Annals of Clinical and Medical Case Reports

Research Article

ISSN 2639-8109 | Volume 7

Rapid Diagnosis of Acute Respiratory Infections by Multiplex Endpoint PCR Technology

Alexandru Giubelan[1], Aurelian Udristioiu[1*] and Nica-Badea Delia[2]

[1]Medicine Faculty, Titu Maiorescu University, Bucharest

[2]Constantin Brancusi University, Faculty of Medical Science and Behaviors, Targu Jiu, Romania

[*]Corresponding author:

Aurelian Udristioiu,
Department of Molecular Biology, Titu Maiorescu University of Bucharest, Damboviciului Street No: 2, Postal Code 040051, District 4, Bucharest, Romania, Tel: 40723565637;
E-mail: aurelianu2007@yahoo.com

Received: 26 Oct 2021
Accepted: 12 Nov 2021
Published: 17 Nov 2021
J Short Name: ACMCR

Keywords:

Antibiotic resistance; PCR; Gene; DNA; Resistance markers; Microbiology report

1. Abstract

Introduction: The multiplex endpoint PCR technology offers a number of potential advantages, results are available in a matter of hours rather than days, the extreme sensibility facilitates de- tection of even minutes the amounts of pathogen DNA in clinical samples and the test is not significantly affected by prior adminis-tration of antibiotics.

Aim: The aim of this work was to rapidly identify the antibiotic resistance the monitoring of pathogen growth at the patients admitted in Hospitalization Intensive Care Unit, with the diagnosisof Community Acquired Pneumonia, (CAP).

Method: The Analyzer Unyvero™ Pneumonia Application was used in detection of pneumonia associated pathogens and their antibiotic resistance genes using the Pneumonia Unyvero™ System, following PCR pathogen species with sequencing of the amplified microbial DNA.

Results: The main pathogens of community acquired pneumonia from the cohort study, 36 cases, (20 males in mean age 35- 66 years and 16 females in mean age 40-55 mean years), were Streptococcus pneumonia, (16 cases), Staphilococcus aureus, (10 cases), Klebsiella pneumonia (5 cases) and other important agents were “atypical”, such as Haemophilus Influenzae, Chlamidophi- la pneumonie and Moraxela cataralis. A case with Acinetobacter baumani and Proteus Sp. was also widely resistance to mefA gene / ermB gene as all cases of analyzed. The more frequency of genes resistant (29 cases) are ermA gene / ermC / ermB for Staphilococcus aureus and the gene tem+shv / gene / ctx-M with the Chromosomal mutation (7 cases), as gyrA83_87 Ecoli / Pseu for Klebsiella

pneumonia agents.

Also, most resistance antibiotics were Makrolides, (29 cases and Lincosamides (6 cases) and these cases have had the chromosomial integrates. The most resistance microbe, Pseudomonas aeruginosa (1 case), has been registered as multi drugs resistance [MDR]*.

Conclusion The Unyvero™ results have been available 2 days be- fore the primary microbiology report and 3 days before the final confirmation results, obtained by microbiology culture. The Un- yvero Analyzer only provides rapid data to support the therapeuticdecision of currant medic.

2. Introduction

The high number of severe acute infections from the whole world raised concerns about growing antibiotic resistance as a major bur- den for today`s health care system. While microbiological culture is likely to remain a gold standard for infection diagnosis, there is growing interest at the potential of PCR technology to provide early, time critical information based on detection and recognition of bacterial or fungal pathogen DNA. By this new modern method, the results are chosen in perquisite for giving adequate antibiotics treatment as early as possible in order to improve the standard of care.

Incorporating the new multiplex molecular analysis, these tech- nologies can combine pathogens identification and antibiotics sensitivity, testing in a single automated procedure within hours after collecting the patient sample. The focus of researches now is to move beyond detecting single analyte to multiplex targets and detect more pathogens from a single specimen, ex. Sputum [1].

