Rapid Diagnosis by Multiplex PCR Technology at Patients with Acute Respiratory Infections

Aurelian Udristioiu, Manole Cojocaru

Introduction: PCR offer a number of potential advantages, results are available in a matter of hours rather than days, the extreme sensibility facilitates detection of even minutes the amounts of pathogen DNA in clinical samples and the test is not significantly affected by prior administration 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 of Emergency County Hospital Targu Jiu starting in December/2013. Method: The Analyzer Unyvero™ Pneumonia Application was used in detection of pneumonia associated pathogens and their antibiotic resistance genes using the Unyvero™ System following PCR, pathogen species with sequencing of the amplified microbial DNA. Pneumonia Cases Report December/2013: –1. Gender male, age 72 years, Sample ID 1311_1, the results was positive for Streptococcus pneumonia(873), Moraxela cataralis (308), Resistance markers of the Unyvero™ Pneumonia Panel: -tem β-Lactams gram-negative bacteria, -ermB Makrolides / Lincosamides, mecA Oxacillin Staphylococcus. Therapeutic failure must be considered when is administering Makrolides / Lincosamides(ermB), Macrolides (mefA), Penicilins (tem), [Figure 1, 2, 3]. -2. Gender male, age 65 years, Sample ID 1310_1, the results was positive for Klebsiella pneumonia(62), resistance marker result ermB ermB Makrolides / Lincosamides. Therapeutic failure must be considered

when is administering Penicilins. -3. Gender Female age 55 years, Sample ID 1310_2, the results was positive Staphylococcus Aureus, tem β-Lactams gram-negative bacteria, -ermB Makrolides. Therapeutic failure must be considered when is administering Penicilins. Conclusions: The Unyvero™ results were available 2 days before the primary microbiology report and 3 days before the final confirmation results, obtained by microbiology culture. The Unyvero Analyzer only provides rapid data to support the therapeutic decision of currant medic.

Introduction

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.

Following PCR, pathogen species present can be identified sequencing of the amplified DNA. The assay for detection and identification of a defined panel of 25 bacterial of 25 bacterial and fungal pathogen known to cause a majority of acute or chronic respiratory infections.

The need of sophisticated arsenal of new technological tools in the microbiology lab is dictated by two recent events. The first is the emergence and growth of though strain of microbe resistant to antibiotics, even the new antibiotics is drying up (ex.

Microorganism such as K. pneumonia and E. coli have become resistant to third generation cephalosporins as well as carbapenems. The focus of researches now is to move beyond detecting single analytes to multiplex targets and detect more pathogens from a single specimen, ex. Sputum, (1).

This application is a semi-quantitative nucleic acid test on the basis of eight multiplex PCR reactions that are performed in parallel. It is tested for the simultaneous detection and identification of multiple pathogen-derived nucleic acids in sputa, respiratory aspirates and bronchiallavage from individuals suspected of pneumonia, in order to provide information on pathogen species and antibiotic resistance genes.

DNA from individuals exhibiting signs and symptoms of pneumonia aids in the diagnosis of respiratory bacterial or fungal infections if used in conjunction with other clinical and laboratory findings. Concomitant cultures are necessary to recover targeted organisms for further susceptibility testing.

It is recommended that specimens found to be negative for pathogens and resistance genes after examination using the Unyvero™ Pneumonia Application must be confirmed by microbiological culture, [Photo 1]. Positive results do not rule out fungal or viral co-infection, or co-infection with other bacteria not present on the Unyvero™ Pneumonia panel and the agent detected may not be the definite cause of disease, (2).

Photo 1. *Analyzer Unyvero™ Pneumonia*

Principle of the Analysis

The Unyvero™ Pneumonia Application automates 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 of patient 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 [Photo 2]. 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

Detection limits for each pathogen was 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 positive 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). Detection limits for resistance analytes can be determined with DNA fragment dilutions. At a concentration of 105

copies / mL all resistance analytes aredetected.

Interfering Substances

Interferences were tested in suitable pools with respiratory drugs, common antibiotics and sample media or individually for example for lysis 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 Interference Testing in Clinical Chemistry". 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 (mefA, ermA, ermB, ermC, tem, shv, dha, oxa51 like, ctxM, mecA, ebc, quinolone resistances in E.coli and P. aeruginosa) in 26 cases out of 32 antibiotic resistant pathogens a

Photo 2. *Analysis of the patient samples shown by grey test bars on the overview screen*

correlation between Unyvero P50™ results with the antibiogram was demonstrated. Curetis is currently

conducting a prospective European multicenter clinical trial to generate more clinical performance data.

Method

Sample type aspirate sputum, at the patients admitted in Hospitalization Intensive Care Unit and The Unyvero™ Pneumonia Application was performed in the day after specimen collection in Department of Microbiology from Clinical Laboratory Analyses of Emergency County Hospital Targu Jiu.

