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Conventional and Molecular Techniques for Diagnosis of Bacterial Pneumonia

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Abstract: This study aimed to detect bacterial causes of pneumonia and antibiotics that can be used for the treatment. In addition to detect the prevalence of atypical pneumonia caused by Mycoplasma pneumoniae, Legionella pneumophila and Chlamydia pneumoniae among strains isolated from patients in different Mansoura University Hospitals, Mansoura City, Egypt, due to its importance in treatment of human infections. Fifty five clinical isolates from 88 samples were detected and identified by morphological and biochemical methods. The result of isolation showed that Citrobacter diversus (41.4%) was the most common pathogen detected in adults and Pseudomonas aeruginosa (42%) was the most common pathogen detected in children 42%. Their antimicrobial susceptibility to 17 antimicrobial agents from 6 antimicrobial categories (aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillins, glycopeptides) was determined by disk diffusion method, according to recommendation of Clinical and Laboratory Standards Institute. The highest resistance was shown to cefaclor and cefuroxime (98%) and the highest susceptibility was shown to imipenem (70%). The result of the study showed that no atypical bacteria were detected in sputum and endotracheal aspirate (ETA) of patients with pneumonia using multiplex PCR.

keywords: *Pneumonia, Atypical Bacteria, Antibiotics,PCR.*

1.Introduction

Pneumonia is a kind of severe respiratory tract infection that affects the lungs, the alveoli in the lungs become filled with pus and fluid, which reduces oxygen intake and makes breathing painful. There are many causes of pneumonia, but bacteria and viruses are the most common [1]. Bacteria that cause pneumonia have classically been classified into two groups on the basis of etiology, "typical" and "atypical" organisms. Typical organisms can be cultured on standard media or seen by Gram stain, but "atypical" organisms do not have such properties [2]. Common typical organisms include *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, Group A *Streptococcus*, and other aerobic and anaerobic Gram-negative organisms. Atypical organisms include *Legionella*, *Mycoplasma* and *Chlamydia* [3].

Diagnosis of pneumonia can be carried out in different ways. A combination of clinical, radiological and laboratory results should be present to increase the possibility of accurate diagnosis. Chest x-rays and laboratory tests can assist confirmation of diagnosis of pneumonia by obtaining certain results, such as consolidation or lung infiltration, that still need to be eligible in conjunction with the clinical picture [4]. The initial treatment for community acquired pneumonia (CAP) depends on the results of the physical examination, laboratory results, and patient characteristics (for example, age, chronic diseases, smoking history, history of illness) [5]. Antibiotics such as beta-lactam, fluoroquinolones, macrolides and doxycycline are broad-spectrum antibiotics used to treat many Gram-negative and Gram-positive bacteria that caused pneumonia [6, 7].

Antibiotic resistance is the ability of microbe to resist the effect of medication where microorganisms can survive exposure to antibiotics. The main cause of antibiotic resistance is the genetic mutation in bacteria [8], which often arises from the overuse and misuse of antibiotics inside and outside the hospital, creating conditions favorable to the emergence, spread, and persistent of resistant microorganisms [9]. The continued rapid spread of resistance mechanisms has led to the development of multidrug-resistant strains, which limits the use of antimicrobials [10].

2. Materials and methods

Bacterial strains

Clinical isolates were obtained from 88 samples from different sources (blood, ETA and sputum) from patients who were admitted to different Mansoura University Hospitals from July 2019 to January 2020.

Clinical samples were collected under aseptic conditions. These samples were cultured using the standard media (Chocolate, Blood and MacConkey's agar media) and incubated aerobically at 37 ºC overnight.

These isolates were identified based on phenotypic methods (morphology by Gram stained film, pigmentation of colony and biochemical reactions).

Antimicrobial susceptibility test

Detection of antimicrobial susceptibility in clinical isolates was done by disk diffusion method on Mueller–Hinton agar medium according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [11]. The following antibiotic disks from MAST Categories Ltd., Merseyside, UK, were used: Gentamicin (CN, 10µg), Amikacin (AK, 30µg), Imipenem (IPM, 10µg), Meropenem(MEM, 10µg), Ceftazidime (CAZ, 30µg), Cefepime (FEP, 30µg), Amoxicillin/clavulanic acid (AMC, 30µg), Cefaclor (CEC, 30µg), Cefoxitin (FOX, 30µg), Cefuroxime (CXM, 30µg), Ciprofloxacin (CIP, 5µg), Levofloxacin (LEV, 5µg), Piperacillin-tazobactam (TPZ, 110µg), Vancomycin (VA, 30µg), Ceftriaxone (CRO, 30µg), Cefoperazone/sulbactam (CES, 105µg), Cefoperazone (CEP, 75µg).

