COMPARISON OF THE SENSITIVITY OF THE POLIMERASE CHAIN REACTION FOR DETECTION OF *CHLAMYDIA PNEUMONIAE* INFECTIONS AFTER TWO DIFFERENT APPLIED METHODS FOR DNA EXTRACTION FROM SPUTA, TRACHEAL AND BRONCHIAL SECRETS

V. Ouzounova, I. Mitov
Department of Microbiology
Medical Faculty, Medical University-Sofia;

SUMMARY

Chlamydia pneumoniae (Chlamydophila pneumoniae) is one of the important pathogens in human respiratory tract infections. The microogranism is an obligatory intracellular gram-negative bacterium that is common cause of bronchitis, sinusitis, pharyngitis and atypical pneumonia. 90% or more of *C. pneumoniae* infections remain asymptomatic. There is suggested a relationship between *C. pneumoniae* and chronic diseases such as chronic obstructive pulmonary disease, coronary heart disease, multiple sclerosis, reactive arthritis, Guillain-Barre' syndrome, Alzheimer's disease, etc. The knowledge for the epidemiology of *C. pneumoniae* infections mainly depends on serological detection received from studies of the population immune status using microimmunofluorescence and cell culture. These methods are time consuming and not very sensitive. Recently the isolation of the microorganism was improved and more frequently the PCR started to be applied as a method of choice to diagnose *C. pneumoniae* infections. The method is performed within several hours and gives the possibility to use a wide variety of patient's specimens.

The aim of this study is to compare the sensitivity of the polimerase chain reaction after two different types of DNA extraction from sputa, tracheal and bronchial secrets, artificially infected with *C. pneumoniae*.

Key words: *Chlamydia pneumoniae*, PCR, phenol/chlorophorm/isoamyl alcohol isolation, sputum, tracheal and bronchial secrets.

INTRODUCTION

Chlamydia pneumoniae (Chlamydophila pneumoniae) is one of the important pathogens in humans and is known to infect the upper and lower respiratory tracts among children and adults **(5)** Pneumonia, bronchitis, sinusitis and pharyngitis are the most common illnesses among the recognized ones associated with *C. pneumoniae*. Recent studies have suggested that the microorganism may have role in the pathogenesis of newly diagnosed and chronic stable asthma **(8)** and atherosclerotic cardiovascular disease **(5)** A considerable number of articles report on a link between *C. pneumoniae* and multiple sclerosis, reactive arthritis, Guillain-Barre' syndrome, Alzheimer's disease, sarcoidosis and hilar lymphadenopathy, lung cancer, chronic fatigue syndrome, etc. However, these data are based on seroepidemiological studies, which can not exactly define if the infection is present or past. Seropositivity in the adult population ranges from 41 to 75% **(10)**, reinfections or activations without an IgM response are common **(9)**, and significant IgG titer rises are frequently delayed or may even be missed. On the other hand the results from the serology may be incorrect because of cross-reactive interference with other Chlamydia species or even more with other Gram negative microorganisms.

Another method widely used for detection of *C. pneumoniae* infections is the cell culture method. But it is difficult to grow the bacterium from clinical specimens in a cell culture.

Conventional assays for the detection of *C. pneumoniae* have limitations, and more accurate diagnostic methods are needed. Recently the isolation of the microorganism was improved and more frequently the Nucleic acid amplification (NAA) techniques started to be applied as a method of choice to diagnose *C. pneumoniae* infections. These methods have the potential to offer clinical laboratories a convenient means of detecting *C. pneumoniae* rapidly and reliably, ensuring optimal clinical decisions and patient care, including choice of appropriate antibiotic therapy.

There is still no gold standard for PCR detection of *Chlamydophila pneumoniae* **(2)**. There are numerous methods available for DNA isolation and PCR detection of the organism. It is obvious from the literature that the detection of *C. pneumoniae* in different clinical specimens

varies from high to low numbers or even negativity of the samples based on the method used. And no single PCR protocol is appropriate for all situations **(1)**.

