การดื้อต่อยาปฏิชีวนะของเชื้อ *Pseudomonas aeruginosa* ที่แยกได้จากสิ่งแวดล้อมในจังหวัดมหาสารคามและจังหวัดหนองบัวลำภู

Antibiotic resistance of environmental Isolates of *Pseudomonas aeruginosa* in Maha Sarakham province and Nong Bua Lamphu province

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บทคัดย่อ

Pseudomonas aeruginosa ที่ดื้อยาหลายชนิดในเวลาเดียวกัน (Multidrug resistant *P. aeruginosa* หรือ MDR *P. aeruginosa*) สามารถที่จะพบได้บนอุปกรณ์เครื่องมือทางการแพทย์, บุคลากรในโรงพยาบาล และสิ่งแวดล้อมภายในโรงพยาบาลซึ่งจะส่งผลให้เกิดความล้มเหลวในการรักษาการติดเชื้อชนิดนี้ด้วยยาปฏิชีวนะ แต่ในปัจจุบันยังไม่มีการศึกษาข้อมูลเกี่ยวกับการกระจายของ MDR *P. aeruginosa* ในสภาพแวดล้อมภายนอกโรงพยาบาลมากนัก ด้วยเหตุนี้คณะผู้วิจัยจึงต้องการเปรียบเทียบการกระจายของ MDR *P. aeruginosa* จากสิ่งแวดล้อมภายในและภายนอกโรงพยาบาลจากสามพื้นที่ ได้แก่ โรงพยาบาลมหาสารคาม โรงพยาบาลหนองบัวลำภู และมหาวิทยาลัยมหาสารคาม โดยทำการศึกษารูปแบบการดื้อยาปฏิชีวนะของเชื้อ *P. aeruginosa* ที่เก็บจากดินและน้ำจากทั้งสามพื้นที่ด้วยวิธี disc diffusion method และทำการคำนวณหาอัตราส่วนของ MDR และดัชนีแสดงการดื้อต่อยาปฏิชีวนะหลายชนิด (Multiple Antibiotic Resistance (MAR) index) ของเชื้อ *P. aeruginosa* จากผลการทดสอบพบว่า *P. aeruginosa* ที่แยกมาได้ดื้อต่อยา trimethoprim/sulfamethoxazole มากที่สุด (ร้อยละ 100.0) ดื้อต่อยา ceftazidime เป็นอันดับสอง (ร้อยละ 43.5-78.3) และดื้อต่อยา imipenem น้อยที่สุด (ร้อยละ 4.3-13.0) โดยที่ความชุกของ MDR *P. aeruginosa* จากทั้งสามพื้นที่จะอยู่ในช่วง 21.0-26.7% และค่า MAR index มากกว่า 0.2 จะพบใน ร้อยละ 62.9, 60.0 และ 91.3 ของ *P. aeruginosa* สายพันธุ์จากมหาวิทยาลัยมหาสารคาม โรงพยาบาลมหาสารคามและโรงพยาบาลหนองบัวลำภูตามลำดับ ผลการศึกษานี้แสดงให้เห็นว่า MDR *P. aeruginosa* สามารถพบได้จากในดินและน้ำที่เก็บจากภายในโรงพยาบาลและพื้นที่นอกโรงพยาบาล

คำสำคัญ : เชื้อดื้อยาหลายชนิดในเวลาเดียวกัน ดัชนีแสดงการดื้อต่อยาปฏิชีวนะหลายชนิด เชื้อ *Pseudomonas aeruginosa* disc diffusion เชื้อแบคทีเรียที่แยกได้จากสิ่งแวดล้อม

Abstract

Hospital surfaces, equipment, and healthcare staff are known sources of multidrug resistant (MDR) *Pseudomonas aeruginosa,* a pathogen capable of causing difficult- and impossible-to-treat infections. However, little information is available on the occurrence and distribution of MDR *P. aeruginosa* in the wider environment. In the present study, therefore, we examined soil samples collected in the vicinity of two hospitals (Mahasarakham Hospital and Nong Bua Lamphu Hospital) for the presence of MDR *P. aeruginosa*. We also examined soil and water samples collected from

