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P.063

Molecular detection of viruses in sputum of community acquired pneumonia (CAP) patients

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Molecular detection of viruses in sputum is complicated by the occurrence of inhibition of PCR reactions. In this study, it was investigated how specimen treatment and RNA isolation can be optimized to avoid this inhibition in sputum in patients suffering from CAP.
The optimal method to treat sputum samples prior to RNA isolation was determined by comparing the different methods to lyse sputum. Subsequently, different protocols for RNA isolation routinely used in molecular detection of respiratory diseases were tested. Different sputa were pooled, lysed and spiked with a mixture of respiratory viruses in different concentrations. Then, the performance of the PCR detecting the different respiratory viruses was evaluated by monitoring the detection limit, CP values and magnitude of fluorescence.
Subsequently, the most optimal method for specimen treatment and RNA isolation was used to investigate the occurrence of different respiratory viruses in sputa and throat and nose swabs in patients with CAP.
It was found that especially the method of RNA isolation highly influences the detection limit. It was also found that introducing molecular detection of respiratory viruses on sputa in patients suffering from pneumoniae results in the detection of respiratory viruses. Part of these respiratory viruses, could not be detected in other respiratory samples.
This study demonstrates that the choice of an appropriate RNA isolation method seems to be a crucial step in the validation of the molecular detection of respiratory viruses in sputa.

P.064

Clinical severity and course of respiratory tract infections and the detection of respiratory viruses detected by multiplex RT-PCR in young hospitalized children

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Purpose: A prospective study was performed to investigate the relationship between the presence and (semi-quantitatively) amount of respiratory viruses and the clinical course of children under 6 years admitted with a respiratory tract infection.
Methods: Nasopharyngeal aspirates (NPA) were tested for the presence of human boca-, respiratory syncytial-, parainfluenza 1–4, influenza A and B, corona-, human metapneumo-, parecho-, entero-, adeno- and rhinoviruses. Clinical severity scores were calculated prospectively using a questionnaire. The presence and (semi-) quantity of the fourteen respiratory viruses were correlated with the severity of clinical symptoms on admission and at day 3. The quantity of a specific virus was deduced from the amplication cycle of the PCR assay at which a sample becomes positive (so-called Cp value). The lower the Cp value, the higher the concentration of the virus present.
Results: In 59% of the 119 included children one or two viruses could be detected. RSV, rhino- and bocavirus were detected in resp. 40%, 26% and 19% of the positive patients. For bocavirus a decrease in clinical symptoms coincided with an increase in CP values. In clinically improved patients, the Cp values of specific respiratory viruses increased, whereas in clinically unimproved patients the CP values remained the same.
Conclusion: Cp values can be used as semi-quantitative measurements to calculate the concentration of a specific virus. The amount of bocavirus present in NPAs correlates with clinical symptoms. Clinical improvement of a patient was correlated with an increase in CP value of a specific respiratory virus.

P.065

Involvement of cellular factors during the respiratory vaccinia virus entry in the lung

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The smallpox was eradicated from the world in 1980. Though, efforts are mounting to replenish the vaccine stocks and develop new drugs as the variola virus (VARV) might be used as a weapon of mass destruction. The study aim is to understand molecular interactions which may occur during the orthopoxvirus entry in the lungs as VARV is infectious in human by the respiratory route. When virus reaches the alveolar spaces, it encounters the lipid-rich surfactant that covers the epithelium. This material is a mixture of phospholipids and proteins. Surfactant proteins could interact with virus to modulate host defense function in the lung whereas virus could interact with phospholipids to facilitate its entry into the epithelium cells. In this study, we determined the correlation between the virulence of vaccinia virus (VACV) strains (as VARV surrogate) and virus-surfactant component interactions. Preliminary results suggest interaction between IMV (intracellular mature virus) particles with sulfatide acid and phosphatidylinositol phosphate. IMV particles weakly interact with DPPC while EEV (extracellular enveloped virus) strongly bind DPPC and DPPG in presence of $Ca2+/Mg2+$ at pH 7.5. Surfactant protein D (SP-D), involved in important roles in innate immunity, is capable to bind both IMV and EEV. This study showed that VACV could interact with lung surfactant factors as phospholipids and SP-D. Further experiments should be performed to determine the role of this interactions in virus entry and the viral protein partner involved in. SP-D may play a role in the defense against VACV entry which should be confirmed in animal model.