We intended to evaluate the sensitivity of PCR assay after two different types of DNA isolation from sputa, bronchial and tracheal secrets.

MATERIALS AND METHODS.

The clinical samples used in the study were sputa, tracheal and bronchial secrets provided to the routine laboratory. Before starting the examination, 1ml of each sputum was diluted in 200µl 2,5% N-acetyl-cystein for 30-60 min at room temperature. This step was not performed for the tracheal and bronchial secrets. All the specimens were examined for the most common pathogens in the respiratory tract. And after negative results including negative PCR for *C. pneumoniae* they were infected with one and the same amount of *C. pneumoniae* strain TWAR-183.

Two different DNA extraction methods were performed. The first one was the phenol/chlorophorm/isoamyl alcohol isolation method and the second was QIAamp DNA Mini Kit (50), Quiagen.

Phenol-chlorophorm-isoamyl alcohol isolation method: 1 ml of each specimen was centrifuged for 40 min at 14 000g. The supernatant was removed and were added 200µl 1xTE buffer. After vortexing the specimens were subjected to phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation. The precipitate was collected by centrifugation, then dried and resuspended in H_2O.

QIAamp DNA Mini Kit (50), Quiagen: 1 ml of each specimen was centrifuged for 40 min at 14 000g. Then the extraction was performed according to the manufacturer's instructions.

The used PCR assay detected *C. pneumoniae* specific 16sRNA 463bp fragment **(4)**.

RESULTS.

A comparison between two extracting procedures (1.phenol-chlorophorm-isoamyl alcohol method and 2.the commercial Quiagen kit) from sputa, tracheal and bronchial secrets was carried out to determine which method yields more reliable results in order to be adopted in the future as a standard procedure for diagnosis of *C. pneumonaie*. With the extracted DNA there was performed PCR with primers CpnA/CpnB and compared its sensitivity when the extraction was done using phenol-chlorophorm and the commercial kit.

The well known classical method of phenol-chlorophorm-isoamyl alcohol extracion showed sensitivity of the PCR 10^3 for the tracheal and bronchial secrets. It was the same sensitivity as with the Quiagen kit.

There was found a difference when the specimen used was sputum - the detected sensitivity of the PCR was 10^2 with the phenol-chlorophorm-isoamyl alcohol extracion method, meanwhile using the commercial kit the sensitivity was $4x10^1$.

DISCUSSION.

The aptitude of PCR to amplify small and at the same time specific amounts of nucleic acids is the reason that this method was introduced in the clinical practice and now plays a leading role in the rapid diagnostic procedure of *C. pneumoniae* infections. A large number of PCR assays have been developed to detect *C. pneumoniae*. There are different protocols for detecting *C. pneumoniae* DNA as like as different targets for the reaction - 16S rRNA, MOMP, pmp4, etc. But the success of the PCR depends even on the quality of the DNA. The DNA must be free of contaminants and nucleases that could impair the amplification process.

There is still no gold standard for PCR detection of *C. pneumoniae*. PCR is a technique theoretically capable of detecting a single copy of purified target DNA, but the procedure often lacks sensitivity, reproducibility, and specificity when applied to direct testing of clinical material **(3)**. The sensitivity of the reaction varies according to the primers, targets, clinical samples used and etc. **(6)**. And the unsatisfactory performance of PCR may be due even to the presence of inhibitors of the polymerase in clinical material.

Some authors report that PCR is 95% sensitive when the specimen is sputum, enzyme immunoassay is 80% sensitive, while culture technique is only 60% sensitive and 100% specific. With nasopharynx and throat samples PCR prove to be between 93-95% sensitive and 100% specific. This makes PCR, especially nested PCR **(7)**, superior to the other available laboratory methodologies. The recommendation of these authors is that in order to increase the sensitivity and specificity of clinical specimens for *C. pneumoniae*, the following combination of tests should be performed:

1. PCR as is the first-choice method for C. pneumoniae detection.
2. PCR results should be followed by southern blot using specific probe to C. pneumoniae.
3. Enzyme immunoassays for detection of IgG, IgM and IgA against specific peptide coding sequence for the major membrane protein of *C. pneumoniae* but not against *C. trachomatis*.