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a non-hospitals site (Mahasarakham University). Drug resistance of the bacteria was assessed by performing the disc diffusion method and calculating both the number of MDR *P. aeruginosa* isolates and the Multiple Antibiotic Resistance (MAR) index of isolates. A high percentage of *P. aeruginosa* isolates was resistant to trimethoprim/sulfamethoxazole (100.0%) and ceftazidime (43.5-78.3%), with fewer exhibiting resistance to imipenem (4.3-13.0%). The prevalence of MDR *P. aeruginosa* ranged from 21.0-26.7%, and a MAR index greater than 0.2 was found in 62.9%, 60.0%, and 91.3% of the isolates from Mahasarakham University, Mahasarakham Hospital and Nong Bua Lamphu Hospital, respectively. These results demonstrate that soil and water, both in the proximity of hospitals and more distant areas, represent a potential source of MDR *P. aeruginosa*.

Keywords: Multidrug resistance (MDR), Multiple Antibiotic Resistance (MAR) index, *Pseudomonas aeruginosa*, disc diffusion, environmental isolates

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium found in soil and water, and on the surfaces of plants, animals and humans. *P. aeruginosa* is an important opportunistic human pathogen, responsible for 10-15% of hospital-acquired infections worldwide.[1] In immunocompromised individuals, for example patients with HIV infection, burns, cancer, or cystic fibrosis, *P. aeruginosa* can cause urinary tract infections, respiratory infections, wound infections including secondary infection of burns, and sepsis.[2] Such infections increase morbidity and mortality rates in these patients. *P. aeruginosa* can also cause infection in immunocompetent persons, for example folliculitis, green nail syndrome, and hot foot syndrome.[2] A major problem with *P. aeruginosa* infections is the lack of treatment options because *P. aeruginosa* is intrinsically resistant to several classes of antibiotic. Selecting an inappropriate antibiotic has important consequences including the development of new resistant or multidrug resistant strains and therapy failure.

A high prevalence of multidrug resistance indicates a serious need for broad-based, local antibiotic resistance surveillance, and planning of effective interventions to prevent spread of the multidrug resistant microorganism.[3, 4] Multiple antibiotic resistance (MAR) in bacteria is most commonly associated either with the presence of plasmids which contain one or more resistance genes, or chromosomal DNA mutations.[5, 6] One good indicator for multiple antibiotic resistance in bacteria is the Multiple Antibiotic Resistance (MAR) index. The MAR index is calculated as the ratio of 'the number of antibiotics to which the organism is resistant' to the 'total number of antibiotics to which the organism is exposed' and it has been shown to be a cost effective and valid method of bacteria source tracking.[4, 7, 8]

Antibiotic resistant strains of *P. aeruginosa* have been reported in several locations[9, 10] including Thailand.[11, 12] In Thailand, however, most studies have focused on clinical isolates of *P. aeruginosa*. The distribution of MDR *P. aeruginosa* in the environment has never been assessed, although inappropriate use of antibiotics, a factor which might increase antibiotic resistance in the wider environment, has been extensively reported.[13-16] In this study, therefore, we examined strains of *P. aeruginosa* isolated both in the vicinity of hospitals and further away for resistance to commonly used antibiotics. This information will permit an evaluation of the current situation of environmental antibiotic resistance in *P. aeruginosa* in Northeast Thailand, enabling hospitals and clinics to make evidence-based decisions in their management of *P. aeruginosa* infections.

