Original Article

A Microbiological Study of *Nocardia, Legionella,* and *Mycoplasma* Isolated from Lower Respiratory Tract Infections in Iraqi Patients

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Abstract

A total of 295 patients suffering from lower respiratory tract infections, including bronchitis (99), pneumonia (156), and bronchiectasis (40), were enrolled in this prospective microbiological study. The patients were of two categories: 173 were immunocompromised (with underlying diseases), and 122 were immunocompetent. Two types of clinical specimens, including sputum (216) and bronchial wash (79), were collected from these patients. Direct examination, culture, and biochemical tests were performed to test for the presence of bacteria. A total of 311 bacterial isolates and 32 fungal growths were detected. The bacterial isolates were encountered from 62.8% of the immunocompromised patients and from 37.2% of the immunocompetent patients. Out of these, 311 isolates, 17 (4.8%) were relatively uncommon pathogens of the respiratory tract, namely 11 (3.5%) were *Nocardia,* 4 (1.3%) were *Mycoplasma,* and 2 (0.6%) were *Legionella.* The *Nocardia* isolates were fully sensitive to amikacin and cotrimoxazole, and *Legionella* isolates were fully sensitive to erythromycin, azithromycin, ciprofloxacin, and levofloxacin. Also, 294 common pathogens were isolated, namely Gram-positive (49.2%), Gram-negative (33.5%), and anaerobic (11.9%) bacteria.

Key words: *Nocardia, Legionella, Mycoplasma,* respiratory tract infections.

Introduction

Certain microorganisms, notably *Nocardia, Legionella,* and *Mycoplasma,* play a significant role in respiratory tract infections (RTIs). These agents cause a wide range of diseases that are not specific in their clinical manifestations and radiological features. In addition, their isolation and determination of drug resistance are not easy tasks.[1,2] Also, these microorganisms are important pathogens in the elderly and in immunocompromised persons.[3,4]

Nocardia is an aerobic, branching, filamentous actinomycete, which fragments into a rod-shaped to coccoid element.[5] It is Gram-positive and partially acid fast and is known as an opportunistic pathogen, particularly in immunocompromised patients, e.g., AIDS patients.[6,7] *Nocardia* has been reported as a cause of variety of infections, particularly pulmonary infections.[6,8]

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Legionella was first identified in 1976 during an outbreak of upper respiratory infections at an American Legion Convention in Philadelphia, Pennsylvania.[9] Since that time it has been established that *Legionella* is an important cause of pneumonia, both in community-acquired (1-15%) and hospital-acquired (up to 50%) infections.[10,11] *Legionella* is a Gram-negative, aerobic, nonspore forming, rod-shaped microorganism. It contains branched chain fatty acids, has nonfermentative metabolism, and requires L-cysteine and iron salts for growth. *Legionella* can be cultured on special media such as buffered charcoal yeast extract (BCYEa) agar.

Mycoplasma is a small prokaryotic cell and lacks a rigid cell wall and hence has a pleomorphic form. This renders it insensitive to the action of b-lactam drugs and prevents it from staining by Gram's method.[3] Colony size varies from 200-500u, with fried-egg appearance.[3] Also, *Mycoplasma* infections can be seen in both the upper and lower respiratory tracts.

The present study identifies *Nocardia*, *Legionella*, and *Mycoplasma* and their antimicrobial susceptibility among Iraqi immunocompetent and immunocompromised patients suffering from lower respiratory tract infections.

Materials and Methods
Patients

This prospective study was conducted from April 2004 to October 2005 on 295 patients suffering from lower respiratory tract infections. There were 174 male (59.0 %) and 121 female (41.0%) subjects. The patients ranged from 21 to 80 (59.5 ± 12.1) years of age. The subjects included 122 (41.4%) apparently immunocompetent (without underlying disease) and 173 (58.6%) immunocompromised patients (with underlying disease). Immunocompromised status was suspected in patients with advanced malignancies undergoing chemotherapeutic treatment, uncontrolled diabetes mellitus type II of > 5 years duration, repeated and frequent pyogenic infections (> 5 attacks per year), and with prolonged hospitalization and patients receiving high doses of corticosteroid therapy for underlying autoimmune diseases.