Principle of the Analysis

The Unyvero™ System Pneumonia Application automates gave us presentations and discussed in latest information on pneumonia diagnosis. The analyzer has integrates in a disposable cartridge, genomic DNA purification, eight parallel multiplex end-point PCR reactions and the qualitative detection of the target amplicons after hybridization onto an array.

Technique

1.The patient sample is pipetted into the Unyvero™ Sample Tube using the Unyvero™ Sample.

2.Transfer Tool, closed with the Unyvero™ Sample Tube Cap, and lysed with the Unyvero™ Lysator.

3.Subsequently, the Unyvero™ Sample Tube and the Unyvero™ Master Mix Tube are inserted into the Unyvero™ Pneumonia Cartridge.

4.The Unyvero™ Pneumonia Cartridge is then inserted into the Unyvero™ Analyzer, which processes it automatically.

The supplied software guides the user through the entire work flow. A bar code reader allows the entry patients data, checks the shelf-life of consumables and stores their lot numbers. The full analysis should take approximately 30 minutes. The analysis of the patient samples is shown by grey test bars on the overview screen. To view the results, tap on the corresponding blue test bar.

A screen opens and shows the following buttons: Summary, Microorganisms, Resistance Markers, information. In the middle of the screen, the respective antibiotic classes for which a therapeutic failure must be considered if they were administered are displayed. On the right side of the screen, the common microbial source of the resistance markers is displayed.

Detection Limits

Following PCR, pathogen species presented can be identified sequencing of the amplified DNA. Detection limits for each pathogen were determined with pathogen dilutions in buffer. At the concentration of 106 pathogens / mL all analytes are detected with the Unyvero™ P50 Pneumonia Cartridge. In addition, the majority of the analytes are positively at a concentration of 104 pathogens / Ml (S. marcescens. S. maltophilia, A. baumannii, L. pneumophila, S. aureus, M. morganii, K. pneumoniae, K. oxytoca, P. aeruginosa).

Interfering Substances

Interferences were tested in suitable pools with respiratory drugs, common antibiotics and sample media or individually for example for lyse buffer, blood, human DNA, and common respiratory pathogens, which might be present in respiratory samples. Worst case concentrations were used according to CSLI guideline "EP7-A2. No interference was observed.

Sensitivity & Specificity

The Unyvero™ Pneumonia Application achieved an overall sensitivity of 75.5 % (sensitivity per analyte between 50% and 100%, depending on the microorganism) at an overall specificity of 95.2% (72.3% to 100%, depending on the microorganism). For rare pathogens, the number of cases was insufficient to establish sensitivity and specificity data. For detected resistance markers (genes like mefA, ermA, ermB, ermC, tem, shv, dha, oxa 51 like, ctx M, mecA, ebc, quinolone resistance in E. coli and P. aeruginosa) in 36 cases out of 32 antibiotic resistant pathogens a correlation between Unyvero P50™ results with the antibiogram was demonstrated. Actually, the firm Curetis is currently conducting a prospective European multicenter clinical trial to generate more clinical performance data.

3. Method

Sample type, aspirates sputum from the cohort study, 36 cases, (20 males in mean age 35-66 years and 16 females in mean age 40-55 mean years), patents admitted in Hospitalization Intensive Care Unit with the diagnosis of Community Acquired Pneumonia, (CAP), were performed on the Analyzer Unyvero™ Pneumonia Application, in the day after specimen collection, in the department of Microbiology, from Clinical Laboratory Analyses of Emergency County Hospital. The selection of the samples at the patients admitted in Intensive Care Unit (ICU) with community acquired pneumonia were based on typical clinical signs of severe infection, in evidences of clinician doctors which were included the symptoms such as increased fever, positive X-ray, presence of purulent sputum and on the results of laboratory blood samples with increased white blood cell count, (>25000/mm³), VSH >40 mm/h), Fibrinogen (>550 mg/dl) and Protein C Reactive (>12 mg/dl). These signs of severe acute infection were primordially for faster results in pneumonia testing. Such quick results from laboratory are perquisite for giving adequate antibiotic treatment as early as possible in order to improve the standard of care.

