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Isolation and Identification of Antimicrobial Resistance of Proteus Mirabilis in Patients with Community-Acquired Urinary Tract Infections

Abd Elrahman Mustafa Abd Elrahman Osman*, Shingray Osman Hashim, Mohammed Abdall Musa, Omer Mohammed Tahir
Microbiology Colleagues, Medical Laboratory Sciences Division, Port Sudan Ahlia College, Sudan
Email address: abdo_rahman96@hotmail.com

***Abstract*—** *This study was carried out in Red sea state, Sudan during the period from November 2016 to March 2017, to investigate antimicrobial resistance of P.mirabilis isolated from patients suffering from community-acquired urinary tract infections. Two hundred and nineteen urine specimens were collected from patients attended Port Sudan Teaching Hospital, Red Sea Diagnostic Centre, Sea port corporation specialized Hospital and Prince Osman Digna Teaching Hospital. Out of the two hundred and nineteen urine specimens examined, only 10 (4.6%) P.mirabilis were recovered. The result indicated that the MIC, MIC50 and MIC90 of amoxyclav were (4 µg/ml each), co-trimoxazole (0.5- 4 µg/ml, 0.5 µg/ml and 4 µg/ml), ceftriaxone (0.008- 0.08 µg/ml, 0.008 µg/ml and 0.08 µg/ml) and ciprofloxacin (0.001-0.01µg/ml, 0.001 µg/ml and 0.0 µg/ml). The study conducted that the responsibility of P.mirabilis to UTIs slightly higher than previously recorded. The antimicrobial resistance of P.mirabilis to traditionally used antibiotics was high too.*

***Keywords*—** *P.mirabilis; MIC; Urinary Tract Infections)UTI); Red Sea state.*

I. INTRODUCTION

Proteus mirabilis (*P. mirabilis*) is a motile gram-negative bacterium belonging to the family *Enterobacteriaceae*. It is indole negative, chloramphenicol and ampicillin sensitive (1), (2).

Members of the *Enterobacteriaceae*, are all oxidase negative, actively motile, non-spore forming, non-capsulated and a recognized by their ability to cause disease (3).

The organism occurs widely in man, animals and in the environment and can be readily recovered from sewage, soil, garden vegetables and many other materials (2).

P. mirabilis is the species most commonly recovered from humans, especially from urinary and wound infections and accounts for 90% of all infections caused by the *Proteus* species (3).

Urinary tract infections (UTIs) are one of the most common infectious diseases, and nearly 10% of people will experience a UTI during their life time. The infections may be symptomatic or asymptomatic, and types of infection can result in serious sequelae if left untreated (4).

Although several different microorganisms can cause UTIs, including fungi and viruses, bacteria are the major causative organisms and are responsible for more than 95% of UTI cases (4).

P. mirabilis is a common cause of hospital acquired UTIs and it can also cause infection in nonhospitalized patients, especially those with staghorn (struvite) calculi, structural urinary tract abnormalities or an indwelling catheter (1).

Like many members of the family *Enterobacteriaceae*, *Proteus* species can harbor numerous plasmid – and integron - mediated antimicrobial resistance determinants (5).

The increase in the number of resistant and multiresistant (resistant to two and more antimicrobials) strains of bacteria is a major concern of health officials worldwide. Recently, bacterial resistance arising through the production of extended – spectrum beta – lactamases (ESBLs) has been recognized as a worldwide therapeutic problem (6). The mechanisms of this resistance are often complex, and include production of β-lactamases, unregulated efflux pumps, and target site mutations (7).

Organisms that produce extended – spectrum beta – lactamases (ESBL) remain an important reason for therapy failure with cephalosporins and have serious consequences for infection control. That clinical microbiology laboratories detect and report ESBL – producing organisms is therefore important. Most ESBLs can be divided into three groups: TEM, SHV, and CTX-M types (8).

Wild type strains of *P. mirabilis* are susceptible to all penicillins and cephalosporins. However, since 1990, a progressive increase of β-lactam. Resistance, mediated by the production of acquired β-lactamases, has occurred in this species. Various ESBLs, such as TEM, CTX-M, PER, and VEB – type ESBLs, have been described in *P. mirabilis* in separate geographic areas (9).