Bacteriological Samples

A total of 295 bacteriological specimens were collected from patients in the Ibn Sina Teaching Hospital (respiratory care and endoscopy units) and in the Oncology and Nuclear Medicine Hospital in Mosul, Iraq. The samples consisted of 216 sputum and 79 bronchial washings (collected by bronchoscopy).

The studied lower respiratory tract infections included 99/295 (33.6%) bronchitis, 156/295 (52.9%) pneumonia, and 40/295 (13.5%) bronchiectasis (Table 1). The diagnoses were made by consultant physicians in these teaching hospitals, based mainly on clinical and radiological findings.

Isolation of Nocardia

The clinical specimens from the sputum and bronchial washings were inoculated onto the selective media including paraffin containing agar[12] and Lowenstein Jensen's "L-J"[13] medium, which were incubated at 35-37ºC for 3-28 days under 5-10% CO_2. Other culture media inoculated included sheep blood agar, chocolate agar, MacConkey's agar, Sabouraud's, tap water, and brain-heart infusion agars (Oxoid, UK). The cultures were examined daily after the third day of incubation. If no growth was obtained after 4 weeks, it was considered negative and discarded.[3,4,13]

Conventional and specific biochemical tests were used for the identification of *Nocardia*.[14]

Nocardia isolates were also tested for their ability to produce the enzyme beta lactamase using the rapid iodometric method.

Isolation of Legionella

The sputum specimens were divided into four categories: untreated, heated at 50ºC for 30 minutes, acid-treated (0.2M KCl-HCl), and diluted (1:10) in trypticase soy broth.[15] All four categories of sputum specimens and the bronchial washing specimens were inoculated on the ordinary BCYEa (Oxoid, UK) without inhibitory agents and on a selective buffered charcoal yeast extract medium BMPAa (Oxoid, UK) containing polymyxine B, anisomycin, and cefamandole and incubated at 35ºC in 5% CO_2 humid atmosphere. The cultures were examined daily after 3-5 days incubation for the presence of growth. The plates were held for a maximum of 2 weeks before being discarded as negative.[3,16,14] In addition, standard culture media, including sheep blood agar, chocolate agar, and MacConkey's agar were inoculat-

Table 1. The numbers of the three clinical conditions studied in relation to the clinical specimens examined.

| | | Number of Clinical Specimens | | | | | |
| | | Sputum | | | Bronchial Wash | | |
Clinical Entities	Total Number	Immuno-compromised	Immuno-competent	Total	Immuno-compromised	Immuno-competent	Total
Bronchitis	99	41	30	71	20	8	28
Pneumonia	156	65	53	118	21	17	38
Bronchiectasis	40	17	10	27	9	4	13
Total	295	123	93	216	50	29	79

ed to confirm the absence of growth of *Legionella* on these media.

Fixed smears were prepared from the isolated colonies and stained according to the standard Gram's technique, then counter-stained by a prepared carbol fuchsin solution for 1 minute.[17] Also, *L. pneumophila* was tested for catalase and hydrolysis of starch, gelatin, and hippurate.[16]

Isolation of Mycoplasma

Mycoplasma-specific agar (Oxoid, UK) plates were inoculated with 0.1-0.2 ml of the examined specimens from the sputum and bronchial washings and incubated in a moist atmosphere with 5% CO_2 at 35oC for up to 4 weeks. The agar surface then was observed under 40X for presence of growth.

The plates containing the suspected *Mycoplasma* colonies were flooded with Dienes stain diluted 1 in 10 in distilled water.[3] Colonies of *Mycoplasma* retained the stain for few days, while those of other bacteria lost the color in 30 minutes.

Definitive identification of *M. pneumoniae* was accomplished by overlaying agar plates showing suspicious colonies with 5% sheep erythrocytes in 1% agar prepared in physiological saline. The 1% agar was melted and cooled to 50ºC, then blood cells were added, and a thin layer was poured over the original agar surface. The plates were reincubated for 24 hours and examined for beta hemolysis around colonies of *M. pneumoniae*.