CONCLUSIONS:

1. The two methods for DNA extraction - phenol-chlorophorm-isoamyl alcohol method and the commercial Quiagen kit show close properties. The sensitivity of PCR performed after these extractions is equal when the specimens are tracheal and bronchial secrets.

2. The higher DNA yields from sputa in comparison with the tracheal or bronchial secrets my be due to the extra preliminarily treatment of the sputa with N-acetyl-cysteine, used to liquid them.

3. The difference in the sensitivity of the PCR with one degree with the commercial Quiagen kit as compared to the phenol-chlorophorm-isoamyl alcohol method may be explained with the higher possibilities of the kit for purification and preservation of the DNA.

4. No matter how sensitive is the PCR using sputa, tracheal and bronchial secrets these specimens are not easy of access and suitable as materials from children. So, examination of the DNA extraction and the sensitivity of the polimerase chain reaction is needed when the specimen used is throat swab.

5. Nested PCR assays are usually more sensitive than single-step PCR assays for the detection of *C. pneumoniae* in respiratory and PBMC specimens.

6. In spite of the high specificity and sensitivity of the PCR this method is still expensive and needs experienced staff.

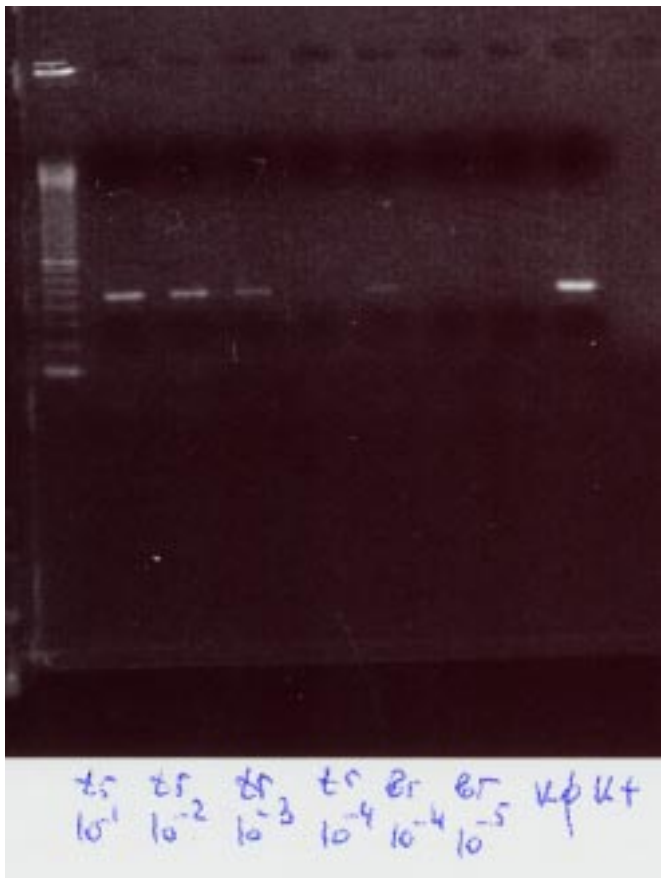
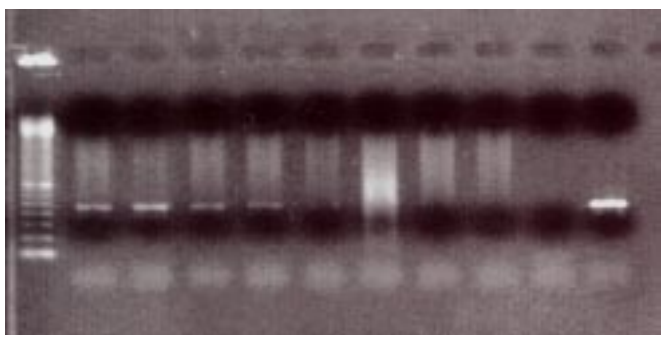


Fig. 1.



