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Antibiotic Resistance of *Pseudomonas aeruginosa* in Well Waters in Irrigated Zone (Middle Atlas-Morocco)

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ABSTRACT

Pseudomonas aeruginosa is a bacteria which can live in freshwater, soil and plants. It is a commensal of the digestive tube, slightly abundant in human body. Its presence in wells water is a result of current anthropogenic activity and pollution loads. It is an important nosocomial pathogenic agent characterized by an intrinsic resistance to multiple antimicrobial agents which can develop high-level multidrug resistance. To assess the contamination of these well waters by this pathogenic germ, two hundred samples were collected for four seasonal campaigns between March 2017 and May 2018 with a rate of forty three (43) samples per season in an irrigated zone. The samples were analysed to identify *P. aeruginosa*, then the isolated serotypes were determined by slid agglutination test using four pools and 20 monovalent Antisera. The detected *P. aeruginosa* were tested for susceptibility to 18 antibiotics. A total of (n=85/139) isolated strains were identified as *P. aeruginosa* representing 61.15 % of prevalence among *Pseudomonas* spp. Antimicrobial resistance revealed that 68% of them are multidrug resistant, while15.09 % of strains resist at least to 7 antibiotics, 30.19% resist at least to 11 antibiotics, 13.21% resist at least to 12 antibiotics, 5.66% resist at least to 13 antibiotics, and 3.77% resist at least to 14 antibiotics. The high level resistance of *P. aeruginosa* is observed with piperacillin tazobactam (100/10µg) (84.91%), ciprofloxacin (5µg) (79.25%), imipenem (10µg) and ceftazidime (30µg) (37.58%). The resistance of phenotypes of *P. aeruginosa* strains allowed to identify (n=20/85) ESBL, (n=31/85) oxacillinase broad spectrum ES-OXA, (n=5/85) phenotype of impermeability to imipenem, (n=12/85) cephalosporinase AMPC, and (n=17/85) wild type. The results showed the high antibiotic resistance levels of *P. aeruginosa* strains from well water samples against antibiotics. Furthermore, based on the results, these well waters can be a source of *P. aeruginosa* and human and animal susceptibility to other opportunistic pathogens.

INTRODUCTION

The well waters are solicited for several uses in households, irrigation, alimentation, and considered as a vehicle for diffusion and dissemination of human associated bacteria (Pitondo-Silva et al. 2014). The World Health Organization (WHO) estimates that 80% of diseases that affect the world population are directly associated to the poor water quality, and poor sanitation system which has defective hygiene (WHO 2010). However, the presence of *Pseudomonas* spp. is the main concern in setting health-based targets for microbial safety of water. *Pseudomonas* spp. are opportunistic pathogens bacteria often found in the environment. Strains of this species generally have a very wide nutritional variance and they can live in various ecological niches (Monteil et al. 2002). These bacteria are a non-fermenting Gram negative aerobic bacilli involved in opportunistic nosocomial infections and in opportunistic infections of immunocompromised hosts (Ender et al. 2017). *Pseudomonas* is a genus of the Gamma proteobacteria class of bacteria, which belongs to the Pseudomonadacea family. This genus regroups more than 140 species including *P. aeruginosa*. The *P. aeruginosa* is highly ubiquitous in water systems and capable to acquire antibiotic resistance due to its low membrane permeability and extensive efflux pump system (Nasreen et al. 2015). The *P. aeruginosa* is a considerable pathogenic agent characterized by an intrinsic resistance to multiple antimicrobial agents and the ability to develop high level multidrug resistance through some mechanisms (El Ouardi et al. 2013). In fact, The *P. aeruginosa* forms biofilms that award a high power of colonization, resistance to antiseptics, and antibiotics (Ckd et al. 2016). Recent studies showed a rapid and worrying evolution of antibiotic resistant strains both in their number and spectrum, more and more extensive with cephalosporin resistant strains of third generation (C3G), and / or resistant to high-level fluo-