A available therapeutic options for the treatment of these antimicrobial -resistant organisms are severely limited because these organisms frequently display a multidrug – resistant phenotype (10).

Treatment of UTI cases is often started empirically. However, because of the evolving and continuing antibiotic resistance phenomenon, regular monitoring of resistance patterns is necessary to improve guidelines for empirical antibiotic therapy.

Minimum inhibitory concentration (MIC) testing is a very valuable quantitative assay tool for evaluating the pathogenic microorganisms degree of susceptibility and to detect the specific resistance mechanism. Selection of the most effective

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antimicrobial agents and dosing regiment for serious infection will help in eliminating the pathogens and minimize resistance selection and decrease mortality.

II. METHODOLOGY

2.1 Study Design

2.1.1 Type of the study

The work is a descriptive and Cross-sectional study of 219 patients with symptoms of community-acquired urinary tract infections.

2.1.2 Study area

This work was carried out in Red Sea state. Urine collection and patients data were done in Port Sudan Teaching Hospital, Red Sea Diagnostic Centre, Sea Port Corporation specialized Hospital and Prince Osman Digna Teaching Hospital. The laboratory investigation was carried out in the Laboratory of Port Sudan Ahlia College section of Medical Laboratory Science.

2.1.3 Target population

Patients suffering from UTI signs and symptoms that attended the above mentioned hospitals were included in this study. The patients age varied from 1 month to 75 years.

2.1.4 Data collection

Data were collected using structural interviewing questionnaire.

2.2 Collection of Specimens

Mid stream urine (MSU) was collected from each patient. The patients were asked to clean the pre urethra area with soap and water. Adult females were instructed to make swabbing for the urethra with sterile swab and avoid any antiseptic. Then the patient were asked to pass the first drops of urine and collect the mid-stream urine in sterile, wide mouth container (10-20ml).

2.3 Cultivation of Specimens

2.3.1. Culture media

The following culture media were obtained from Hi media laboratories PVT. Ltd. India. and use throughout the study: MacConkey's agar medium, Blood agar, Mueller Hinton agar medium and Nutrient agar.

2.3.2. Inoculation of urine samples

Under aseptic conditions near Bunsen burner, urine specimens were inoculated onto MacConkey's and blood agar by using sterile loop, then incubated aerobically at 37°C over night.

2.4 Examination of Bacterial Growth

The primary culture on MacConkey's agar and blood agar that showed significant growth was examined for fermentation on to MacConkey's agar and haemolysis on blood agar. The morphological character, size, shape, colour and haemolysis were observed and recorded.

2.4.1. Interpretation of culture growth

The culture growth obtained was interpreted as significant (>10^5CFU/ml) according to Gupta and Joshi, (14). Culture of less than (10^3CFU/ml) of urine was considered insignificant, while culture with no growth were considered negative. Significant culture were further investigated.

2.5. Purification of Bacterial Growth

The isolates were streaked onto Nutrient agar and incubated overnight at 37°C. The resultant growth was checked for purity and stored in Bijou bottle for further investigation.

2.6. Identification of P. mirabilis

2.6.1. Primary identification

2.6.1.1. Colonial morphology

Bacterial colonies were examined from above for round, irregular, crenated or branching appearance also either transparent or opaque, smooth or rough and dull or shiny appearance. The colonies were showed flat or raised appearance when examined from the side. The color of the colonies was examined, which depend on indicators contained in media (11).

2.6.1.2. Grams stain

Asmear was prepared from bacterial colony and allowed to air-dry completely in a safe place protected from dust, and direct sunlight, the smear was fixed by rapid passing over flame of a Bunsen burner and allowed to cool before staining. The fixed smear was then covered with crystal violet stain for 30-60 seconds and the stain was washed off rapidly with clean tap water, the water was then tipped off and the smear was covered with lugol's iodine for 30-60 seconds, the iodine was then washed off with clean tap water and decolorized rapidly (few seconds) with acetone-alcohol and washed immediately with clean water. Then the smear was covered with neutral red stain for 2 minutes and washed off with clean tap water, the back of slide was wiped clean and placed in a draining rack for air dry, the smear was then examined microscopically, first with the 40x objective to check the staining and then with the oil immersion objective to report the bacteria (11).