4. Results

The main pathogens of community acquired pneumonia from the cohort study were Streptococcus pneumonia (16 cases), Staphylococcus aureus, (10 cases), Klebsiella pneumonia (5 cases), and other important agents were "atypical", such as Haemophilus Influenzae, Chlamidophila pneumonie and Moraxela cataralis. A case with Acinetobacter baumani and Proteus Sp. was also widely resistance to mefA gene / ermB gene as all cases of analyzed. The more frequency of genes resistant (29 cases) are ermA gene / ermC / ermB for Staphilococcus aureus and the gene tem+shv / gene / ctx-M with the Chromosomal mutation (7 cases), as gyrA83_87 Ecoli / Pseu for Klebsiella pneumonia agents.

Also, most resistance antibiotics were Makrolides, (29 cases and Lincosamides (6 cases) and these cases have had the chromosomial integrates. The most resistance microbe, Pseudomonas aeruginosa, (1 case), has been registered as multi drugs resistance [MDR]*, (Table 1).

Table 1. Resistance markers of the Pneumonia panel and the resulting antibiotic resistance.

No. IDL	Microorganisms detected	Antibiotic resistance	Gene resistance
1310_1	Klebsiella pneumonia	Makrolides /], [ermB],].Lincosamides	ermB gene
1310_2	Streptoccocus Sp.	Makrolides.	ermB gene/tem gene
1310_3	Staphilococcus aureus Other/Fungi: Chlamidophila pneumonie	Penicilins (tem)	ermB gene/tem gene Chromosomal mutation; Pseud. aeruginosa, (gyrA83-Ecoli)
1310_4	Klebsiella pneumonia	Makrolides / Lincosamides	ermB gene
1310_5	Proteus Sp. Other/Fungi: Haemophilus Influenzae, Chlamidophila pneumonie	Makrolides Oxacilin	ermB gene/oxa51 Chromosomal mutation; Escherichia Coli (gyrA83-87_Ecoli).
1311_1	Streptococcus pneumonia Moraxela cataralis	Makrolides, Oxacillin Lincosamides,	ermB gene/tem gene/ mecA gene
1311_2	Streptococcus pneumonia	Makrolides / [mefA], [ermB],].Lincosamides Penicilins (tem)	mefA gene / ermB gene / tem
1311_3	Streptococcus pneumonia Pseudomonas aeruginosa	Makrolides / [mefA], [ermB],].Lincosamides [MDR]* [int1].	mefA gene / ermB gene [int1gene].
1311_4	Klebsiella pneumonia	Penicilins (shv)	mefA gene / shv gene [int1gene] /sul 1 gene Chromosomal mutation; (gyrA83_3Pseu).
1311_5	Streptococcus pneumonia Acinetobacter baumani	Makrolides / [mefA], [ermB],].Lincosamides	mefA gene / ermB gene
1312_1	Staphilococcus aureus Klebsiella pneumonia	Makrolides / [mefA], [ermB],].Lincosamides 3rd Gen Cephalosporins [tem+shv], [ctx-M].	ermA gene / ermC / ermB tem+shv / gene / ctx-M g Chromosomal mutation; gyrA83_87 Ecoli / Pseu

4. Interpret Results

The green boxes on the Analyzer of the Unyvero™ Pneumonia Application and values do loosely correlate with the amount of detected DNA and therefore with the number of pathogens in a given patient sample, however the number of pathogens obtained by culture does not always correlate with the number of pathogens in a sample due to limitations of growth. These numbers are reflecting a threshold value depending on the species and serve to give an aid to quantification. (< 250 no green box; 250 – 499 one green box, 500 – 999 two green boxes, >= 1000 three green boxes). It was a specific customer demand to have some form of number. The clinician may still take this data into consideration e.g. in an immune compromised patient and with certain pathogens. Still, a clinician may take this data into consideration e.g. in an immune compromised patient and with certain pathogens. These results were confirmed by microbiology culture; however, the final microbiology results were available 3 days after the Unyvero™ results [2].