Antibacterial Susceptibility Test

The isolates were tested for their sensitivity to selected antibacterial agents using the standard disc diffusion method.[18] A sterile cotton swab soaked in the bacterial suspension was used to inoculate the organisms onto the surface of Mueller-Hinton agar plates, except *Legionella*, in which case BCYE agar was used.[19] The plates were incubated at 35ºC for 48 hours (*Nocardia*) and at 35ºC for 72 hours (*Legionella*). The resultant inhibition zone diameter for each disc was measured and compared with the control plates.[19] An antibacterial susceptibility test for mycoplasma was not done for technical reasons.

Results

Among the 295 cases studied, a total of 311 bacterial isolates and 32 fungal isolates were detected. The bacterial isolates were categorized into two main groups. The first was composed of 17 uncommon bacterial isolates that were categorized into 11 *Nocardia*, 2 *Legionella*, and 4 *Mycoplasma* species. These organisms were more commonly seen in immunocompromised patients (58.8%) than in immunocompetent patients (41.2%) (Table 2). The second group involved 294 common bacterial isolates that belong to 14 different species. Gram-positive bacteria constituted 49.2%, Gram-negative bacteria constituted 33.5%, and anaerobic bacteria constituted 11.9% of the total bacterial isolates (Table 3).

Uncommon bacterial pathogens

Eleven *Nocardia* isolates (3.7%) were identified from a total of 216 sputum, and 79 bronchial wash samples collected from patients suffering from lower respiratory tract infections. The 11 *Nocardia* isolates

Table 2. The uncommon bacteria isolated from the sputum and bronchial wash of the studied patients.

			Patients							
			Immunocompromised (N=173)				Immunocompromised (N=122)			
	Total		Sputum		Bronchial wash		Sputum		Bronchial wash	
Isolates	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Nocardia	11	64.7	3	17.7	4	23.5	1	5.9	3	17.7
Legionella	2	11.8	1	5.9	1	5.9	0	0.0	0	0.0
Mycoplasma	4	23.6	0	0.0	1	5.9	3	17.7	0	0.0
Total	17 (100%)		10 (58.8%)				7 (41.2%)			

Table 3. The microorganisms isolated from the sputum and bronchial wash of immunocompromised and immunocompetent patients.

			Patients			
	Total		Immunocompromised		Immunocompetent	
Isolates	No.	%	No.	%	No.	%
Nocardia	11	3.5	7	2.3	4	1.3
Legionella	2	0.6	2	0.6	0	0.0
Mycoplasma	4	1.3	1	0.3	3	1.0
Gram-positive bacteria	153	49.2	93	29.9	60	19.3
Gram-negative bacteria	104	33.5	70	22.5	34	10.9
Anaerobes	37	11.9	23	7.4	14	4.5
Total	311	100.0	196	63.0	115	37.0

belong to the *N. asteroides* complex group. Further differentiation of *N. asteroides* complex was achieved by complementary tests, which included growth at 35ºC and 45ºC, acid formation from rhamnose, aryl-sulfatase production, acetamide utilization, and antibiotic sensitivity to tobramycin, cefamandole, and erthromycin. Out of the 11 tested *N. asteroides*, 10 (90.9%) were able to produce the enzyme beta lacta-mase. Respiratory nocardiosis was found in 11 patients, 8 with pneumonia, 2 with bronchitis, and 1 with bronchiactasis. In most cases the patients (7/11) were > 50 years old and were immunocompro-mised. Generally, the patients presented with gener-al signs and symptoms of respiratory tract infection including fever, productive cough, dyspnea, chest pain, malaise, anorexia, night sweating, and weight loss. The radiological findings of patients with pneu-monia included nodules, lung masses, infiltrates, and lobar consolidations, while that of bronchitis were nonspecific. Dilatation of the bronchial tree and lung tissue were seen in bronchiactasis.

Two isolates of *Legionella* were detected, one from sputum and the other from bronchial wash samples (Table 2). Both isolates were obtained from two eld-erly (68 and 72 years) immunocompromised male patients. The major symptoms present in these patients were cough, fever, dyspnea, chest pain, chills, myalgia, arthralgia, nausea, vomiting, and

diarrhea. Chest X-rays showed that both patients had pneumonia in the left upper lobe.