2.6.2. Confirmatory identification

2.6.2.1. Oxidase test

In this test organism that produce the enzyme Cytochrome Oxidase oxidize phenylenediamine to a deep purple color. Oxidase reagent disc were placed on sterile Petri plates and rubbed with colony of the test organism using a piece of stick, and looked for a red-purple color within 20 min.

2.6.2.2. API 20 E (Analytical Profile Index) (REF:20100 / 20160)

2.6.2.2.1. Procedure

An incubation box (tray and lid) was prepared and about 5 ml of distilled water was distributed into the honey combed wells of the tray to create a humid atmosphere. The strip was removed from it is packaging and placed in the incubation box. A tube containing 5 ml of sterile saline was used. From isolation plate a single well isolated colony was removed by using a pipette and carefully emulsified to achieve a homogeneous bacterial suspension which was used immediately after preparation. With the same pipette the bacterial suspension was distributed into the tubes of the strip (the formation of bubbles at the base of the strip was avoided through tilting the strip slightly forward and the tip of the

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pipette was placed against the side of the cupule): for the CIT, VP, and GEL tests both tube and cupule were filled, for the other tests only the tubes were filled. For the ADH, LDC, ODC, H_2S and URE tests anaerobiosis was created by overlaying with mineral oil, then the incubation box was closed and incubated at 38 ± 2°C for 18-24 hours. After the incubation period the strip was read by referring to the reading table. When 3 or more tests were positive, all the spontaneous reaction on the result sheet were recorded and the reagents were added to the reveal tests: for the TDA test 1 drop of TDA reagent was added. A reddish brown color indicates a positive reaction that was recorded on the result sheet. For IND test 1 drop of James reagent was added and pink color development in the whole cupule indicated a positive reaction that was recorded on the result sheet. For VP test 1 drop of each VP1 and VP2 reagents were added and wait at least 10 minutes for development of a pink or red color indicated a positive reaction that was recorded on the result sheet, the reaction was considered negative, if a slightly pink color was appeared after 10 minutes, the strip was reincubated for further 24 hours without adding any reagents when the number of positive tests (including GLU test) was less than 3 while the tests requiring the addition of reagents were revealed.

2.6.2.2.2. Interpretation

For the determination of the numerical profile on the result sheet, the tests were separated into groups of 3 and a value 1, 2 or 4 was indicated for each. By adding together the values corresponding to positive reactions with in each group, a 7-digit profile number was obtained for the 20 tests of the API 20 E strip. Identification was performed using the database with the analytical profile index.

e.g: The 7 digit profile number: 0736000 indicates *P. mirabilis*

The organism is: P . mirabilis.

2.7 Antimicrobial Susceptibility Test

2.7.1. Preperation of Mueller Hinton Agar

Plates with Mueller Hinton Agar were prepared according to the directions specified on the label, the sterilized molten medium was cooled to 45-50 ˚C and poured into sterile, dry Petri plates on a leveled surface, to a depth of 4 mm.

The PH of the Mueller Hinton Agar was checked (7.4±2 at room temperature) and the presence of any excess surface moisture on the medium was removed by keeping the plates inverted in an incubator (35-37˚C). Some representative plates after solidified were incubated at 35˚C for 24-72 hours to check sterility.

2.7.2. Seeding of the discs

Using sterile forceps the antimicrobial discs was placed evenly distribution on the inculated plate, the plate was then inverted and incubated aerobically at 37 ˚C for 24 hours. After overnight incubation the control and test plates were examined to ensure that the growth was confluent or near confluent. Using a Ruler on the underside of the plate the diameter of each zone of inhibition was measured in mm. The end point of inhibition is where growth starts (12), (13).

2.7.3. Interpretation of the zone size

Using the interpretive chart, the zone of each antibiotic disc was measured and reported as sensitive, resistant or intermediate.

Sensitive: Sensitive suggests that the infection caused by the pathogen in respond to treatment when the drug is used in normal recommended doses and administered by an appropriate route.

Resistant: A pathogen reported as resistant implies that the infection it has caused would not respond to treatment with the drug to which it is resistant irrespective of dose or site of infection. (12,13).