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 cel count(>15000/mm³), VSH (>40 mm/h), Fibrinogen (>450 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.

Interpret Results

The green boxes on the Anlizer 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.

The numbers next to the green boxes are artificially created and normalized numbers without a measurable unit. 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

Figure 1. *Microorganism results in Pneumonia Panel*

boxes). It was a specific customer demand to have some form of number. However, a 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.

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−1. Gender male, age 72 years, Sample ID 1311_1, the results was positive for Streptococcus pneumonia (873), Moraxela cataralis (308), [Figure 1]. Resistance markers of the Unyvero™ Pneumonia Panel, [Figure 2], were: -tem β-Lactams gram-negative bacteria, -ermB Makrolides / Lincosamides, mecA Oxacillin Staphylococcus,. Therapeutic failure must be considered when is administered Makrolides / Lincosamides(ermB), Macrolides (mefA), Penicilins (tem), [Figure3].

-2. Gender male, age 65 years, Sample ID 1310_1, the results was positive for Klebsiella pneumonia(62), resistance marker result ermB ermB Makrolides / Lincosamides. Therapeutic failure must be considered when is administering Penicilins

-3. Gender Female age 55 years, Sample ID 1310_2, the results was positive Staphylococcus Aureus, tem β-Lactams gram-negative bacteria, -ermB Makrolides. Therapeutic failure must be considered when is administering Penicilins.

These results was confirmed by microbiology culture; however, the final microbiology result was available 3 days after the Unyvero™ result only. Gram-Stain: Microscopy exam: Leukocytes(+), Gram-negative rods (+) on gram stain of microscopic slide.
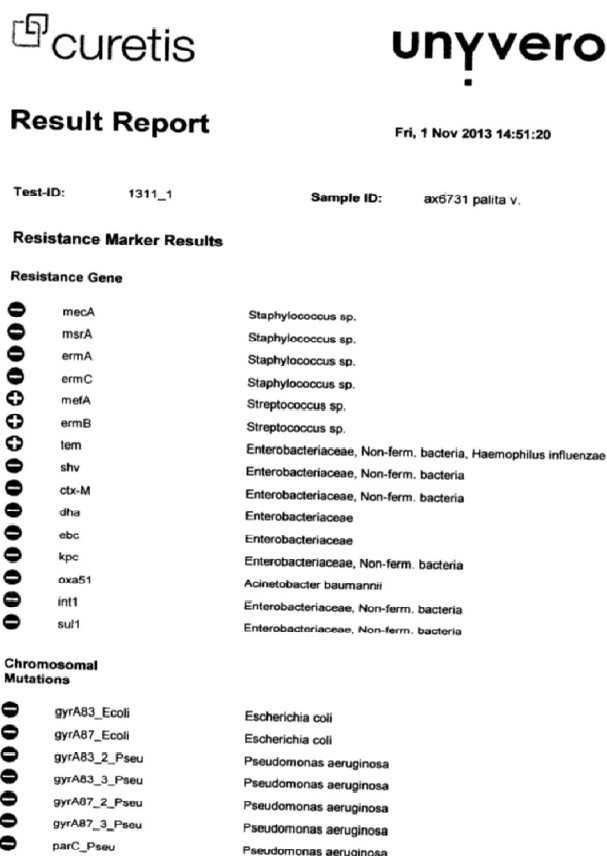
Figure 2. Resistance genes Pneumonia Panel

The Classical Method Kirby Bauer Disc Diffusion Method

In Culture at 1310_1 raised Klebsiella pneumoniae (+++), Sensible Antibiotics, Amoxicillin, Sulfonamide Cotrimoxazol, Quinolone Moxifloxacin, Monobactame Aztreonam, Chephalosporin Cefotaxim AND Ampicillin R, Amoxic./Clavulanacid R, Piperacillin R, Piperac/Tazobactam R, resistance spread especially on our intensive care unit.

Discussions

The Unyvero™ Pneumonia Cartridge detects the following microorganisms: Acinetobacter baumannii, Chlamydophila pneumoniae, Enterobacter sp., Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Klebsiella oxytoca, Legionella 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, (Cephalosporines, Penicillines), dha (Cephalosporines), ebc (3rd Gen. Cephalosporines), ermA(Makrolides / Lincosamides), ermB (Makrolides / Lincosamides) , ermC (Makrolides / Lincosamides) , gyrA83, (E. coli), gyrA87, int1, kpc, mecA, mefA/E, msrA, oxa51 like, parC, shv, sul1, and tem (Fluoroquinolones, E. coli). The resistance markers of the Pneumonia panel and the resulting possible antibiotic resistances are shown in Table 1, (3).