Molecular study on atypical bacteria:

Fifty bacterial isolates were used to detect the presence of atypical pneumonia caused by *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydia pneumoniae* using multiplex PCR.

Bacterial DNA Extraction:

Genomic DNA extracts of atypical bacteria to be used as templates in this study was done by G-spinTM Total Scientific kits in accordance with the manufacturer's recommendations. Genomic DNA was stored at -20^{o}C until used.

Primers and multiplex PCR:

The presence or absence of atypical bacteria was detected by multiplex PCR reaction using primers as summarized in table (1) [12]. The reaction mixture (25µl) contained 10 µl of master mix, 1 µl forward primer and 1 µl reverse primer, 5 µl extracted DNA and 4 µl of nuclease free water. The samples were gently vortexed, the PCR tubes were centrifuged for few seconds in a micro centrifuge. Amplification was carried out using thermal Controller (MJ Research, INC., USA). After first denaturation at 94°C for 10 minutes, the reaction was subjected to 36 cycles. Each cycle composed of denaturation at 94°C for one minute, annealing at 60°C for one minute and elongation at 72°C for 90 seconds followed by eventual extension at 72°C for 10 minutes. Then the product was kept at 4°C. Amplified products were visualized on 2% agarose gel stained with ethidium bromide under UV light [13].

3. Results and Discussion

Table (2) recorded the morphological and biochemical characteristics of bacterial isolates.

The results of isolation of 88 clinical samples, showed that *Citrobacter diversus* (19.3%) was the most common pathogen detected followed by *P. aeruginosa* (13.6%), *Klebsiella pneumoniae* (8%), *Enterobacter agglomerans* and *Candida* sp. (5.7%), *E. coli* (4.5%), *Proteus vulgaris* (3.4%), Vancomycin-resistant *Staphylococcus aureus* (VRSA) and *Streptococcus* sp. (1.1%). Table (3) and table (4) recorded the distribution of isolated microorganisms in children and adults

respectively among different clinical samples

Table (1): Primer sequences for PCR assays

Organism	Primer Sequence	Product size (bp)
C.pneumoniae	F5'-GTTGTTCATGAAGGCCTCT-3' R5'-TGCATAACCTACGGTGTGTT-3'	437
M.pneumoniae	F5'-AGG GTT GAT AGG TTA AGA GC-3' R5'-CCA ACA GCT AGT TGA CATCG-3'	386
L.pneumophila	F5'-TCAATCTGGCGTGGATCTCT-3' R5'-GTCACTGGTTAAACGGACTA-3'	180

Table (2): Morphological and biochemical characteristics of bacterial isolates

Bacterial isolate	Morphological characteristics			Biochemical characteristics								
	Gram stain	Cell shape	Arrangement	Hydrogen sulfide production	Lysine decarboxylase	Lysine deaminate	Indole	Ornithine decarboxylase	Citrate	Urease	Catalase test	Oxidase test
P. aeruginosa	Gram negative	Bacilli	Pairs or single short rods	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-	+ve
Staphylococcus aureus	Gram positive	Cocci	Grape-like clusters	-	-	-	-	-	-	-	+ve	-ve
Streptococcus sp.	Gram positive	Cocci	A chain of round cells that may appear bent or twisted	-	-	-	-	-	-	-	-ve	-ve
Proteus vulgaris	Gram negative	Bacilli	Pairs or single short rods	+ve	-ve	+ve	+ve	-ve	-ve	+ve	-	-
Klebsiella pneumoniae	Gram negative	Bacilli	Pairs or single short rods	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-	-
Citrobacter diversus	Gram negative	Bacilli	Pairs or single short rods	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-	-
Enterobacter agglomerans	Gram negative	Bacilli	Pairs or single short rods	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-	-

Table (3): Distribution of isolated microorganisms in children among different clinical samples