roquinolones and / or resistant to alternative therapies such as carbapenems (El Ouardi et al. 2013a). A wide range of antibiotics are used against bacterial infections. Beta-lactamases, Carbapenems, Fluoroquinolons and Aminosids are the most classes of antibiotics used clinically. However, *P. aeruginosa*, as other (extended spectrum β-lactamase: ESBL) bacteria can have a high level of activities against many antibiotics (Nedeljković et al. 2015). Carbapenems are often used as a drug of choice against the *P. aeruginosa* multidrug-resistant (MDR), but their activity is increasingly compromised by the emergence and the worldwide dissemination of resistant strains, which are implicated in numerous nosocomial infections (Maroui et al. 2015). Previous studies showed the great potential of clinical isolates of *P. aeruginosa* to acquire antimicrobial resistance. However, few studies report the resistance profile and its acquisition mechanisms in isolated environments, especially those obtained from water wells. This study aims to determine prevalence, serotype and antibiotic resistance profile of isolated *P. aeruginosa* from well waters in Middle Atlas region in Morocco.

MATERIALS AND METHODS

Site Selection and Sampling Campaign

The study area is located in Beni Mellal-Khenifra region (Fig.1), stretched out on two water tables (Beni Aamir and Beni Moussa). It covers a surface of 3600 km² in the middle basin of Oum Errbiaa. The water samples were collected directly from 43 wells in irrigated zone, during four campaigns (Table 1).

Isolation and Identification of *P. aeruginosa* Strains

From each water sample, *P. aeruginosa* was isolated and characterized according to the official Moroccan method (NM 2012). The enumeration of *Pseudomonas* spp. was carried out after the filtration of 100 mL of water sample. It was performed on Pseudomonas Cetrimide Agar (USP-EP, Oxoid CM0579) with 10 mL glycerol/L incubated at 42°C for 24-48 h. The typical colonies, that clearly showed pyocyanin production (blue green colonies and fluorescence at 360 nm), are considered as positive for *P. aeruginosa*. All colonies were confirmed using King A Agar and King

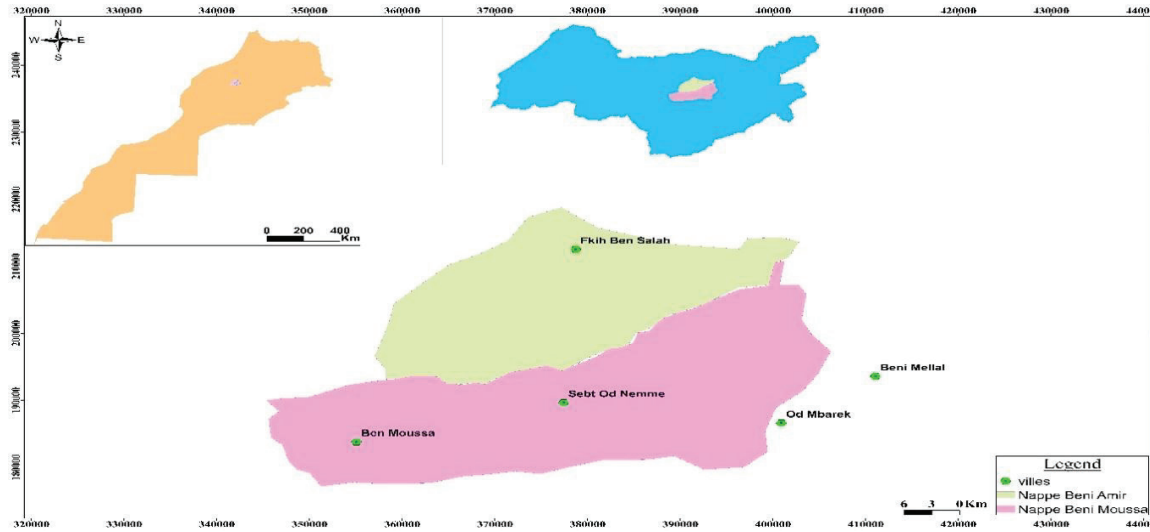

Fig.1: Location of the study area.

Table 1: Distribution of samples by zone and period of sampling.

	Campaign 1		Campaign 2		Campaign 3		Campaign 4		Campaign 5	
Zone	BM	BA	BM	BA	BM	BA	BM	BA	BM	BA
Date	13/03/2017	03/04/2017	08/05/2017	29/05/2017	10/07/2017	24/07/2017	Terrain inaccessible	Terrain inaccessible	23/04/2018	07/05/2018
Samples	20	23	21	22	23	20	0	0	23	18

B Agar (Bio-life) incubated at 42°C for 24 h. The production of specific pigments allowed the differentiation of *P. aeruginosa* and other *Pseudomonas* spp. The preliminary biochemical characterization of the strains was confirmed by using API 20 NE (Bio Mérieux).