Materials and Methods

Sample collection

A total of 180 environmental samples were randomly collected from Mahasarakham and Nong Bua Lamphu provinces, Thailand. One hundred and twelve samples (8 samples from pond water and 104 from soil) originated from Mahasarakham University's Khamriang Campus, which is 9.3 km away from Mahasarakham Hospital. Another 28 soil samples were collected from Mahasarakham Hospital, Mueang District, Maha

Sarakham province and 40 soil samples were from Nong Bua Lamphu Hospital, Mueang District, Nong Bua Lamphu province. We collected only soil samples from the two hospitals, because there was no surface water at these sites. The sample sizes were determined by the area available for collection. The larger the area of soil and water available, the larger the number of samples collected. Sample collection was conducted between June and December 2011. The soil samples were stored in sealed plastic bags and the water samples kept in sterile bottles (room temperature) during transport to the laboratory. All samples were processed immediately upon arrival at the laboratory.

Isolation and identification of *P. aeruginosa*

Ten grams of each soil sample and 10 ml of each water sample were diluted with 90 ml sterile water, and plated on differential medium (*Pseudomonas* agar P) in tripicate. Plates were incubated at 37°C for 18-24 hours and observed for colonies of *P. aeruginosa*. *P. aeruginosa* was further identified by routine bacteriological methods (i.e. colonial morphology, Gram stain, motility test, growth at 42°C) and traditional biochemical methods (i.e. oxidase test, urease test, nitrate reduction test, indole test, pyocyanin production, TSI agar test, DNase test, O/F test, ONPG test). [17]

Antimicrobial susceptibility testing

Antibiotic susceptibility was determined using the NCCLS disc diffusion method with *P. aeruginosa* ATCC 27853 as the control strain. [18] Antibiotic discs [ceftazidime (30 µg), gentamicin (10 µg), norfloxacin (10 µg), imipenem (10 µg), amikacin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), and ciprofloxacin (5 µg)] were purchased from Becton Dickinson and Company (BD). Isolates were identified as multidrug resistant (MDR) *P. aeruginosa* if they were resistant to 3 or more of the following antibiotic classes: penicillins / cephalosporins / monobactams, carbapenems, aminoglycosides, and fluoroquinolones.[19]

Multiple Antibiotic Resistance (MAR) index

The MAR index of each isolate was calculated as the ratio *a/b*, where *a* represents the number of antibiotics the isolate is resistant to, and *b* represents the total number of antibiotics against which the isolate is tested. Typically, a MAR index value greater than 0.2 is observed when isolates are commonly exposed to several antibiotics, whereas a MAR index value lower than 0.2 occurs when isolates are rarely or never exposed to antibiotics.[4, 7, 8]

Results

A total of 100 *P. aeruginosa* isolates (98 from soil samples and 2 from water samples) were recovered from the 172 soil samples and 8 water samples. Of the 100 isolates, 62 were obtained from Mahasarakham University, 23 were from Nong Bua Lamphu Hospital, and 15 were from Mahasarakham Hospital.

Antibiotic resistant *P. aeruginosa* was detected at all of our study locations, with all of the isolates shown to be resistant to at least one of the tested antibiotics. Table 1 shows how the antibiotic resistance profiles varied between the different locations. Among the *P. aeruginosa* isolates from these three locations, trimethoprim/sulfamethoxazole resistance was detected with the highest frequency (100.0%), followed by ceftazidime (43.5-78.3%) and gentamycin (24.2-40.0%). Imipenem resistance was detected with the lowest frequency (4.3-13.0%).

The prevalence of MDR *P. aeruginosa* ranged from 21.0-26.7% depending on the location (Table 2). Interestingly, the prevalence of MDR *P. aeruginosa* at the two hospitals (21.7% for Nong Bua Lamphu Hospital and 26.7 % for Mahasarakham Hospital) was only slightly higher than that detected at the non-hospital site (21.0% for Mahasarakham University).