The Classical Method Kirby Bauer Disc Diffusion Method

As an example, in the Culture 1310_1 raised Klebsiella pneumoniae (+++) and sensible antibiotics were Amoxicillin, Sulfonamide Cotrimoxazol, Quinolone Moxifloxacin, Monobactame Aztreonam, Chephalosporin Cefotaxim and Ampicillin resistance, (R), Amoxic./Clavulanacid(R), Piperacillin (R) , Piperac/Tazobactam (R), resistance spread especially on our intensive care unit.

5. Discussions

The Unyvero™ Pneumonia Cartridge can detects the following microorganisms: Acinetobacter baumannii, Chlamydophila pneumoniae, Enterobacter sp., Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Klebsiella oxytoca, Legio-

nella pneumophila, Moraxella catarrhalis, Morganella morganii, Pneumocystis jirovecii, Proteus sp., Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, Stenotrophomonas maltophilia und Streptococcus pneumoniae. Simultaneously, the following genes associated with antibiotic resistance are detected in the same cartridge: ctx-M, (Cephalosporins, Penicillins), dha (Cephalosporins), ebc (3rd Gen. Cephalosporins), ermA(Macrolides / Lincosamides), ermB (Makrolides / Lincosamides) , ermC (Macrolides / Lincosamides) , gyrA 83, (E. coli), gyrA87, int1, kpc, mecA, mefA/E, msrA, oxa51 like, parC, shv, sul1, and tem (Fluoroquinolones, E. coli) [3]. In our cases from the study, the main pathogens of community acquired pneumonia are Streptococcus pneumoniae, and Haemophilus influenzae but inappropriate therapy is linked to the presence of resistance organism such as Pseudomonas aeruginosa, Staphylococcus aureus and Acinetobacter. In Streptococcus pneumoniae resistance to macrolides and tetracycline depends largely on acquired genes, as doses tetracycline and trimethoprim resistance in Haemophilus influenza whereas, in both species, quinolone resistance is a mutation. In case of S.aureus methicillin resistance, (MRSA), is determined by the acquired mecA gene which is easy detected by multiplex endpoint PCR technology, respectively Unyvero™ Pneumonia Analyze. A few resistances in Enterobacteriaceae are largely or entirely mutational, the best example being quinolone resistance, which mostly occurs by mutation of gyrA genes, like in the present study Acinetobacter baumannii acquires antibiotics resistance by o number of mechanisms. Resistance is often associated with acquired genes, with these various compromising penicillins, aminoglycosides, tetracyclines, antifolates and chloramphenicol. In some cases these genes are carried on plasmids, as in Enterobacteriaceae, in others they are chromosomally integrated, often in large" resistance islands". Resistance to carbapenems may involve acquired carbapenems genes, but can also arise by activating "bla gene" which occurs in all A. baumannii isolates but is not ordinarily expressed. In Pseudomonas aeruginosa (1 case), the detection of multi drugs resistance [MDR]*, was achieved by two family of plasmid encoded ampC genes and by three classical Amber class A beta-lactamase (tem, shy, ctx-M).

Transferable Antibiotics Resistance (Resistance Genes)

Most resistance markers that are detected by the Unyvero™ Pneumonia Application are genes, which are transferred by mobile genetic elements like plasmids or integrons 6. Most resistance in Enterobacteriaceae is associated with acquired resistance genes often carried by plasmids, variously encoding β-lactamases, aminoglycosides, modifying enzymes, r-RNA methylases or efflux pomp. Presence of such a gene correlates with a resistance against a particular antibiotic class7 [4].