Serotyping and Agglutination of *P. aeruginosa*

P. aeruginosa serotype isolate was determined by slide agglutination test using polyvalent and monovalent antisera (BIO-RAD). The positive agglutination is described as positive when strains cause a positive slide agglutination reaction. The monovalent is used when the corresponding polyvalent produce agglutinations.

Antibiotic Susceptibility of *P. aeruginosa* Isolates

The isolated *P. aeruginosa* was tested for susceptibility to 18 antibiotics. The susceptibility was tested by the disk diffusion method according to the recommendations of the French Society of Microbiology Antibiogram Committee CASFM/EUCAST (EUCAST/CASFM 2017). From each colony of each test strain, a suspension of 0.5 McFarland density was prepared and poured dried into the Müller-Hinton agar (OXOID, CM0337). Eighteen antibiotics were tested, including Ticarcillin (75 mg), Piperacillin (100 mg), Ticarcillin/Clavulanic acid (85 mg), Piperacillin/Tazobactam (10 mg), Ceftazidim (30 mg), Cefepim (30 mg), cefotaxime (30 mg), Imipenem (10 mg), Meropenem (10 mg), Aztreonam (30 mg), Fosfomycin (30 mg), Amikacin (30 mg), Tobramycin (10 mg), Gentamicin (10 mg), Nétilmycin (10 mg), Ciprofloxacin (5 mg), Lévofloxacin (5 mg), and Colistin (10 mg). The *P. aeruginosa* ATCC 27853 was used as a control reference strain. The isolated *P. aeruginosa* were classified as susceptible, intermediate or resistant according to the clinical interpretative criteria recommended by the CASFM/EUCAST (EUCAST/CASFM 2017). The resistance to antibiotics was determined after 24 hours, based on the inhibition zone diameter. Briefly, the detection of ESBL phenotype was performed by antagonism effect between the three different antibiotic disks (aztreonam, ticarcillin/clavulanic acid and ceftazidime) separated by 2 mm (EUCAST/CASFM,2017). Finally, to detect an ESBL phenotype in presence of cephalosporinase, combination disk test with cefepime was used and inhibition diameters were compared (EUCAST/CASFM,2017).

Statistical Analysis

The data were treated with the data processing software R Statistics 3.4.2, and XLSTAT.

RESULTS

Prevalence of *Pseudomonas* in Well Waters

Out of 200 water samples examined, the *Pseudomonas* spp. was isolated in 139 samples. Among the isolated bacterial strains, a total of n=58/139 was identified as *P. aeruginosa*. The antimicrobial activity of *P. aeruginosa* strains revealed a high level of resistance among all anti pseudomonal drugs tested. The generated results are shown in (Fig. 2).

The analysis of isolated bacterial strains showed a high resistance degree (Fig. 2) to Pepiracillin Tazobactam with (84.21%), Ciprofloxacin with (79.25%), Ceftazidim and Imipenem with (73.58%), a middle resistance degree to by Tobramycin, Levofloxacin, Amikacin with (67.92%), Gentamycin with (66.04%) and Netilemycin with (62.26%); and a low resistance degree to Aztreonam with (56.60%), Fosfoycin with (47.17%), and Meropenem with (35.85%).

