Molecular diagnostic investigation of human pathogen chlamydiae

Doctoral thesis

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1. Introduction

Chlamydiae are obligate intracellular bacteria with a unique biphasic lifecycle among the prokaryotes. In medical aspect three human pathogen species are described for causing widespread infections. *Chlamydia trachomatis* is one of the most prevalent sexually transmitted bacterium worldwide. The pathogen has been classified into 15 different serovars based on typing of its immunodominant antigen, the major outer membrane protein (MOMP). Serovars D-K represent the largest group causing urogenital and neonatal infections worldwide. The prevalence of urogenital *C. trachomatis* infections varies in different countries, while many risk factors have an influence on it. Age and the number of sex partners are the most common risk factors together with the infrequent or lack of condom use, and the fact of an earlier infection. Characterizing certain serovars is based on genotyping methods, which can be helpful primarily in epidemiological studies. These genotyping techniques are also used in clinical studies determining the relationship between certain serovars and clinical manifestations as well as in the research of the pathogenetic differences among the known serovars. In Hungary *C. trachomatis* infection is not a notifiable disease and there is limited information on its prevalence. Moreover the distribution of certain *C. trachomatis* serovars has not been studied previously in Hungary.

C. psittaci is a pathogen of several wild bird species and poultry birds. The infection spreading from birds to humans causes respiratory disease, which can be occasionally fatal. Psittacosis is an occupational disease, that primarily occurs in breeders, poultry processing plant employees, but pet birds may also serve as the source of infection.

During 2005, two major outbreaks have been reported in Hungary, infections occurred in poultry processing plants in both cases. Based on the clinical picture psittacosis could not be differentiated from other community-acquired pneumonias, therefore a history of exposure to birds is the most important clue for obtaining the correct diagnosis. In the laboratory diagnosis, besides the specific serological methods, quick and direct detection of the pathogen could be helpful to confirm the diagnosis in severe cases.

C. pneumoniae usually causes mild respiratory infections and it is responsible for about 10% of community-acquired pneumonia cases in developed countries. The pathogen has gained a significant role in the research field of the development of atherosclerosis. Evidence on the association of *C. pneumoniae* and atherosclerosis is primarly based on seroepidemiological studies, direct detection of the pathogen from the atherosclerotic vessel wall, and animal studies.

2. Aims of the thesis

2.1. Determination of the prevalence of *C. trachomatis* infection in a Hungarian high-risk population and molecular typing

There are limited epidemiological data on the prevalence of *C. trachomatis* infection in the general population. The aims of the study were to detect *C. trachomatis* and to determine the prevalence of the infection in a high-risk group of female sex workers (FSW), as well as to characterize and to determine the distribution of certain *C. trachomatis* serovars by the genotyping method of *omp1* PCR-based restriction fragment length polymorphism (RFLP) analysis.

2.2. Confirmation of *C. psittaci* infection in potential patients by molecular methods

Based on the clinical symptoms *C. psittaci* infection is indistinguishable from other types of atypical respiratory infections. The aim of the work was to develop some molecular methods for the direct detection of *C. psittaci* that confirm the serological results, and to verify the etiological role of *C. psittaci* in two cases of fatal psittacosis.

2.3. Investigation of the association between *C. pneumoniae* and atherosclerosis in patients who underwent percutaneous coronary angioplasty

Atherosclerotic narrowing in the coronary arteries can be treated by percutaneous transluminal coronary angioplasty (PTCA). The

objective of the work was to detect the reactivation of persistent *C. pneumoniae* and certain viruses as the result of the intervention.

3. Methods

3.1. Determination of the prevalence of *C. trachomatis* infection in a Hungarian high-risk population and molecular typing

Endocervical samples were obtained from 484 FSWs during an anonymous screening in Budapest and the suburban area from January 2006 to July 2006. For the detection of *C. trachomatis* DNA in the samples, a PCR method was performed targeting the extrachromosomal plasmid of the pathogen.

C. trachomatis serovars were further differentiated by a genotyping method based on PCR-based RFLP analysis. *C. trachomatis*-positive samples detected by the plasmid PCR were analysed by a seminested PCR targeting the *omp1* gene. The PCR products were digested with *Alu*I (Promega) restriction endonuclease. Serovars belonging to group C with the same restriction patterns were further digested with *Hinf*I, *Eco*RI, *Dde*I (Promega, Madison, USA). The variants of serovar D were differentiated with *Cfo*I (Promega). Extracted DNA samples from *C. trachomatis* strains representing certain serovars were used as reference controls.