Table 1 Percentage of *P. aeruginosa* isolates resistant to each antibiotic from water and soil samples at the different locations

Class of antibiotic	Antibiotic	Percentage of resistant isolates					
		MSU (n=62)		HpMs (n=15)		HpNb (n=23)	
		S (%)	I/R (%)	S (%)	I/R (%)	S (%)	I/R (%)
Aminoglycoside	Amikacin	72.6	27.4	73.3	26.7	47.8	52.2
	Gentamycin	75.8	24.2	60.0	40.0	60.9	39.1
Carbapenem	Imipenem	93.5	6.5	86.7	13.0	95.7	4.3
Cephalosporin	Ceftazidime	56.5	43.5	46.7	53.3	21.7	78.3
Fluoroquinolone	Ciprofloxacin	75.8	24.2	73.3	26.7	60.9	39.1
	Norfloxacin	88.7	11.3	73.3	26.7	87.0	13.0
Sulfonamide	Trimethoprim /Sulfamethoxazole	0.0	100.0	0.0	100.0	0.0	100.0

The number of isolates is shown in parentheses at the top of each column; MSU: Mahasarakham University; HpMs: Mahasarakham Hospital; HpNb: Nong Bua Lamphu Hospital; S: sensitive; I: intermediate; R: resistant.

Table 2 Percentage of MDR *P. aeruginosa* isolates obtained from the three studied locations

Location	MDR *P. aerugionosa* (%)
Mahasarakham University (n=62)	21.0
Mahasarakham Hospital (n=15)	26.7
Nong Bua Lamphu Hospital (n=23)	21.7

The MAR index values determined for P. aeruginosa isolates in this study ranged from 0.14 to 1.00 (Table 3). The proportion of isolates with a MAR value greater than 0.2 were, respectively, 62.9%, 60.0%, and 91.3% for the sites at Mahasarakham University, Mahasarakham Hospital and Nong Bua Lamphu Hospital (Table 3). This indicates that a large proportion of the isolates examined are likely to originate from humans or animals, where antibiotic use is common.

Table 3 Multiple Antibiotic Resistance (MAR) index of *P. aeruginosa* isolates obtained from the three studied locations

MAR index	Mahasarakham University (%)	Mahasarakham Hospital (%)	Nong Bua Lamphu Hospital (%)
1(0.14)	37.1	40.0	8.7
2(0.29)	**27.4**	**20.0**	**21.7**
3(0.43)	**12.9**	**13.3**	**39.1**
4(0.57)	**9.7**	**0.0**	**17.4**
5(0.71)	**6.5**	**0.0**	**8.7**
6(0.86)	**4.8**	**13.3**	**0.0**
7(1.00)	**1.6**	**13.3**	**4.3**
Total MAR>2	**62.9**	**60.0**	**91.3**

Discussion and Conclusions

In this study, researchers determined the resistance profiles of environmental isolates of *P. aeruginosa* from Northeast Thailand to a range of antibiotics in common national use. Strains of antibiotic resistant *P. aeruginosa* were detected at hospital sites (Mahasarakham Hospital and Nong Bua Lamphu Hospital), and also at a non-hospital site (Mahasarakham University). The antibiotic resistance profiles of these isolates varied between the sites.

P. aeruginosa is intrinsically resistant to trimethoprim/sulfamethoxazole[20, 21], therefore it was not surprising to find that all of the isolates in this study, without exception, were resistant to this drug combination. We also found that, at all three sites, a high proportion of the *P. aeruginosa* isolates were resistant to ceftazidime. Ceftazidime is a treatment currently recommended for many types of infection including melioidosis, which is caused by another bacterium present in these areas, *Burkholderia pseudomallei*.[22, 23] Our detection of ceftazidime resistance in *P. aeruginosa* suggests that other soil bacteria, including *B. pseudomallei*, might be ceftazidime resistant. This is potentially problematic as there is a high prevalence of melioidosis across Northeast Thailand.[24, 25] We also found that the proportion of antibiotic resistant bacteria isolated from the hospital sites was slightly higher than at the non-hospital site, probably because these isolates have been exposed to antibiotics more recently or more often, as suggested by Moges *et al.*[26]