Isolated microorganism	Samples						Total	
	Blood		ETA		Sputum			
	NO.	%	NO	%	NO	%	NO	%
Klebsiella pneumoniae	0	0	5	19	0	0	5	19
Citrobacter diversus	0	0	5	19	0	0	5	19
E. coli	0	0	1	4	0	0	1	4
VRSA	0	0	1	4	0	0	1	4
P. aeruginosa	0	0	11	42	0	0	11	42
Enterobacter agglomerans	0	0	2	8	0	0	2	8
Candida sp.	0	0	1	4	0	0	1	4
Total	0	0	26	100	0	0	26	100

Table (4): Distribution of isolated microorganisms in adults among different clinical samples

Isolated microorganism	Samples						Total	
	Blood		ETA		Sputum			
	NO.	%	NO.	%	NO.	%	NO.	%
Klebsiella pneumoniae	0	0	0	0	2	6.9	2	6.9
Citrobacter diversus	0	0	6	20.7	6	20.7	12	41.4
E. coli	0	0	0	0	3	10.3	3	10.3
Proteus vulgaris	0	0	2	6.9	1	3.4	3	10.3
P. aeruginosa	0	0	0	0	1	3.4	1	3.4
Enterobacteragglomerans	0	0	0	0	3	10.3	3	10.3
Streptococcus sp.	0	0	0	0	1	3.4	1	3.4
Candida sp.	0	0	0	0	4	14	4	14
Total	0	0	8	27.6	21	72.4	29	100

Table (5) recorded the data of antimicrobial susceptibility of 50 isolates against 17 agents from 6 antimicrobial categories. The highest resistance was shown to cefaclor and cefuroxime (98%) and the highest susceptibility was shown to imipenem (70%).

Table (5): Antimicrobial susceptibility of 50 isolates from patients in different Mansoura University Hospitals, Egypt.

Antimicrobial categories	Antimicrobial Agents (Antibiotics)	Symbol	Resistance (R)		Intermediate (I)		Susceptible (S)	
			No.	%	No.	%	No.	%
Aminoglycosides	Amikacin	AK	23	46	3	6	24	48
Carbapenems	Imipenem	IPM	12	24	3	6	35	70
	Meropenem	MEM	39	78	0	0	11	22
Cephalosporins	Ceftazidime	CAZ	45	90	3	6	2	4
	Cefepime	FEP	10	20	0	0	5	10
	Gentamicin	CN	34	68	3	6	13	26
	Cefaclor	CEC	49	98	0	0	1	2
	Cefuroxime	CXM	49	98	0	0	1	2
	Cefoperazone/sulbactam	CES	8	16	1	2	6	12
	Cefoperazone	CEP	39	78	3	6	8	16
	Ceftriaxone	CRO	41	82	3	6	6	12
	Cefoxitin	FOX	1	2	0	0	1	2
Fluoroquinolones	Ciprofloxacin	CIP	8	16	1	2	6	12
	Levofloxacin	LEV	3	6	1	2	11	22
Penicillins/ß-lactamase Inhibitors	Piperacillin- tazobactam	TPZ	4	8	11	2	101	20
	Amoxicillin/clavulanic acid	AMC	42	84	5	10	3	6
Glycopeptides	Vancomycin	VA	1	2	0	0	1	2

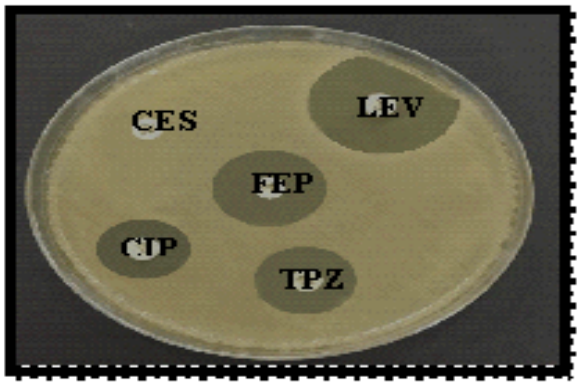

Fig. (1): P. aeruginosa isolate

P. aeruginosa sample in fig. (1) susceptible to IPM and intermediate resistance to AK and resistance to MEM, CAZ, CN, CXM, CRO, CEC, CEP and AMC