3.2. Confirmation of *C. psittaci* infection in potential patients by molecular methods

In the first case postmortem blood and lung tissue samples of a 69-year-old woman were analysed. The patient was admitted to hospital with severe dyspnoea. Amoxicillin was prescribed by the family physician, but her symptoms worsened. The chest radiograph revealed extensive pleuropneumonia. She received ciprofloxacin intravenously on the same day. The next day, her dyspnoea deteriorated and she required mechanical ventilation. The antibiotic treatment was continued with levofloxacin. Later in the evening she became bradycardic, her respiration stopped, and the patient died. Psittacosis was assumed as an etiological cause, because the patient had been working at the plucking unit of a poultry processing plant in Békés county.

In the second case samples were obtained from a 48-year-old woman who was admitted to hospital with a 2-week history of cough and fever. The chest radiograph showed infiltrates in the lung. Antibiotic therapy was initiated with moxifloxacin and co-amoxiclav upon admission. The patient was transferred to the intensive care unit with development of severe respiratory failure and hypoxia. She required mechanical ventilation. The antibiotic therapy was completed with clindamycin, rifampicin, and ceftriaxone. The patient had been working at the plucking unit of a poultry processing plant in Bács-Kiskun county. Psittacosis was assumed, therefore blood and

bronchial fluid samples were sent for microbiological testing. The patient's status worsened, she had multiorgan-failure, tahcycardia and hypoxia, and finally she died. Post-mortem parenchymal tissue samples (taken from brain, lung, liver, spleen and kidney) were stored frozen until examination.

Serological testing of the blood samples was performed with a commercial microimmunofluorescence (MIF) assay (Focus Diagnostics, Cypress, USA). Detection of *C. psittaci* DNA from the post-mortem tissues and the bronchial fluid was performed with two PCR methods targeting two different genes. The target sequences were the *ompA* gene coding MOMP, and *Omp2* gene coding the 60-kDa cysteine-rich outer membrane protein.

3.3. Investigation of the association between *C. pneumoniae* and atherosclerosis in patients who underwent percutaneous coronary angioplasty

During 2003, blood samples of 28 patients admitted to the Cardiology Unit of the Railway Hospital with unstable angina and underwent PTCA intervention were tested. Blood samples were obtained prior to and on days 4 and 14 after PTCA.

Detection of *C. pneumoniae* DNA was performed with a nested PCR targeting the *ompA* gene. For the detection of CMV DNA a nested PCR was performed targeting the immediate early-exon-4 gene. Each sample was tested in five replicates for both pathogens to increase the sensitivity of the assays. Serum antibody levels of *C. pneumoniae*

IgA and IgG were determined by a commercial ELISA test (NovaTec, Dietzenbach, Németország). Detection of CMV IgG, human herpes simplex virus (HSV) IgG and Epstein-Barr virus (EBV) IgG antibodies was performed by commercial ELISA methods.

4. Results

4.1. Determination of the prevalence of *C. trachomatis* infection in a Hungarian high-risk population and molecular typing

Of the 484-endocervical samples 32 (6,6%) were found to be positive by *C. trachomatis* plasmid PCR. Data on age were obtained from 275 individuals for analysing the effect of age on *C. trachomatis* infection. The FSWs were aged from 18-59 years and the mean age was 27.5 years. A significant association was observed between age and the prevalence of *C. trachomatis* infection. Younger women had a higher prevalence rate.

Among the 32 positive samples seven different urogenital *C. trachomatis* serovars could be identified by the *omp1* PCR-RFLP, showing the same restriction patterns as the control strains. Serovar D was the most prevalent (11/32, 34,4%), followed by E (7/32, 21,9%), F (6/32, 18,8%), G (3/32, 9,4%), J (3/32, 9,4%), H (1/32, 3,1%), and I (1/32, 3,1%). The *Cfo*I digestion of samples identified as serovar D showed a distinct restriction pattern in one sample, which referred to the D^- variant.

4.2. Confirmation of *C. psittaci* infection in potential patients by molecular methods

In the first case specific *C. psittaci* antibodies were detected in the serum sample in an IgM titre of 1:16, IgG titre of 1:512, and IgA titre of 1:32, indicating an acute infection. In the second case no specific IgM was detected, the serum had an IgG titre of 1:256 and IgA titre of 1:32.