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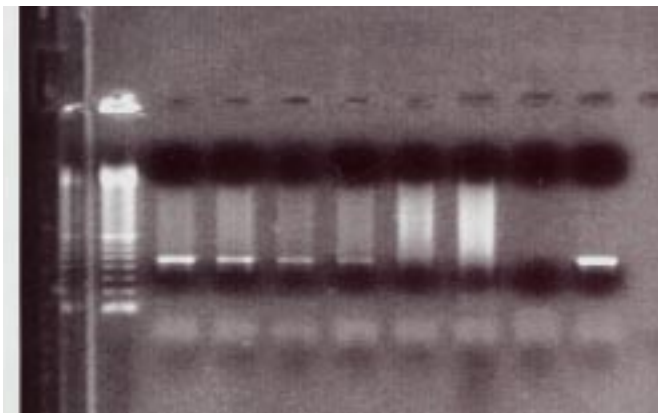

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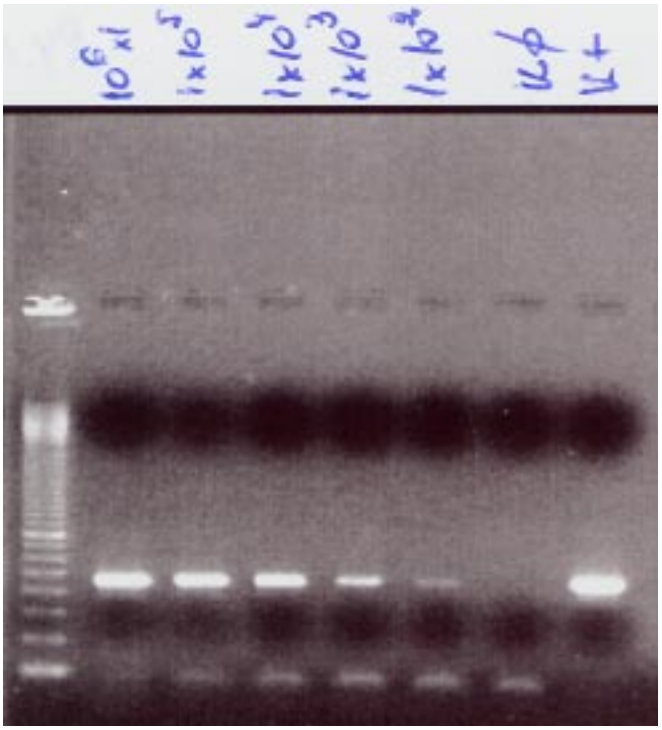

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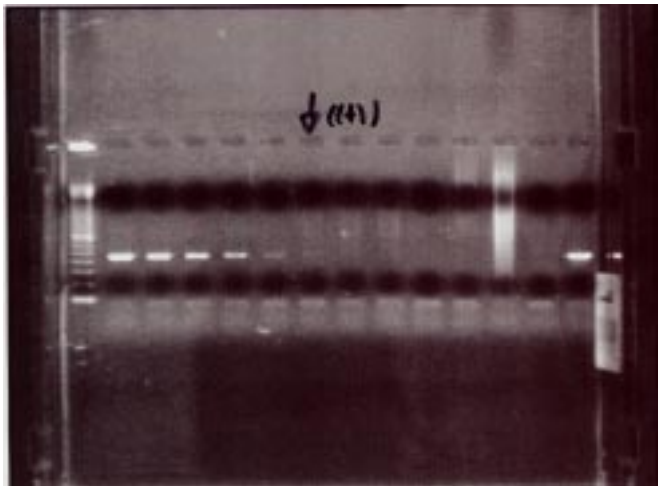

Fig. 5.

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Corresponding author:
Dr. Vessela Ouzounova
Department of Microbiology, Medical Faculty,
Medical University-Sofia;
2, Zdrave street,
Sofia 1431, Bulgaria
tel.: +359/2/ 9172580
mobile: +359/888/830274
e-mail: pumpi@abv.bg