P.066

Consequences of combinations of mutations in the neuraminidase active site on the cell surface expression, the activity and the sensibility of the neuraminidase to the neuraminidase inhibitors

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Introduction: The neuraminidase (NA) is critical for the spread of the influenza virus. Neuraminidase inhibitors (NAIs), zanamivir and oseltamivir, are structural analogs from sialic acid which target conserved residues in the neuraminidase active site. The introduction in clinic of new molecules requires a tight attention to monitor the emergence of resistance.
Purposes: After a first study [1], we wanted to study combinations of mutations with the R292K and the I222L mutations to assay if the synergy of multiple amino acid change may confer a stronger resistance while conserving a great replicative capacity.
Methods: In the A/Moscow/10/99 (H3N2) virus background, viruses were constructed by reverse genetic. They were grown on MDCK cells. Fluorimetric assays were conduced to assay the NA activity and the drug concentration required to inhibit 50% of the NA activity (IC50).
Results and Conclusion: The R292K/C42F mutant conserved the same activity and the same rate of resistance as the R292K mutant. The E119V/I222L H274Y/I222L H274Y/R292K mutants presented a drop in NA activity. We were not able to detect by phenotypic tests the virus containing the D151N/R292K, R152K/R292K, W178L/R292K, I222L/R292K, E276D/R292K, E433K/R292K, E119D/I222L, W178L/I222L, D198N/I222L, E277G/I222L mutations. To study the impact of those mutations on the neuraminidase, transfections on 293T cells and confocal studies were done. Western blot and fluorimetric tests after cellular fractionation allowed us to study the expression of the mutant neuraminidases at the cell surface and to determine their activity.

Reference(s)

[1] Richard M et al. J Clin Virol. 2008 Jan;41(1):20–4.

P.067

Real-time PCR for the detection of respiratory viruses

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Purpose: Viruses are responsible for 80% of respiratory infections. New respiratory viruses were recently described like human Metapneumovirus (hMPV) and human Bocavirus (hBoV). The objective of this study is to evaluate the sensitivity of real-time PCR for the detection of respiratory viruses, comparing results with those obtained using direct examination with fluorescent anti-viral antibodies and viral culture.
Methods: Real-time RT-PCR and PCR were done on the Roche LC480 using a TaqMan system. Primers and probes were furnished by Argene (Varihles, France). 169 respiratory samples from 146 patients (54% adults, 44% less than 2-years-old, 2% between 2 and 15 years), were extracted using Qiagen columns (Qiagen, Westburg bv, Nl) and amplified according to the recommendations

of the manufacturer. Samples were inoculated on appropriate cell lines and viral antigens were sought using fluorescent antibodies.
Results: Using PCR or RT-PCR, adenovirus was detected in 29 samples (17%), RSV in 27 (16%), Influenza viruses in 17 (10%), hBoV in 16 (9.5%), and hMPV in 6 (3.5%). Two viruses were detected together in 13 samples and 3 viruses in 5 samples. HboV were associated with other viruses in 62% of the cases. For adenovirus, RSV and influenza, real-time PCR allowed a gain of sensitivity of 24% and 26% compared to direct examination and culture, respectively. The most appropriate sample was nasopharyngeal aspiration.
Conclusion: Real-time PCR is a rapid and more sensitive technique for detection of respiratory viruses than direct examination and culture. It detects viral coinfections that are not detected by classic techniques.