C. psittaci DNA was detected in lung tissue of the first patient, as well as in the bronchial fluid of the second patient by the *ompA* PCR and also by the *Omp2* PCR. In the second case *C. psittaci* DNA was detected by both methods only in the lung tissue among the post-mortem tissues.

4.3. Investigation of the association between *C. pneumoniae* and atherosclerosis in patients who underwent percutaneous coronary angioplasty

C. pneumoniae DNA was detected from the blood samples obtained before PTCA in one sample, and from three samples obtained after PTCA. CMV DNA was detected in one sample obtained before PTCA, and in five samples obtained after PTCA. None of these samples were positive for the DNA of both pathogens. A significant association was observed between the PTCA intervention and the combined detectability of the DNA of both pathogens.

No significant differences were observed in the levels of serum antibodies for *C. pneumoniae* and the other pathogens obtained before or 4. and 14. days after the PTCA intervention.

5. Conclusions

5.1. Determination of the prevalence of *C. trachomatis* infection in a Hungarian high-risk population and molecular typing

According to the results of several studies in the European countries, the prevalence of urogenital *C. trachomatis* infection in FSWs is less than 10% based on the detection of *C. trachomatis* DNA. These findings correspond well with the observed prevalence rate of 6,6% in Hungary. Despite being a high-risk population, the higher prevalence in younger FSWs -18,8% under 20 years and 9,4% between 20-29 years- supported the fact that age is a risk factor for chlamydial infection. According to several other studies, serovars D, E, and F appeared to be the most prevalent by genotyping and these serovars comprise 75% of the *C. trachomatis* positive samples. These results indicate that most urogenital *C. trachomatis* infections are caused by a small number of serovars. A probable explanation for the predominance of serovars D, E, and F might be that these serovars can escape more successfully from the immune system, may persist for longer periods, and have different virulence. The genotyping method based on RFLP analysis presented in this thesis proved to be a valuable tool for further epidemiological surveys and diagnostic

purposes. The applied molecular techniques presented in this work have good advantages for further epidemiological studies to determine the prevalence of the circulating *C. trachomatis* serovars in different cohorts.

5.2. Confirmation of *C. psittaci* infection in potential patients by molecular methods

Psittacosis causing severe respiratory failure requiring mechanical ventilation is uncommon but can be found in several case reports. Delayed anamnesis of the history of exposure to birds is the main reason for developing severe disease. Doxycycline is considered to be the treatment of choice in *C. psittaci* infections. In the presented cases, however, ineffective empirical antibiotic therapies were initiated for the treatment of the severe pneumonias. The first patient's increased age and the second patient's weak health status could have been additional risk factors affecting the outcome.

In the first case, only postmortem samples could have been tested, nevertheless, according to the clinical findings, serological results with the presence of specific IgM and high titre of IgG supported the diagnosis. Detection of *C. psittaci* DNA in lung tissue confirmed the serological results. In the second case in the absence of the specific IgM, the presence of the specific IgA not always indicates an acute infection. Therefore a second blood sample should have been tested

for the detection of the changes in antibody titres, while the detection of *C. psittaci* DNA in the bronchial fluid confirmed the diagnosis.

Poultry processing plant employees are considered a high-risk group for psittacosis infections. In this work the clinical suspect of psittacosis was confirmed by molecular diagnostic methods for the first time in Hungary, which also supported the serological results. *C. psittaci* infection should be taken into account in the differential diagnosis, especially in rural areas where handling of domestic fowl is common.

5.3. Investigation of the association between *C. pneumoniae* and atherosclerosis in patients who underwent percutaneous coronary angioplasty

The results revealed for the first time a higher prevalence of *C. pneumoniae* and CMV DNS in peripheral blood samples of patients after PTCA that might be associated with the pathogen reactivation. However, the prevalence of these pathogens was low which could be explained by the low pathogen concentration in the plaque, inappropriate sampling time, or the lack of the pathogen in the plaque. Other reasons could be that different interventions, such as atherectomy might cause greater injury in the vascular wall leading to better conditions for pathogen reactivation.

Serological results did not support the hypothesis of pathogen reactivation following PTCA intervention. A possible explanation could be the high prevalence of *C. pneumoniae* and CMV antibodies

in the general population, and the high antibody levels among the seropositive individuals. These facts suggest that slight increase in the antibody levels after PTCA cannot be detected. Further studies are needed to confirm these observations and larger patient groups should be tested. To examine the possible changes in antibody levels after PTCA more frequent sampling may provide more information.