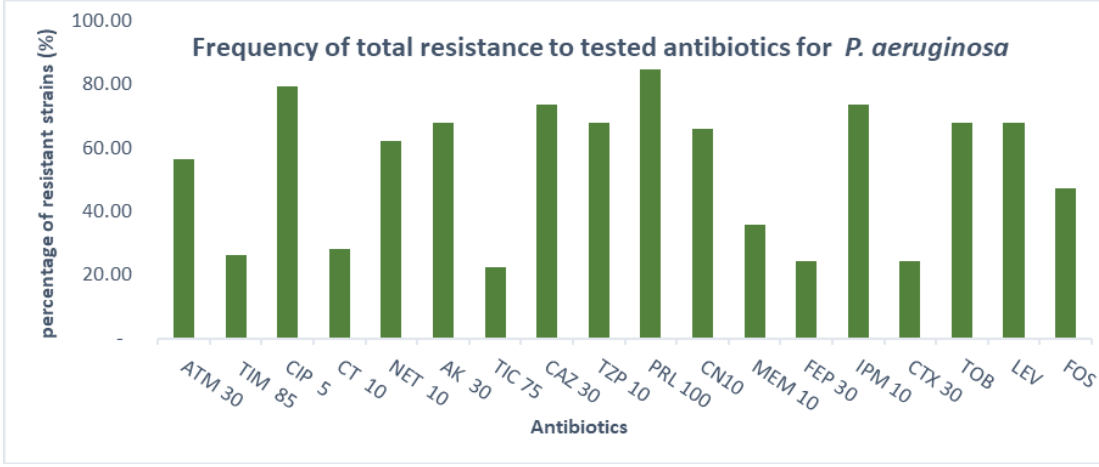

Fig. 2: Antimicrobial resistance pattern of *P. aeruginosa* isolated from wells.

The largest manifested sensitivity (Fig. 2) concerns the carboxypenicillins classes: Ticarcillin (77.36%), ticarcillin acid clavulanic (73.58%), followed by cephalosporins the 3rd and 4th generation with cefepim and cefotaxim (75.47%, 71.70%) respectively. At the end, the sensitivity was noted to Polymyxin classes: Colistin (71.70%).

A multidrug resistant strain of *P. aeruginosa* is defined as a strain which resists to more than three antimicrobial drug classes. The results revealed that 68% of strains were multidrug resistant, whereas 30.19% of strains resist at least to 11 antibiotics, 15.09 % of strains resist at least to 7 and 10 antibiotics, 13.21% of strains resist at least to 12 antibiotics, 5.66% of strains resist at least to (4, 8 and 13) antibiotics, 3.77 % of strains resist at least to 5 and 14 antibiotics, and finally 1.89% of strains resist to 9 antibiotics (Table 2).

Phenotype Antibiotics Resistance

With the disc diffusion test, the phenotypic detection marker antibiotics was sufficient to identify the wild-type phenotype and the common β-lactam resistance phenotypes of *P. aeruginosa* previously described.

Wild type phenotype: The 20% of *P. aeruginosa* strains detected wild type have natural resistance mechanism, with an absence of synergy among the aztreonam, ceftazidim and the ticarcillin acid clavulanic. Also, these isolated bacteria are susceptible to the phenotypic detection markers as the ticarcillin, imipenem, cefepim, ceftazidim, ticarcillin acid clavulanic, and the aztreonam with a diameter zone of 30mm±3. However, a smaller zone diameter given by cefotaxime is less active on these strains.

Penicillinase phenotype: The 36.5% of strains have a phenotype of the Oxacillinase broad spectrum ES-OXA class D, according to the classification of Bush Jacoby, which is resistant to the ticarcillin or intermediate/resistant to the cefepime. The activities of the cefotaxim, ceftazidim, aztreonam and the imipenem against these strains are susceptible. Consequently, when OXA-type enzymes are produced the clavulanic acid does not generally restore a susceptibility to the ticarcillin. Non-synergic effect is observed between clavulanic acid and other β-lactams, only the imipenem antagonized of cefepim/or ceftazidim activity. Finally, the activity of the cefepim was reduced.

Phenotype due to ESBL production: The 23.5% isolates of *P aeruginosa* strains contain ESBL (extended spectrum β-lactamase) phenotype class A according to the classification of Bush Jacoby which is resistant to the aztreonam, ceftazidim and all β-lactamase tested, but sensitive to the imipenem. However, a synergy effect was detected between ceftazidim, aztreonam, and the ticarcillin acid clavulanic. Consequently, the clavulanic acid can not restore susceptibility to the ticarcillin. Finally, the activity and diameter zone of the ceftazidime (≤22mm) and aztreonam (diameter ≤ 27 mm was reduced.

Phenotype cephalosporinase AMPC: The 14.1% strains of *P. aeruginosa* were detected as phenotype cephalosporinase AMPC due to constitutive depressed mutants "high-level cephalosporinase". Only the imipenem is active against these strains of *Pseudomonas aeruginosa*. However, an absence of synergy among the aztreonam, ceftazidime and the ticarcillin acid clavulanic was noted with a presence of antagonistic effect between the imipenem and cefepime. In addition, the presence of zone edges, consisting of scatter colonies was observed. Consequently, the clavulanic acid did not restore susceptibility to ticarcillin.