P.068

Use of a respiratory virus panel test for the diagnosis of viral respiratory infections: impact of rhinoviruses

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Introduction: Acute respiratory virus infections are among the most common causes of human diseases. Numerous viruses display a respiratory tropism. These viruses usually present with similar signs and symptoms that are nearly undistinguishable by clinical diagnosis. In recent years, many new respiratory virus pathogens have been identified: metapneumovirus, coronaviruses NL63 and HKU1, bocavirus. Our aim was to address the impact of the detection of these new emerging viruses and of common cold associated viruses not routinely tested, on the diagnosis of respiratory virus infections.
Materials and Methods: A total of 212 respiratory specimens (173 nasopharyngeal swabs and 39 brochoalveolar lavage) were prospectively tested with the Luminex respiratory viral panel (RVP). This assay is based on multiplex reverse transcriptase-PCR coupled to target-specific primer extension. TSPE products are sorted and identified by using a fluid microsphere-based array and the Luminex x-MAP system. It can detect 20 different respiratory virus types/subtypes in a single 5 hours-test.
Results: RVP detected at least one viral pathogen in 127 (60%) specimens, including 55 (43%) enterovirus/rhinovirus, 28 (22%) respiratory syncytial virus (RSV) A and B, and 17 metapneumovirus. Nineteen (15%) specimens showed coinfection with two viruses. Enterorhinoviruses were frequently associated with lower respiratory tract infections as well as parainfluenza viruses. Bronchiolitis were associated to RSV, metapneumovirus or enterorhinoviruses.
Conclusion: RVP assay provides an excellent tool for the simultaneous rapid detection of 20 respiratory viruses with reduced manpower. This assay is highly requested by clinicians and has a strong impact on patient care and infection control.

P.069

Comparison of one and two step RT-PCR in the detection of RNA respiratory viruses

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The poor sensitivity and slow turnaround time of virus culture and immunofluorescence for the detection of respiratory virus infection has led to the development of molecular assays which offer the opportunity of rapid turnaround and high sensitivity. Many respiratory virus infections, being caused by RNA viruses, require an initial reverse transcription (RT) step to generate the cDNA for PCR.
Aim: We compared the performance of four one-step RT-PCR kits (Invitrogen Superscript, Ambion AgPath, ABI RNA to Ct and Qiagen Quantitect) against an established two step multiplex PCR method. Sensitivity was assessed by endpoint dilution of positive material and routine samples identified as positive by the two step method were retrospectively tested using one step RT-PCR. The Ct values of an internal control were also examined with two of the kits.
Results: The ABI and AgPath kits demonstrated comparable relative sensitivities and approximately equal sensitivity to the two-step method when tested on dilution series for nine respiratory viruses. The Superscript assay was up to 1 log less sensitive than ABI and AgPath and commonly showed higher Ct values.
Preliminary results indicate that the Quantitect assay is at least as sensitive as the ABI but gives greater consistency in Ct values with an internal control, indicating its potential superiority in multiplex assays. Quantitect also gave rise to significantly lower ΔRn values than other assays, requiring manual alteration of threshold values.
Conclusion: One-step RT-PCR offers significant advantages over a two-step method both in terms of complexity and turnaround times, without loss in sensitivity.

P.070

A rapid real-time PCR assay for detection of oseltamivir resistance mutations in influenza A/H1N1 and H3N2 neuraminidase

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Purpose: The incidence of influenza viruses resistant to oseltamivir (Tamiflu®) has increased dramatically in the influenza epidemic of 2007/2008. In the Netherlands, 27% of the influenza A/H1N1 viruses appeared unsusceptible to oseltamivir (0–70% worldwide), all carrying a H274Y single point mutation in the neuraminidase protein. Screening for associated oseltamivir resistance mutations is useful for treatment of vulnerable influenza infected patients.
Methods: Multiplex real-time assays were developed by designing primers and locked-nucleic-acid-incorporated (LNA) probes around the H274Y (N1), E119V (N2) and R292K (N2) regions of the neuraminidase gene. LNA-bases were incorporated in the probes to increase hybridization efficiency and discriminative characteristics.
Results: RNA isolated from influenza A/H1N1 cultures (96) were screened for presence of wild type and mutant viral RNA using a LightCycler 480 and TaqGold reagents kit (Roche). In 38 samples the H274Y mutation was detected, 58 for having a wild type genotype. All positive scores were confirmed by sequencing of the neuraminidase gene (100% score). The low limit of detection of the assay was determined to be 500 copies per millilitre and up to 5% of mutants could be detected in a mixing experiment (1x10E6 viral input). Similar results were obtained for the E119V and R292K assays screening H3N2 isolates.
Conclusion: These rapid real-time PCR assays are rapid, robust and may be useful in influenza resistance surveillance and clinical diagnostics.