Growth of *Legionella* on BCYE agar (ordinary and selective) appeared after 4-5 days incubation at 35-36°C in 5% CO_2 atmosphere and under humid conditions. Colonies were 3-4 mm in diameter, circular, low convex, with an entire edge, soft butyrous consistency, and gray to gray-blue color.

Biochemically, *Legionella* did not ferment carbohydrates, reduce nitrate, or produce urease, but did produce catalase, liquefy gelatin, and hydrolyze starch. The two isolates of *Legionella* were tested for hippurate hydrolysis, and both yielded a positive result.

Four isolates of *M. pneumoniae* were detected from the 295 cases studied (Table 2). Three were isolated from sputum samples of immunocompetent patients, while the fourth was from a bronchial wash specimen of an immunocompromised patient. Their ages ranged from 32 to 63 years. The oldest patient suffered from pneumonia, while the other three patients had bronchitis. The main symptoms seen in the four patients were fever, sore throat, malaise, nasal congestion, and cough.

The specimens of sputum and bronchial wash were inoculated onto the specific *Mycoplasma* agar. The plates were incubated at 35-36°C, and colonies were inspected at intervals of 3 to 5 days. Plates were held for up to 3 weeks before being discarded as negative. The colonies developed after 2 weeks incubation, appeared tiny, spherical, yellowish, and were embedded in the agar surface. Positive results were obtained for both Dienes stain and a hemolysis production test.

Antibacterial sensitivity test

The antibiogram profiles of the isolated *Nocardia* and *Legionella* are shown in Figures 1 and 2. Amikacin, cotrimoxazole, and trimethoprim showed a full sensitivity pattern against the tested 11 *Nocardia* isolates. Also, both of the two *Legionella* isolates were sensitive to azithromycin, erythromycin, levofloxacillin, and ciprofloxacillin.

Discussion

In this study three uncommon but important pathogens (*Nocardia*, *Legionella*, and *Mycoplasma*) were looked at microbiologically in lower respiratory tract infections among immocompromised and immunocompetent patients.

The 11 *Nocardia* isolates constituted 3.7% of the 295 cases examined. Comparable percentages were obtained by local studies, 2.72%[20], 0.93%[21], and 2%[22]. The relatively low isolation rates noted in most reports may be explained by technical difficulties and the relatively slow growth of *Nocardia* that often results in the culture being discarded before visualization of the microorganism.

Among the 11 *Nocardia* isolates, 7 (63.6%) were detected from immunocompromised patients, while only 4 (36.4%) were isolated from immunocompetent patients. It was reported that most of nocardiosis cases were seen in immunocompromised patients, although immunocompetent persons might be infected.

Out of the 11 *Nocardia* isolates, 8 (72.7%) were detected in males and 3 (27.3%) in females. The male-to-female ratio was found to be 2.7:1. Also, several investigators have reported that *Nocardia* infections were more frequently recognized in males than in females.[8,23] This higher incidence of nocardiosis in males may be attributed to factors such as smoking, which was more frequently seen in males (158/174; 90.8%) than in females (9/121; 7.4%) in our area. The other factor may be that male and female sex hormones differently affect the growth or virulence of *Nocardia*.[24]

The incidence of respiratory tract nocardiosis may also be age-related. It was found that most of the cases (7/11; 63.6%) were seen among patients older than 50 years of age. Four of the cases (36.4%) were seen in patients 61-70 years old, and 3 (27.3%) were detected among patients in the age group of 51-60 years. This may be explained on the basis that impairment of both humoral and cellular immunity is found with progressive aging. Other factors that could play an important role in the susceptibility of elderly individuals to nocardiosis are increased number and severity of underlying diseases or repeated hospitalizations.