2.7.4 Minimum Inhibitory Concentration (MIC) Test

2.7.4.1. Procedure

The MIC was defined as the lowest concentration of an antimicrobial agents that visually inhibits growth of a microorganism under defined experimental condition. Hicomb consistis of a strip made of an inert material with 8 extensions that carry the discs of 4 mm, a defined concentration of antibiotic is loaded on each of the disc so as to form a gradient when placed on agar plate, Hicomb based on the principle of dilution and diffution, cosists of a gradient that covers a continuous range of 16 two-fold dilutions on 2 different strips (part A&B) as per the conventional MIC method, when applied to the agar surface the antibiotic diffuse into the surrounding medium in high to low amounts from one end of the strip to the other and the gradient remains stable after diffusion. Preparation of medium, preparation of inoculum and inoculation of medium was as above. The pack of Hicomb was opened under aseptic condition, using sterile forceps the Hicomb MIC strip was picked up and applied to the agar surface with the MIC scale facing upwards, with it's higher concentration facing the edge of plate and the markings on strip facing upwards, the handle of the strip was pressed gently and ensured that all the discs were in full contact with the medium, on the opposite side of plate other strip was placed with higher concentration towards the edge of plate and lower concentration towards the center, the plate was closed and inverted to check wether all the discs in full contact with the medium, strips 3 of A and 3 of B can be placed on a 200 mm plate with the markings facings up ward, the plate was incubated at 35-37 ˚C and examined after 18-24 hours.

2.7.4.2. Interpretation of the test results

The zone of inhibition created has taken the form of ellipse.

MIC value: the value at which the zone was convenesed the comb-like projections of the strips and not at the handle.

The MIC was reported as greater than the highest concentration on the strip when no zone of inhibition was observed.

The MIC was reported as less than the lowest concentration on the strip when the zone of inhibition was below the lowest concentration.

The MIC value interpreted from part A might not necessarily be the same for part B and the MIC value is reported that as lowest of the two.

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III. RESULTS

Two hundred and ninteen urine specimens were collected from patients suffering from UTI during the period from November 2016 to March 2017. The patients constitute 107 (48.9%) males and 112 (51.1%) females (Table 1). The age of the patients ranged from one month to 75 years. Out of 219 patients 12 (5.5%) were infants, 57 (26%) were children, 49 (22.3%) were young adults, 31 (14.2%) were adults and 70 (32%) were elderly (Table 2). 120 (54%) of specimens showed bacterial growth when cultured on Blood and MacConkey agar. Among these 83 (73.4%) were lactose fermenters (LF) and the rest 37 (26.6%) were non-lactose fermenters.

3.1. Criteria of Isolation of P. mirabilis

Urine culture yielded more than 10^5 CFU/ml, gram-negative rods was considered significant for further investigation.

3.2. Identification of P. mirabilis

3.2.1 Colonial morphology

Colonial characteristic were observed on blood and MacConkey`s agar after overnight incubation. 10 out of 37 NLF were found suggestive *P. mirabilis* as they produced large, grey-white colonies on blood agar and large yellow colonies on MacConkey`s agar.

3.2.2 Conventional method

P. mirabilis was gram negative rods and Oxidase negative.

3.2.3 API 20 E

ONPG, ADH, and LDC were negative, ODC, CIT, H_2S, URE, TDA and IND were positive, VP, GEL and all sugars GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY and ARA were negative (3).

3.2.4 Antimicrobial sensitivity test

All isolated *P. mirabilis* were subjected to antimicrobial sensitivity test using Modified Kirby-Bauer disc diffusion method. The result revealed that the resistance rate of *P. mirabilis* was (100%) to nitrofuratoin and amoxycillin, (40%) to co-trimoxazole, (20%) to ceftriaxone, ciprofloxacin, and nalidixic Acid, (0%) to ticarcillin, amoxyclav, and amikacin (Table 3), and the Minimum Inhibitory Concentration (MIC) value was (0.5) to amikacin and (>240) to nitrofuratoin, nalidixic Acid and co-trimoxazole .

TABLE 1. Distribution of specimens according to patients gender.

Gender	No of specimen	%
Male	107	48.9
Female	112	51.1
Total	219	100

TABLE 2. Distribution of specimens according to patients age group.

Age group	No of specimens
Infant (up to 1 yr)	12 (5.5)
Children (1-15 yr)	57 (26)
Young adult (16-30 yr)	49 (22.3)
Adult (31-45 yr)	31 (14.2)
Elderly (more than 46 yr)	70 (32)
Total	219

TABLE 3. Rate of antimicrobial Resistance using *P. mirabilis* (n = 10) as indicator organism.