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. Presence of such a gene correlates with a resistance against a particular antibiotic class7. Gene transfer is also more likely in environments where bacteria are in close proximity to each other and in relatively high density such as the gut and oral cavity, (5). In order to control the spread of resistance it is important to have an understanding of

Figure 3. Therapeutic failure for positive microorganism

the molecular biology of the different mobile genetic elements and of the ecology of the environments in which spread is likely, (6).

Bacteria have become resistant to antimicrobials through a number of mechanism; I, Permeability changes in the bacterial cell wall which restricts antimicrobial access to target sites, II, Active efflux of the antibiotic from the microbial cell, III, Enzymatic modification of the antibiotic, IV, Degradation of the antimicrobial agent, V. Acquisition of alternative metabolic pathways to those inhibited by the drug, VI, Modification of antibiotic targets, VII, Overproduction of the target enzyme, (7).

The major encountered aminoglycoside resistance mechanism is the modification of enzymes. These proteins are classified into three major classes according to the type of modification: AAC (acetyltransferases), ANT (nucleotidyltransferases or adenyltrans- ferases). Macrolides have a similar mode of antibacterial action and comparable antibacterial spectra as two other antibiotic classes, i.e., lincosamides and streptogramins B. Consequently, these antibiotics, although chemically distinct, have been clustered together as Macrolide–Lincosamide–StreptograminB(MLS) antibiotics (8).

The β-lactam antibiotics work by inhibiting the cell wall synthesis by binding to so-called penicillin-binding proteins (PBPs) in bacteria and interfering with the structural crosslinking of peptidoglycans and as such preventing terminal transpeptidation in the bacterial cell wall. At Staphilococcus meticilin resistance (MLS) the resistance is due to the presence of r-RNA methylases, encoded by the "erm "genes. The other two mechanisms efflux pumps and inactivating genes are encoded by 'msr "and "ere" determinants, respectively (9, 10, 11).

Gene transfer is also more likely in environments where bacteria are in close proximity to each other and in relatively high density such as the gut and oral cavity. 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.

The Unyvero™ result was available 2 days before the primary microbiology report and 3 days before the final classical antibiogram 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).

Conclusion

The Unyvero only provides data to support the therapeutic decision. The presence of these additional pathogens was verified by sequencing analysis. The most important feature in this case is the correct detection of Klebsiella pneumoniae since this result potentially would have had great impact on the antibiotic therapy regime as well as hygiene measures (patient isolation, etc.), respectively. ∎

Aurelian Udristioiu
Clinical Laboratory, Department of Hematology, Emergency County Hospital Targu Jiu & UCB University, Romania
aurelianu2007@yahoo.com

Manole Cojocaru
Faculty of Medicine, Physiology Department, Titu Maiorescu University, Bucharest, Romania
mancojocaru@yahoo.com

References

[1] Caliendo AM. Multiplex PCR and emerging technologies for the detection of respiratory pathogen.Clin Infect 2011; (4):5326-5330.

[2] Rulls© Curetis AG, 2013 Unyvero ™ P50 Pneumonia Application Guide 00118 V2.0, Hamburg, Germany, 2013, http://www.curetis.com/ .

[3] Kugelberg et al. Antimicrobials, Resistance J Antimicrob Chemother 2005; 55(1): 1-5

[4] Yu et al. Antimicrobials, Resistance J ClinMicrobiol 2004; 42(9): 1-4

[5] VanHoek A, Mevius D, Guerra B, Mullany P and al. Acquired antibiotic resistance genes a new review article published 2011; 1: 1-28.

[6] Depardieu A et al. Frontiers in Microbiology. Antimicrobials, Resistance and Chemotherapy. 2011;203: 1-13.

[7] Wright G.D. Bacterialresis-tancetoantibiotics:enzymatic degradationandmodification. Adv. DrugDeliv.Rev 2005; 57: 1451–1470.

[8] Roberts M.C. Update on macrolide-lincosamide, treptogramin, ketolide,and oxazolidinone resistance genes, -ermA, ermB, ermC Makrolides / Lincosamides, gyrA83 of E. coli . FEMS Microbiol.Lett 2008; 282: 147–159.

[9] Bonnet et al. ctx-M 3rdGen, Cephalosporines, Penicillines, Antimicrob Agents. Chemother 2001; 45(8): 1-8.

[10] Pérez-Pérez et al. dha 3rdGen. Cephalosporines, J Clin Microbiol 2002; 40(6): 1-6.

[11] Pérez-Pérez et al. ebc 3rd Gen. Cephalosporines Pérez-Pérez et al, J Clin Microbiol 2002; 40(6): 1-6.

[12] Oliviera JE, Silva CA, Oliviera GM, Zaneta DM et al. Antimicrob Agents. Chemoter 2009; 53(7): 2887-289.