The 11 *Nocardia* isolates of the present study were all found to be of the *N. asteroides* species because they were sensitive to both tobramycin and cefamandole and resistant to erythromycin. Also, all of the 11 *Nocardia* isolates showed a full sensitivity to amikacin, cotrimoxazole, and trimethoprim. This is in agreement with the results of other investigators.[22,25,26,27] They were resistant to amoxicillin, ampi-
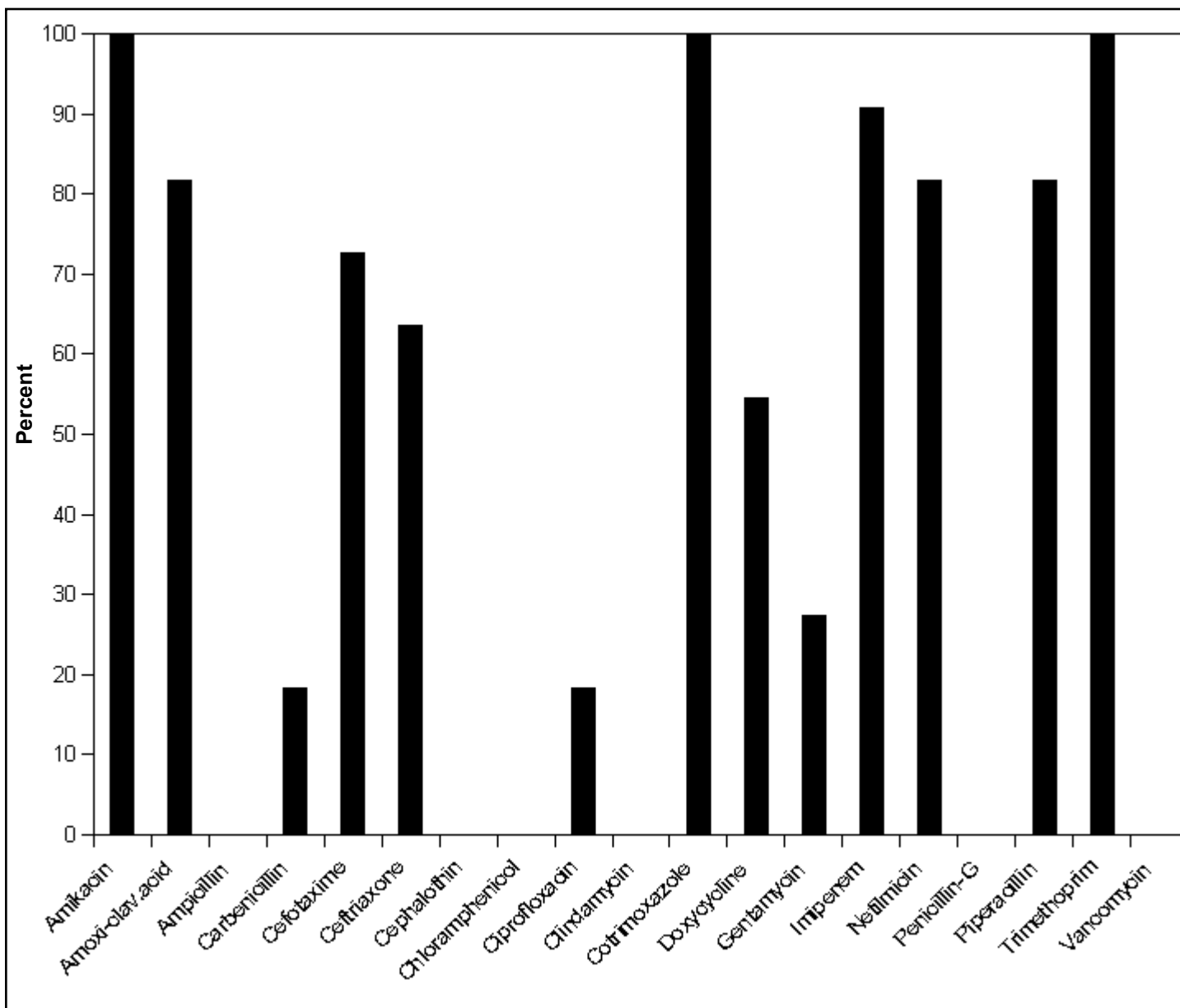
Figure 1: Antibacterial susceptibility test for the isolated *Nocardia*.

cillin, cephalothin, chloramphenicol, penicillin G, and vancomycin.[22,26,28] It was found that 90.9% of our *Nocardia* isolates were beta lactamase producers, which is in keeping with the other reports.[22,29]