Antimicrobial agents	Resistance(%)
Nitrofuratoin	(100 %)
Amoxycillin	(100 %)
Nalidixic Acid	(20 %)
Co-trimoxazole	(40 %)
Amoxyclav	(0 %)
Ticarcillin	(0 %)
Ciprofloxacin	(20 %)
Ceftriaxone	(20 %)
Amikacin	(0 %)

TABLE 4. Minimum inhibitory concentration of antimicrobial agents

Isolate Code	Ci (240-0.001) µg/ml	CF (240-0.001) µg/ml	CO (240-0.001) µg/ml	AC (240-0.001) µg/ml
P.m 109	0.008	0.001	4	4
P.m 110	0.08	0.01	4	4
P.m 112	0.008	0.001	0.5	4
P.m 113	0,008	0.001	4	4
P.m 114	0.008	0.001	0.5	4
P.m 115	0.008	0.01	0.5	4
P.m117	0.008	0.01	4	4
P.m 118	0.08	0.01	0.5	4

Key: (P.m) *P. mirabilis*, (CO) Co-trimoxazole, (AC) Amoxyclav, (Ci) Ceftriaxone, (CF) Ciprofloxacin.

TABLE 5. MIC range, MIC_{50} and MIC_{90} of antimicrobial agents to *P. mirabilis*

Antibiotics	No of tested	MIC µg/ml		
		MIC range	MIC_{50}	MIC_{90}
Nitrofuratoin	N.T	N.T	N.T	N.T
Amoxycillin	N.T	N.T	N.T	N.T
Nalidixic Acid	N.T	N.T	N.T	N.T
Co-trimoxazole	8	0.5 to 4	o.5	4
Amoxyclav	8	4	4	4
Ticarcillin	N.T	N.T	N.T	N.T
Ceftriaxone	8	0.008 to 0.8	0.008	0.08
Ciprofloxacin	8	0.001 to 0.1	0.001	0.01
Amikacin	N.T	N.T	0.5	>30

Key: (N.T) Not Tested, (MIC) Minimum Inhibitory Concentration

IV. DISCUSSION

This study was conducted to determine antimicrobials resistance profile of *P. mirabilis* isolated. In this study out of two hundred and nineteen urine specimens investigated, 120 (54.7%) showed significant bacterial growth, while 99 (46.3%) showed no growth.

P. mirabilis isolated here in showed characteristics of swarming mobility on blood agar and large yellow colonies o MacConkey`s agar and were gram negative rods.

P. mirabilis was isolated and represented (4.6%) of UTI cases. This result disagree with result obtained by Farajnia, *et al.,* (4) and Sorya, *et al.,* (15) they reported that *P. mirabilis* was responsible of (8.1) of UTIs cases in Iran and (6.4%) of UTI cases in Latin America respectively.

Furthermore the result showed that the age group (up to 1 year) coast infected by *P. mirabilis* (5.5%) while the age group (more than 46 years) was the more infected by *P. mirabilis* (32%) (Table 2).

The result showed that UTI cases among females (51.1%) and males (48.9%) were almost equal in Sudan. This result disagree with the result obtained by Gerge, *et al.,* (16) who

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reported that UTI cases among females and males in North America were (79.5%) and (20.5%) respectively.

In vitro activities of antimicrobial agents showed that the resistance rate of *P. mirabilis* to amoxycillin was (100%). This result is similar to the result obtained by Raphael and Moses (2008) who found that the resistance rate of amoxicillin in Nigeria was (100%). The high resistance of *P. mirbilis* to amoxycillin may be due to production of β-lactamase enzyme.

On other hand, the result showed that the resistance rate of *P. mirabilis* to nitrofuratoin was (100%). This result is typical to the result obtained by Helio, *et al.,* (17) who found that the resistance rate of nitrofuratoin in North America was (100%).

The result showed that the resistance to co-trimoxazole was (40%). This result disagrees with the result obtained in Nigeria (12 %) by Raphael and Moses (18).