Researchers determined that more than 20% of the isolates collected from sites at Mahasarakham Hospital and Nong Bua Lamphu Hospital are MDR *P. aeruginosa*. Since a high prevalence of multidrug resistance indicates the need for an antibiotic surveillance program, we also calculated the MAR index of these isolates to antibiotics in common use to differentiate bacteria from different sources. This analysis shows that 60.0% and 91.3% of *P. aeruginosa* isolates from Mahasarakham Hospital and Nong Bua Lamphu Hospital, respectively, had a MAR index greater than 0.2. This suggests that these strains had been exposed to antibiotics previously. Practical methods are therefore needed to control bacterial contamination not just within hospitals, but also to stop spread from hospitals to the nearby environment. Nosocomial infections caused by *P. aeruginosa* are frequently life-threatening and difficult to control. Antibiotic resistant *P. aeruginosa* can be traced to human sources, such as direct shedding from colonized humans, especially during hospitalization, and often following inappropriate empirical antibiotic therapy.[27-29] Additionally, horizontal gene transfer between hospital isolates can contribute to widespread distribution of multidrug resistant strains.[30, 31] A patient colonized or infected with multidrug resistant *P. aeruginosa* can lead to in-room and neighboring area contamination, and contaminated water, fomites and aerosols. This can result in further infections in susceptible individuals, the clinical treatment and containment of these infections becoming an ever more challenging problem.[32-34]

The study also found that about 21.0% of environmental isolates from the non-hospital site, Mahasarakham University were MDR *P. aeruginosa*. This result reveals there is minimal difference in the distribution of MDR strains between hospital and non-hospital sites, suggesting the existence of MDR strains in outpatients and the community. This finding adds to a growing number of reports showing that environmental isolates of *P. aeruginosa* can sustain not just antibiotic resistance, but multiple drug resistance.[8, 17, 26, 29, 35] Interestingly, our results also show that 62.9% of environmental isolates from the non-hospital site had a MAR index greater than 0.2. This suggests that not just hospital sites but also non-hospital sites represent reservoirs for antibiotic resistant *P. aeruginosa*. In theory, the absence of selective pressure at non-hospital sites should reduce antimicrobial resistance levels, since antibiotic resistant strains typically have lower fitness than wild-type susceptible strains.[36-37] However, antibiotics and other soil and water pollutants, such as heavy metals, might create a sufficiently strong selection pressure to maintain antibiotic resistance genes in environmental *P. aeruginosa*.[8, 9, 38]

In Thailand, the inappropriate use of antibiotics has been widely reported. Examples include the use of

antibiotics in patients without evidence of infection, the use of antibiotics in patients who do not need antibiotics[13, 15, 16], the use of antibiotics against which the target strain is not susceptible, and the use of broad-spectrum antibiotics where a narrow-spectrum alternative would have been effective.[14] This overuse creates a strong selective pressure for the emergence of new antibiotic resistant, multiple drug resistant and extensively drug resistant pathogens in Thailand, increasing the risk of untreatable bacterial infections. The findings in this study suggest that antibiotics may now be polluting the environment, creating a selection pressure that enables *P. aeruginosa* in the soil and water to maintain antibiotic resistance genes. It is therefore important to determine regional resistance patterns of clinical and environmental isolates of *P. aeruginosa* to enable physicians to choose the most appropriate antibiotic, improving patient prognosis and reducing unnecessary use of last resort antibiotics.

This study detected MDR *P. aeruginosa*, strains of bacteria with the potential to cause difficult- and impossible-to-treat infections, in soil and water samples collected from hospital and non-hospital sites in Northeast Thailand. This finding underlines the need to raise public awareness about inappropriate antibiotic use, a likely driving factor for the existence of MDR *P. aeruginosa* at these locations. Due to the prevalence of MDR *P. aeruginosa* in the studied areas, it is important that antibiotic surveillance programs as well as phylogenetic studies of environmental and clinical samples are now carried out to understand the origin and movement of resistant isolates and genes.

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