Phenotype impermeability to imipenem: 6% strains of *P.*

Table 2: Classification of Multi drug Resistant strains of *P. aeruginosa* isolated from wells.

	Multi Drug Resistant strains (MDR)	Percentage of Multi Drug Resistant (MDR) %
StrainsResistant to 4 Antibiotics	3	5,66
StrainsResistant to 5 Antibiotics	2	3,77
Strains Resistant to 7 Antibiotics	8	15,09
Strains Resistant to 8 Antibiotics	3	5,66
Strains Resistant to 9 Antibiotics	1	1,89
Strains Resistant to 10 Antibiotics	8	15,09
Strains Resistant to 11 Antibiotics	16	30,19
Strains Resistant to 12 Antibiotics	7	13,21
Strains Resistant to 13 Antibiotics	3	5,66
Strains Resistant to 14 Antibiotics	2	3,77

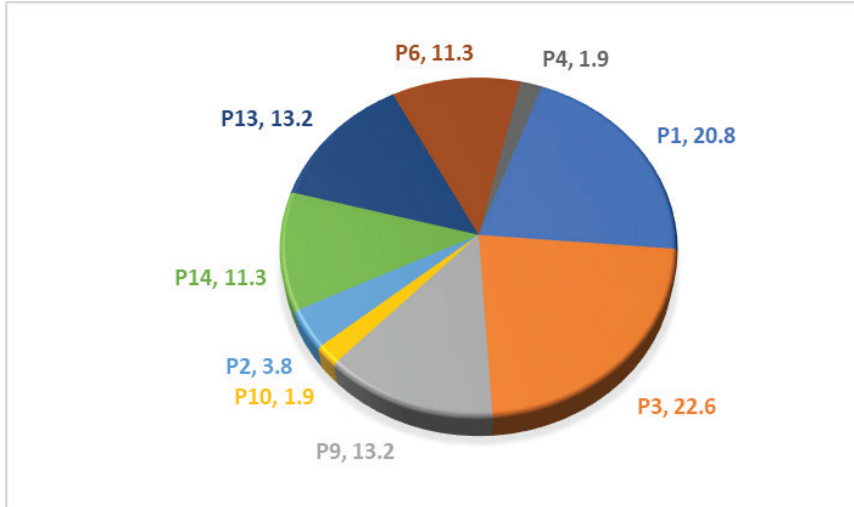

Fig. 3: Serotype distribution of *P. aeruginosa* strains isolated from wells according to the prevalence.

aeruginosa were detected using phenotype, the impermeability to the imipenem, that is showed by resistance strains to imipenem. However, this phenotype was characterized by an absence of image of synergy between the ticarcillin clavulanic acid and those of C3G and/or aztreonam. In addition, the presence of antagonistic effect between imipenem and cefepime, the diameter zone of both IPM and FEP is substantially equal.

Serogroups prevalence of *Pseudomonas aeruginosa*: In this study area, some isolates are poly-agglutinative, while others agglutinated only by polyvalent, but not monovalent antisera. Some isolates are not agglutinated with any serum, such isolates are described as non-typical. So, the following serotypes of *P. aeruginosa* are serologically identified: P1, P2, P4, P10, P3, P6, P9, P13 and P14. The most frequent serotypes are P3 with 22.6% of prevalence, followed by the serotype P1 with 20.8%. However, the serotypes P13 and P9 have (13.2%) of prevalence. While, the serotypes P6 and P14 have prevalence 11.3%, 3.8% for P2 serogroups, and finally P10 and P4 have a low percentage of prevalence with 1.9% (Fig. 3).

Cluster Analysis of *P. aeruginosa* Isolates

All *P. aeruginosa* isolates are generated to a dendrogram using Ward's method (Euclidean distances) and subjected to cluster analysis based on their inhibition zone diameter (IZD). This approach was used as a tool to resolve the difference between the MDR phenotypes of different isolates (Fig. 4). From all isolated sites, the *P. aeruginosa* strains were subjected to cluster analysis and two main clusters were observed (Fig. 4) with the number of isolates from different wells. Both clusters have sub clusters. The phenotype of oxacillinase was found only in cluster 1. The second cluster is a large cluster isolates, mixed cluster with phenotypes oxacillinase, ESBL β-lactamase and cephalosporinases, while the first small cluster detected only the ESBL isolates. However, the second cluster is a very mixed one and contains a large variance between sample areas.