The efficiency of nalidixic acid was determined and the result showed the resistance of *P. mirabilis* to nalidixic acid was (20 %). This result is almost similar to the result obtained in Taiwan by Tsai, *et al.,* (19) who reported the resistance rate to nalidixic acid as (25 %).

Ceftriaxone as the drug of choice for UTIs and ciprofloxacin as an alternative drug were also tested. The results showed that the resistance to both was 20%. This disagrees with the results obtained by Gerge, *et al.,* (10) who found the resistance rate to ceftriaxone and ciprofloxacin in North America were (94%) and (81%) respectively.

The present study demonstrated that the resistance to amikacin was (0%). This result confirmed the result obtained by Gerge, *et al.,* (10) who reported the same resistance (0 %) to amikacin in North America.

Moreover, the result showed that resistance rate to ticarcillin and amoxiclav was (0%). This result disagrees with the result obtained by Nijssen, *et al.,* (20) who reported that the resistant rate to ticarcillin in Europa was (66.8%), but similar to the result obtained by Sita, *et al,* (21) who reported resistant rate to amoxiclav in Netherland was (0%).

Minimum Inhibitory Concentration (MIC) was evaluated by using E-test. The result showed the MICs of ciprofloxacin was (0.001 to 0.01µg/ml) and ceftriaxone was (0.008 to 0.08µg/ml). This results disagree with results obtained by Gerge, *et al.,* (10) who reported that the MIC of ciprofloxacin was (>0.06 to 16µg/ml) and ceftriaxone was (>1 to 256). Furthermore the present study determined the MIC of amoxiclav as (4 µg/ml) in agreement with Sita, *et al*., (21) who reported MIC of amikacin to be 1 to 8 µg/ml.

Also the result showed that the MICs of co-trimoxazole was (0.5 - 4 µg/ml).

This result disagrees with result obtained by Sita, *et al*., (21) who reported MIC of co-trimoxazole as (0.12 to 128).

The present study showed the MIC_{50} and MIC_{90} of ciprofloxacin was 0.001 and 0.01 µg/ml, Ceftriaxone was 0.008 and 0.08 µg/ml. This result disagree with result obtained by Gerge, *et al.* (10) who reported that the (MIC_{50} and MIC_{90}) of ciprofloxacin was >0.06 and 16 µg/ml, ceftriaxone was (>1 µg/ml each).

On the other hand, the study revealed the (MIC_{50}) and (MIC_{90}) of amoxiclav were (4 µg/ml) for each and co-trimoxazole were 0.5 µg/ml and 4µg/ml. This results disagree with results obtained by Sita, *et al*., (21) who reported the (MIC_{50}) and (MIC_{90}) of amoxiclav were (1 µg/ml) and (8 µg/ml) and co-trimoxazole were 0.12 µg/ml and 128µg/ml.

V. Conclusion and Recommendations

5.1. Conclusion

The study concluded that:

1. UTIs in young children, adults and elders were more than infant.
2. Most effective antibiotics for *P. mirabilis* was amikacin, Amoxyclav and ticarcillin due to low resistance rate (0 %).
3. Ciprofloxacin, ceftriaxone and nalidixic Acid are the second choice of antimicrobial therapy with resistance rate (20 %).
4. Co-trimoxazole is less effective antimicrobial agents due to high resistance rate (40%).
5. Nitofurantoin and amoxycillin were not effective have resistance rate (100%).
6. MIC of co-trimoxazole was (0.5 to 4 µg/ml), amoxyclav was 4, ciprofloxacin was (0.001 to > 0,01µg/ml) and ceftriaxone was (0.08 to 0.008µg/ml) are high ranges for treatment.
7. Resistance rate of *P. mirabilis* increased to commonly used antimicrobial agents.

5.2. Recommendations

1. Urine specimens should investigated for culture and susceptibility test.
2. API 20 E is a standardized system for E*nterobacteriaceae* strongly recomended in all Microbiology Laboratories.
3. MIC test is very important technique to evaluate pathogenic microorganism degree of susceptibilities and to detect specific resistance mechanism, so reference laboratories must determine MIC as reference points in the evaluation and comparison of new and existing antimicrobial agents.
4. Establishment of antimicrobial policies and treatment guidelines.
5. *Uses of* nitrofuratoin and amoxicillin as aroutine treatment for *P. mirabilis* infection should be avoided.

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