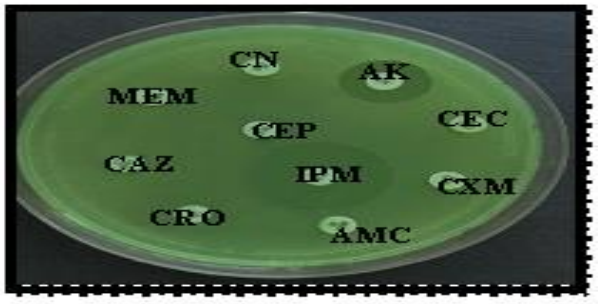

Fig. (2): *Enterobacter agglomerans* isolate

Enterobacter agglomerans sample in fig. (2) susceptible to LEV, FEP, TPZ, CIP and resistance to CES

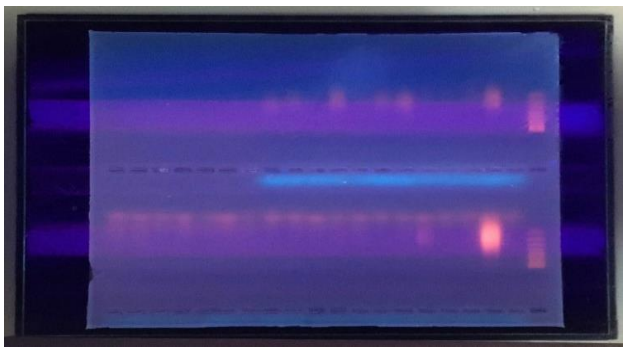

Fig. (3): Multiplex PCR for atypical bacteria

Detection of atypical bacteria:

Fifty bacterial isolates obtained from sputum and ETA were used to detect the presence of atypical pneumonia caused by Mycoplasma pneumoniae, Legionella pneumophila and Chlamydia pneumoniae using multiplex PCR. The results of multiplex PCR, showed that all sputum and ETA samples did not contain atypical bacteria as showed in fig. (3).

Discussion

In this study, the antimicrobial susceptibility of 50 bacterial isolates against 17 agents from 6

antimicrobial categories was determined. Altogether, the highest susceptibility was shown for carbapenems (imipenem 70%). Results of this study showed that Pseudomonas aeruginosa was the most common pathogen detected in ETA culture in positive cases of children 42%, followed by Citrobacter diversus and Klebsiella pneumoniae 19%, Enterobacter agglomerans 8%, E.coli, VRSA and Candida 4%. This is almost similar to study conducted by Ashkenazi-Hoffnung et al. [14] who found that the most common pathogen was Pseudomonas aeruginosa, 51%, followed by Klebsiella sp. 24.5%, Enterobacter sp. 15.5%, E. coli 13.6%, Citrobacter sp. 2% and Staphylococcus aureus 11%**.** Citrobacter diversus was the most common pathogen detected in ETA and sputum culture in positive cases of adults 41.4%, followed by Candida 14%, Enterobacter agglomerans, Proteus vulgaris and E.coli 10.3%, Klebsiella pneumoniae 6.9%, Streptococcus sp. and Pseudomonas aeruginosa 3.4%. This is similar to study conducted by Villafuerte et al. [15] who found that the most frequently isolated pathogens were K. pneumoniae (56%), followed by E. coli (28%), Enterobacter spp. (13%), Proteus spp. (4%) and Serratia spp. (2%).

Results of this study show that no atypical bacteria were detected by multiplex PCR and this result is close to that of El Basha et al. [16] who found that multiplex PCR reaction was positive for atypical bacteria in 12 (3%)

out of 400 patients, 8/400 (2%) were positive for Bordetella pertussis, and 4/400 (1%) were positive for Mycoplasma pneumoniae. None were positive for either C. pneumoniae or Legionella pneumophilia. In another study by Mokhless et al. [17] who reported that PCR reaction was positive foratypical bacteria in 9 (15%) out of 60 samples, five were positive for Mycoplasma, three for Legionella, and one was positive for Chlamydia.

Conclusion

Sputum and ETA samples were the commonest samples giving positive result than blood samples for identification of clinical isolates. P. aeruginosa was the most common pathogen detected in children while Citrobacter diversus was the most common pathogen detected in adults. The highest antibiotics resistance of isolated microorganisms was shown to cefaclor and cefuroxime and the highest susceptibility was shown to imipenem. No atypical bacteria were detected by multiplex PCR.