6. List of publications

Included in the thesis:

Petrovay F, Balla E, Németh I, Gönczöl E. (2009) Genotyping of *Chlamydia trachomatis* from the endocervical specimens of high-risk women in Hungary. *J Med Microbiol* **58**, 760-764.

Petrovay F, Balla E. (2008) Two fatal cases of psittacosis caused by *Chlamydophila psittaci*. *J Med Microbiol* **57**, 1296-1268.

Petrovay F, Heltai K, Kis Z, Treso B, Gonczol E, Burian K, Endresz V, Valyi-Nagy I. (2007) Chronic infections and histamine, CRP and IL-6 levels after percutaneous transluminal coronary angioplasty. *Inflamm Res* **56**, 362-367.

Related to the thesis:

Kis Z, Sas K, Gyulai Z, Treso B, **Petrovay F**, Kapusinszky B, Csire M, Endresz V, Burian K, Mandi Y, Vecsei L, Gonczol E. (2007) Chronic infections and genetic factors in the development of ischemic stroke. *New Microbiol* **30**, 213-220.

Balla E, **Petrovay F**. (2007) Az ornithosis laboratóriumi diagnosztikai módszereinek fejlesztése I. *Mikrobiológiai Körlevél* **7**, 2.

Balla E, **Petrovay F**. (2007) Az ornithosis laboratóriumi diagnosztikai módszereinek fejlesztése II. A laboratóriumi eredmények interpretációja. *Mikrobiológiai Körlevél* **7**, 3.

Not related to the thesis:

Balla E, **Petrovay F**, Boross K, Koós-Hutás P. (2008) Urogenitális mycoplasmák előfordulási gyakorisága és potenciális kóroki szerepe terhes nők körében. *STD és Genitális Infektológia* **2**, 53-58.

Balla E, **Petrovay F**, Boross K. (2004) *Mycoplasma pneumoniae* és *Chlamydia pneumoniae* fertőzések szerodiagnosztikája. *Mikrobiológiai Körlevél* **4**, 1.

Petrovay F, Balla E, Boross K. (2008) *Treponema pallidum* nested PCR gyakorlati jelentősége a szifilisz diagnosztikában. *STD és Genitális Infektológia* **2**, 167-169.

List of presentations related to the thesis:

Petrovay F, Balla E. *Chlamydia trachomatis* szerotípusok meghatározása molekuláris módszerekkel. 2006.11.17-18. Magyar STD Társaság Nagygyűlése, Budapest. Összefoglalók Könyve 41. oldal.

Petrovay F, Balla E. Humán psittacosis: a korai laboratóriumi diagnózis jelentősége. 2006.10.18-20. Magyar Mikrobiológiai Társaság Nagygyűlése. *Acta Microbiol Immunol Hung*, 2006, 53(3), 331-332.

Petrovay F, Heltai K, Endrész V, Ludwig E, Kiss R, Gönczöl É, Vályi-Nagy I. Összefüggés a ballonkatéteres koszorúér-tágítás (PTCA) után bekövetkező restenosis és a *Chlamydia pneumoniae* illetve cytomegalovirus ellenanyagszint emelkedése között. 2004. 10. 7-9. Magyar Mikrobiológiai Társaság Nagygyűlése, Keszthely. Összefoglalók Könyve 98. oldal.

Petrovay F, Heltai K, Endrész V, Ludwig E, Kiss R, Gönczöl É, Vályi-Nagy I. Összefüggés a ballonkatéteres koszorúér-tágítás (PTCA) után bekövetkező restenosis és a *Chlamydia pneumoniae* illetve cytomegalovirus ellenanyagszint emelkedése között. 2005. 04. 14-15. Semmelweis Egyetem PhD Tudományos Napok, Budapest.

Balla E, **Petrovay F**. Ascendáló *Chlamydia trachomatis* fertőzés diagnosztikája-tapasztalataink Western Blot vizsgálattal. 2006.11.17-18. Magyar STD Társaság Nagygyűlése, Budapest. Összefoglalók Könyve 31. oldal.

Balla E, **Petrovay F**. Neonatalis pneumonia- a kórkép diagnosztikája, epidemiológiai jelentősége. 2006.11.17-18. Magyar STD Társaság Nagygyűlése, Budapest. Összefoglalók Könyve 42. oldal.

Hartwell D, Christiansen OB, **Petrovay F**, Lidegaard O, Gönczöl E, Garred P: Genetic factors determining innate immunity and risk of tubal infertility. 2006. 05. 20-23. 35. Nordic Congress of Obstetrics and Gynecology, Göteborg, Sweden.