfortunately, according to our knowledge, there wasn't any published data about their phenotypic and molecular identification in Iran. Detecting the pattern of antibiotic resistance, for an appropriate way to limit the spread of these bacteria, needs the accurate detection of bacteria at the first steps. Regarding

these facts, it seems that accurate detection of these infections and determination of antibiotic resistance of them, should be considered more than previous, especially in terms of controlling nosocomial infections in Iran.

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Ethics approval

This research has been registered at the appropriate board of the Hamadan University of Medical Sciences (ethical code: IR.UMSHA.REC.1396.443)

Conflicts of Interest

The authors declare no conflict of interest regarding this paper.

Author Contribution

M.A and S.H.H designed the project and did the troubleshooting. S.B did the lab experiment of the research. M.A wrote the final article.

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