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4. References

1. UNICEF. (2012). Pneumonia and Diarrhea: Tackling the Deadliest Diseases for the World's Poorest Children. New York: United Nations Children's Fund.
2. Çalık, Ş., Arı, A., Bilgir, O., Cetintepe, T., Yis, R., Sonmez, U. and Tosun, S. (2018). The relationship between mortality and microbiological parameters in febrile neutropenic patients with hematological malignancies. *Saudi Medical Journal*. **39 (9)**: 878.
3. Sattar, S.B.A. and Sharma, S. (2019). Bacterial Pneumonia. In StatPearls [Internet]. Treasure Island (FL). StatPearls Publishing.
4. Unicef and WHO. (2006). Pneumonia: the forgotten killer of children. Geneva: World Health Organization.
5. Fine, M.J., Auble, T.E., Yealy, D.M., Hanusa, B.H., Weissfeld, L.A., Singer, D.E., Coley, C.M., Marrie, T.J. and Kapoor, W.N. (1997). A prediction rule to identify low-risk patients with community-acquired pneumonia. *The New England journal of medicine.* **336(4):** 243–250.
6. Mandell, L.A., Wunderink, R.G., Anzueto, A., Bartlett, J.G., Campbell, G.D., Dean, N.C., Dowell, S.F., File, T.M., Jr, Musher, D.M., Niederman, M.S., Torres, A., Whitney, C.G., (2007) Infectious Diseases Society of America, & American Thoracic Society. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired

pneumonia in adults. Clinical infectious diseases. 44(Supplement_2): S27–S72.
7. Mbata, G.C., Chukwuka, C.J., Onyedum, C.C. and Onwubere, B.J. (2013). The CURB-65 scoring system in severity assessment of Eastern Nigerian patients with community-acquired pneumonia: a prospective observational study. *Primary Care Respiratory Journal*. **22(2)**: 175-180.
8. Laxminarayan, R. (2003). Battling resistance to antibiotics and pesticides: An economic approach. Washington, DC: Resources for the Future.
9. Bonten, M.J., Austin, D.J. and Lipsitch, M. (2001). Understanding the spread of antibiotic resistant pathogens in hospitals: mathematical models as tools for control. Clinical Infectious Diseases. **33(10)**: 1739-1746.
10. Kaye, K.S., and Pogue, J.M. (2015). Infections caused by resistant Gram-negative bacteria: epidemiology and management. Pharmacotherapy: The *Journal of Human Pharmacology and Drug Therapy*. **35(10)**: 949-962.
11. CLSI. (2013). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. CLSI document M100–S23. Wayne, PA: Clinical and Laboratory Standards Institute.
12. Wellinghausen, N., Frost, C. and Marre, R. (2001). Detection of legionellae in hospital water samples by quantitative real-time LightCycler PCR. Applied and environmental microbiology. **67(9)**: 3985–3993.
13. Akter, S., Khatun, R. and Shamsuzzaman, S.M. (2015). Molecular detection of atypical microorganisms in patients with ventilator associated pneumonia. *Ibrahim Medical College Journal*. **9(1)**: 22-25.
14 Ashkenazi-Hoffnung, L., Ari, A., Bilavsky, E., Scheuerman, O., Amir, J. and Prais, D. (2016). Pseudomonas aeruginosa identified as a key pathogen in hospitalised children with aspiration pneumonia and a high aspiration risk. Acta paediatrica. **105(12)**: e588-e592.
15 Villafuerte, D., Aliberti, S., Soni, N.J., Faverio, P., Marcos, P.J., Wunderink, R.G., Rodriguez, A., Sibila, O., Sanz, F., Martin-Loeches, I., Menzella, F., Reyes, L.F., Jankovic, M., Spielmanns, M., Restrepo, M.I., and GLIMP Investigators (2020). Prevalence and risk factors for Enterobacteriaceae in patients hospitalized with community-acquired pneumonia. Respirology (Carlton, Vic.). 25(5): 543–551.
16 Mokhless, N.A., El-Mofty, M.F., Hanafi, N.F., Fayed, A.M. and Asser, S.L. (2010). Atypical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience. *Journal of American Science.* **6(12**): 1074-1079.
17 El Basha, N.R., Shaaban, H.H., El Atroush, H.A., Sherif, M.M. and El Kholy, A.A. (2019). The use of multiplex PCR for the detection of atypical pathogens in Egyptian children with CAP: a high rate of *Bordetella pertussis* in early infancy. The *Journal of the Egyptian Public Health Association.* **94(1)**: 5.