Gene acquisitions mutations and gene of mosaic are all important. The general pattern is that resistance mostly arises by gene acquisition in Enterobacteriaceae [5]. In order to control the spread of resistance it is important to have an understanding of the molecular biology of the different mobile genetic elements and of the ecology of the environments in which spread is likely [6].

Antibiotic resistance occurs by different mechanisms in different pathogens. Mutational changes conferring important resistances can include reduced cytoplasmic membrane transport, resulting in resistance to aminoglycosides, up-regulation of efflux, compromising antibiotics depending on particular pumps affected, DNA mutation resulting in resistance to quinolones [7]. Modification of enzymes is major aminoglycoside resistance mechanism.

According to this modification, the involved proteins are classified into the 3 major classes of acetyltransferases (AACs), nucleotidyltransferases, and adenyltransferases (ANTs). Macrolides share a similar mode of antibacterial action with, and have comparable antibacterial spectra as, the antibiotic classes of streptogramins B and lincosamides. As a result, despite the fact that they are chemically distinct, these antibiotics have been described as the single class of macrolide-lincosamide-streptogramin B (MLS) antibiotics [8].

Resistance to β-lactam antibiotics involve a complex mixture of mutation and mosaic gene formation in Streptococcus peumopnie. Also, β-lactam antibiotics inhibit cell-wall synthesis by binding to penicillin-binding proteins (PBPs) within bacteria and by interfering with structural crosslinkage of peptidoglycans. These actions prevent terminal transpeptidation in the cell walls of bacteria.

Staphilococcus meticilin (MLS) resistance occurs because of the presence of r-RNA methylases, encoded by ERM genes. Inactivating genes and efflux pumps which constitute the other 2 mechanisms for this result, are encoded by ERE and MSR determinants, respectively [9, 10]. Also, in environments in which bacteria reside closely together and in relatively high density, such as the oral cavity and the gut, gene transfer is more likely. To control the spread of resistance, laboratory professionals must comprehend the molecular biological characteristics of the ecology of the environments in which spread is likely and of the different mobile genetic elements [11].

The Unyvero™ result was available 2 days before the primary microbiology report and 3 days before the final classical culture method, confirmation test. A more adequate and result guided antibiotic therapy regime with the usage of an ESBL active carbapenem would have been made possible much earlier. In addition, appropriate hygiene measures could have been taken earlier decreasing the risk of antibiotic [12]. The carbapenen resistance in emerging in Klebsiella pneumonia involves the genes Oxa-48, Pseu, detectable by PCR [13]. The isolates were identified by conventional methods [14]. All isolates were non duplicate. Escherichia coli ATCC 25922 was used as quality control strain.

In the present studies, the majority of the isolates (95%) from bronchial secretions were carbapenem resistant. High resistance rates to carbapenems have been observed in previous studies, ranging from 75% to 100% for imipenem and from 61% to 77% for meropenem [15]. The emergence of A. baumannii strains with increased carbapenem resistance in this area of the world may be due to the extensive misuse of carbapenems [16]. The most prevalent mechanism of carbapenem resistance in A. baumannii is the enzymatic degradation by carbapenem-hydrolyzing b-lactamases, MBL, mostly VIM and IMP, has been reported sporadically in some parts of the world [17].

6. Conclusions

Rapid molecular diagnosis assays has the potential to speed up pathogen and resistance identification, which may enable clinicians to make an early, informed diagnosis in patients with pneumonia. The Unyvero only provides data to support the therapeutic decision. While microbiological culture is likely to remain a gold standard for infection diagnosis, there is growing interest at the potential of PCR technology to provide early, time critical information based on detection and recognition of bacterial pathogen DNA. The most important feature in our cases of this study, is the correct detection for Klebsiella pneumoniae and A. baumanni, these results potentially would have had great impact on the antibiotic therapy regime as well as hygiene measures, patient isolation and preventing of nosocomial infections.

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