The two *Legionella* isolates were obtained from two immunocompromised patients: one from sputum and the other from bronchial wash specimens. Because *L. pneumophila* is the only species in the genus that hydrolyzes sodium hippurate, it was evident that the two *Legionella* isolates detected in the present study belong to the species *L. pneumophila*. Other researchers reported that approximately 90% of *Legionella* infections were caused by *L. pneumophila*.[14,30]

In the current study the sensitivity/resistance profile of the two *Legionella* isolates was tested for nine antibacterial agents namely, azithromycin, erythromycin, levofloxacin, ciprofloxacin, doxycycline, tetracycline, cotrimoxazole, imipenem, and clindamycin. It was found that both of the isolates were sensitive to azithromycin, erythromycin succinate, levofloxacin, and ciprofloxacin. In vitro data suggest that azithromycin and many flouroquinolones have superior activity against Legionella species.[31]

M. pneumoniae is one of the most common causes of atypical pneumonia in studies from the United States and other parts of the world.[32] In the present study, 4 (1.4%) isolates of *M. pneumoniae* were detected among the 295 patients examined. Three of the

Mycoplasma isolates were detected in sputum samples of middle age (32, 43, and 48 years) immunocompetent patients complaining of bronchitis. The fourth isolate was obtained from the bronchial wash sample of an elderly immunocompromised patient suffering from pneumonia.

Due to the difficulty in culturing *Mycoplasma*, its slow growth rate, and the lack of a readily available method, antimicrobial susceptibility testing of this microorganism is neither necessary nor appropriate.[4] In addition, most *M. pneumoniae* infections are self-limiting and usually do not require treatment.

Conclusion

Nocardia, *Legionella*, and *Mycoplasma* are important but uncommon pathogens in lower respiratory tract infections that are worth studying microbiologically. Amikacin and cotrimoxazole are the best antimicrobial agents against *Nocardia*, while macrolides and quinolones are the most efficient agents against *Legionella*.

Figure 2. Antibacterial susceptibility test for the isolated Legionella.

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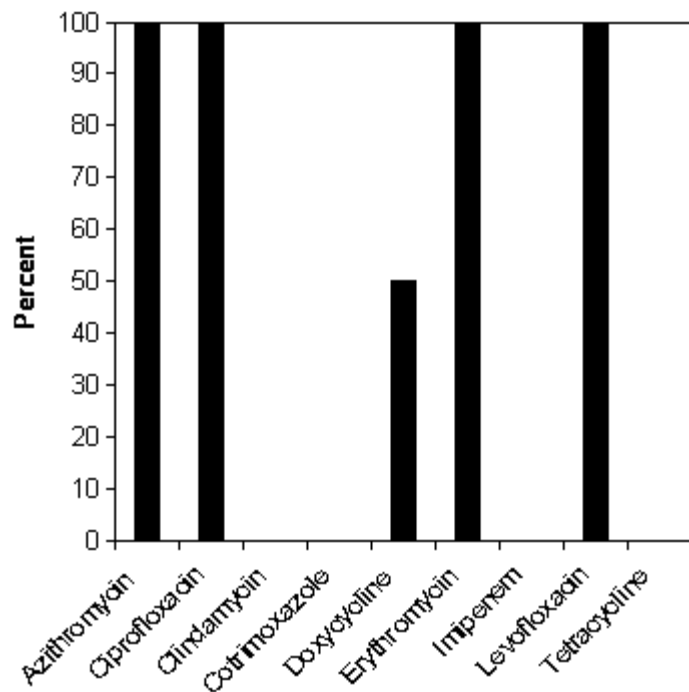8. Boiron P, Provost F, Chevrier G, et al. Review of nocardial infection in France 1987 to 1990. Eur J Clin Microbiol

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Erratum

Correction to Khan TS, Hafiz AM, Nazir B, et al. A retrospective study of sigmoidoscopic examination with rigid sigmoidoscope in a medical school sigmoidoscopy center. J Islam Med Assoc. 2008;40:76-81:

On page 80, the article incorrectly states that the patients were followed for the next 3-6 weeks. The correct time period is 3-6 months.