DISCUSSION

P. aeruginosa has a great ability of survival strategies as the production of biofilm and pigments. It is a distributed bacterium in nature and known as a ubiquitous bacterium. However, few authors reported the presence of resistance profile and its acquisition mechanisms in the environmental isolates (Nola et al. 2017, Kawecki et al. 2017, Moussé et al. 2016).

Pathogenic species such as *Pseudomonas aeruginosa*, found in water (piped water systems, hot water systems and spa pools), may contribute to constitution of biofilm-forming microbial communities (Ugo et al. 2018), the same result were found by Ouardi et al. (2013), Marti et al. (2014), which proved that 95% of the overall biomass appear as a biofilm of *P. aeruginosa* in water of the pipe wells (El Ouardi et al. 2013, Marti et al. 2014).

Our study shows the great potential of *P. aeruginosa* isolated from well water to acquire antimicrobial resistance. The same finding was reported by Koro et al. (2016), and Marti et al. (2014) in several studies in which they highlighted the role of the environment for the dissemination of MDR organisms within human and animal, that can establish the need for great risk assessment to be fulfilled in

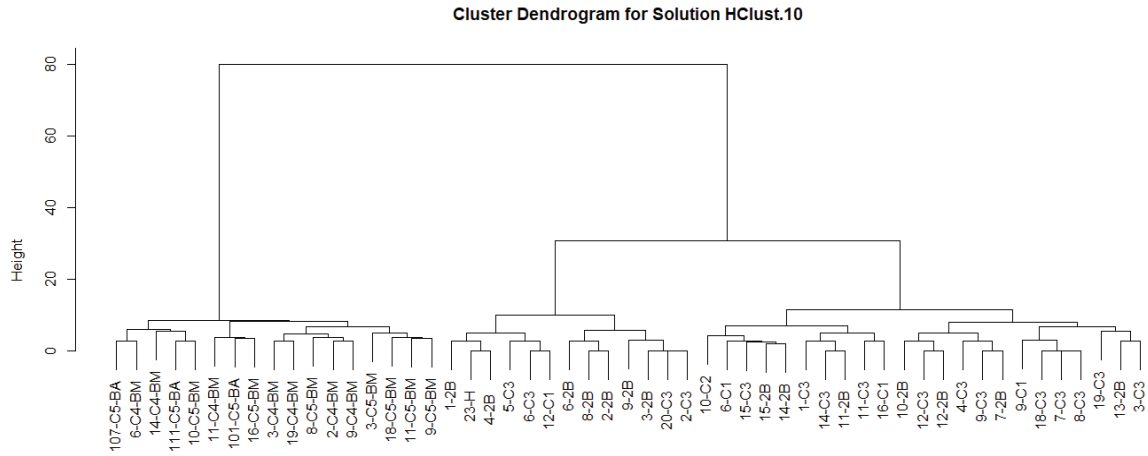

Fig. 4: Cluster analysis of *P. aeruginosa* isolated from wells water.

order to identify the presence of the *P. aeruginosa* in water, thus act as reservoir for MDR strains reported by Kümmerer (Moore et al. 2010, Kümmerer et al. 2009, Penna et al. 2009).

This study aims to evaluate the diversity of MDR phenotypes of *P. aeruginosa* found in well waters in irrigated zone of TADLA plane in Béni-Mellal region. In this study, 68% of *P. aeruginosa* strains were multidrug resistant, with 30.19% of strains resistant to at least 11 antibiotics, and 15.09 % of strains resistant to at least (7 and 10) antibiotics. Also, 61.15 % of prevalence among *P. aeruginosa* strains are resistant to all antibiotics tested. The same study was done in surface waters by Moore et al. (2010) and Kawecki et al. (2017).

The *P. aeruginosa* is widely distributed in well waters in this study area; out of 200 water samples examined, *Pseudomonas* spp. was successfully isolated from 139 samples, whose 85 strains were identified as *P. aeruginosa*. The same results were demonstrated by Alaoui et al. (2008) in Morocco, Rigas et al. (1998) in Greece, and Moore et al. (2002) in Ireland. The *P. aeruginosa* was isolated from 30.8% of the hydrotherapy pools, 72.5% of the Jacuzzis/spas, and 38.2% of the swimming pools (Alaoui et al. 2008, Moore et al. 2002).

The *P. aeruginosa* is naturally resistant to penicillin, to 1st and 2nd generation of cephalosporins, and most of the cephalosporins in 3rd generation. It has always been viewed as a microorganism which is difficult to treat because of its resistance to antibiotics. Then the treatment of the infection of the *P. aeruginosa* is based on the association of the beta-lactam and the aminoglycoside, or the fluoroquinolone and the aminoglycoside, or the beta-lactam and the fluoroquinolone. We have, therefore, chosen the antibiotics that are most frequently prescribed to course of treatment of the infection to *P. aeruginosa*.

The present study is also extended to classify *P. aeruginosa* strains according to the beta-lactamases production, 36.5% of strains present a phenotype of oxacillinase broad spectrum ES- OXA, 23.5% isolates of *P. aeruginosa* strains showed ESBL, 20 % of strains detected wild type, 14.1% strains detected as phenotype cephalosporinase AMPC and 6% strains detected with phenotype the impermeability to the imipenem. This variety of resistant phenotype strains may explain the larger number and diversity of isolates from this water; the same results were found by El Ouardi et al. (2013), Mulamattathil et al. (2014), Nasreen et al. (2015) and Nedeljković et al. (2015). The results from our study and those found in previous studies show that water acquire a great potential of resistant phenotype those are widely distributed in all water wells, thus, should be further taken with seriousness to provide an ideal treatment against the *P. aeruginosa*.

Regardless of all the results of resistant phenotypes from wells that were examined, two classes of Uréidopenicillins (84.11%) and fluoquinolons (73.6%) have the greatest antimicrobial potency of resistance. The same results showed high potency of susceptibility to the carboxypenicillins classes (75.47%), followed by the 3rd and 4th generation of cephalosporins with (73.6%). In fact, 75% of resistance to cefepim was reported by Akhabue et al. (2012). Also, a largest sensitivity is noted to polymyxin with colistin (71.70%). At the end, a low sensitivity was manifested to carbapenems (30.19%). Similar results are obtained by Moore et al. (2010) from surface water and El Ouardi et al. (2013) from groundwater, and Mulamattathil et al. (2014) and Nasreen et al. (2015) from surface and drinking water.

These results about antibiotics classes activity corroborate with the results of Ben Abdallah et al. (2008) indicated that the most active antibiotic is the colistin. It is proved by the results of studies by Maroui et al. (2015), Minchella et al. (2010) in Nimes in France on patients in the CHU.

The study highlights a variety of serogroups of *P. aeruginosa* isolated from water wells. The predominant serogroups isolated in this study are P1, P3, P6, P9, P13 and P14. These results are original and unique in the area and there are no similar results obtained from water wells. The serotype P3 is the most resistant to almost all tested antibiotics, except the colistin. The P3 serotype is widely spread due to highest resistance to antibiotics.

The fact that strains isolated in this study should be in contact with environmental, aquatic and animal strains. These results are in agreement with those from surface water and swimming pool (Stirling et al. 2008, Nola et al. 2017, Mérens et al. 2012, Ckd et al. 2016).

In addition, the importance of emergence of MDR strains could be justified by the phenotype resistant to action of carbapenems drug and polymyxins, which are the greatest choice treatment of infections by the *P. aeruginosa* resistant to multiple beta-lactam antibiotics.

CONCLUSION

This study shows a high prevalence, with a great diversity of the isolated *P. aeruginosa* from water wells and gives an idea about their antibiotic resistance profile. The presence of *P. aeruginosa* would provide information about the health risks associated with the consumption of contaminated water.

Moreover, after some investigations and various analyses, we found that different well waters used for drinking and housework and livestock beverage are not suitable. These results, which are out of standards in the analysed well waters can cause minor or severe infections to consumers. The importance of assessment and urgent actions to treat well water is clear, and the authorities need to take action against the risks related to the contamination of water in order to prevent or decrease the spread of *P. aeruginosa* in environment and well waters.

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