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Research Paper

GENETIC CHARACTERIZATION OF *CHLAMYDIA PNEUMONIAE* USING RESTRICTION ENDONUCLEASE ENZYME (XBAL) IN DELTANS NIGERIA

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Restriction endonuclease enzyme (Xbal) is a cutting enzyme and as such was used to characterize the organism. Of the 124 samples collected from different locations in Mid-Western Nigeria, 95(76.61%) were positive while 29(23.38%) were negative for *Chlamydia pneumoniae* by DNA isolation. The enzyme cut the DNA of *Chlamydia pneumoniae* at several points as visualized on agarose gel placed on ultra-violet illuminator. Only 3 DNA could not be cut by this enzyme. This shows strain variation.

Keywords: DNA Isolation, Polymerase chain reaction, Chlamydia trachomatis, *Chlamydia pneumoniae*

INTRODUCTION

The order chlamydiales has one family chlamydiacease with the genus Chlamydia. The generic name Bedsonia has also been used in recognition of the pioneering work of Bedson and characterized the agent of psittacosis during it's prevalence in the early 1993's, however, by the rules of nomenclature Chlamydia has priority (Grayston *et al*., 1986). Chlamydia are non motile, coccoid, gram negative bacteria, ranging in sizes from 0.2 to 1.5 micrometer chlamydia lacking mechanisms for the production of metabolic energy, hence this restricts them to an intracellular existence where the host supplies energy rich immediate. Thus Chlamydia are intracellular parasite. Chlamydia can only reproduce within cytoplasmic vesicles of host cell by a developmental cycle. The small rigid, highly infective, extracellular elementary bodies are about 0.3 micrometre in diameter. The elementary bodies is then re-organised into a large one measuring 0.5-1micrometre and this lack electron dense necleod, within the membrane bound vacuole,the reticulate body grows in size, divides by binary fusions. In the inclusion elementary body may leave the host cell to infect a new cell (sGraston *et al*., 1986).

Chlamydia pneumoniae (Cpn) is a tiny bacterium which is often noted for causing a form of pneumonia. Up until the 1970's it was not even

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isolated and was mistaken for a virus. It was not until 1989 that J. Thomas Grayston and his associates named it as a separate species of the chlamydiae. Cpn is very difficult to culture and so, without modern laboratory techniques, also to study. It is an intracellular bacterium, which means that it invades the body cells, and it is obligate parasite, which means that it cannot supply it's own energy source and so takes over the energy machinery of the body cells it invades, depleting them and leaving the host cell less functional. Chlamydia is a genus of bacteria that are obligate intracellular parasites. Chlamydia infections are the most common bacterial sexually transmitted infections in humans and are the leading cause of infectious blindness worldwide. The three Chlamydia species include Chlamydia trachomatis (a human pathogen), Chlamydia sius (affects only swine), and Chlamydia muridarium (affects only mice and hamsters). Prior to 1999, the Chlamydia genus also included the species that are presently in the genus Chlamydophila: Two clinically relevant species, chlamydophila pneumoniae and chlamydophila pssitaci were moved to the chlamydophila genus.

Chlamydia pneumoniae is also refers to a common obligate intracellular bacterium that causes upper and lower respiratory infections worldwide (Grayston et al. 1990). In addition to acute infections,several chronic inflammatory diseases have been presumptively associated with *C. pneumoniae* infection. Increasing evidence implicates that a persistent lung infection caused by *C. pneumoniae* may contribute to the initiation, exacerbation and promotion of asthma symptoms (reviewed by Hahn 1999). A causal association between *C. pneumoniae* infection and asthma is biologically plausible based on the observations that asthma is a chronic inflammatory disease of the airways, and that chlamydiae are known to produce chronic inflammatory damage in target organs. Whether *C. pneumoniae* lung infections activate the same immunopathologic mechanisms that have been demonstrated for other chlamydial diseases has not been explored systematically.

C. pneumoniae infection has also been linked with atherosclerosis—another chronic inflammatory disease. Evidence of a serological association of *C.pneumoniae* with atherosclerosis and its complications was first presented by Saikku *et al*. (1988). Since then, a large number of seroepidemiological studies have confirmed these findings (reviewed by Leinonen and Saikku 2002). The actual presence of *C. pneumoniae* in atheroslerosis and its lesions has also been demonstrated in a number of studies methods and by various (reviewed by kuo and Campbell 2000, Taylor-Robinson and Thomas 2000). Moreover, the presence of C. pneumoniae-specific T lymphocytes in the atherosclerotic tissue specimens suggests that C. pneumoniae participates in the maintenance of the inflammatory response in the tissue and may thus be involved in the progression of the disease (Halme *et al*., 1999, Curry *et al*., 2000, Mosorin et al. 2000). In experimental animals, C.pneumoniae infection has been found to induce inflammatory changes and calcified lesions containing Chlamydia (Fong et al.1997, Laitinen et al. 1997) and to accelerate the development of atherosclerosis (Moazed *et al*., 1999).

Both asthma and atherosclerosis are chronic inflammatory diseases. C-reactive protein (CRP) is a typical acute phase reactant, whose concentration increases within hours of any tissues-damaging, inflammatory event, including acute

bacterial infection (Ablij and Meinders, 2002). Slightly elevated CRP levels, measured by high-sensitivity assays, have been shown to predict cardiovascular events (Rifai and Ridker 2001, Ridker *et al*., 2002). The role of CRP in asthma has been investigated.

Therefore heat shock proteins (Hsp) are a class of evolutionary highly conserved proteins, which are produced by all organisms in response to injury or stress, including infectio, to protect cells from unfavourable conditions (Kaufmann, 1990). It has been suggested that Hsp60 may play a role in the pathologenesis of chlamydial infections. It is overproduced in persistent infections (Beatty *et al*., 1994a), and it has been associated with the hypersensitivity and immunopathology seen in these infections (Morrison *et al*., 1989b). there is increasing evidence on the association between antibodies to chlamydial Hsp60 and the development of immunopathological damage following *C. trachomatis* infections (Peeling and Mabey 1999), but the role of Hsp60 in *C. pnumoniae* infections has not been clarified yet. Due to the high sequence homology between bacterial and human Hsps, these proteins have been postulated to be critical antigens in autoimmune diseases. There are studies suggesting that the host immune response to the development of atherosclerosis (Kol *et al*., 1998, Mosorin *et al*., 2002; and Wick *et al*., 2001).

MATERIALS AND METHODS

Area of Study

The area under study in this project work was ukwani local government area of Delta state.

Sample Collection

Early morning sputum samples were collected into universal containers from 124 patients in four selected zones within mid-western Nigeria. The categories of patients from whom samples were collected from were hospitalised patients confirmed to be suffering from upper and lower respiratory tracts infections, for example pneumonia,bronchitis, pharryngitis etc. and preserve in the refrigerator.

Sample Processing

Embroyonated eggs were incubated for 15 days to allow development of blood capillaries and or embryo. After which candling was done to detect the presence of capillaries and or embryo. Embryonated eggs were sterilized by cleaning the surfaces with formalin and a piece of cotton wool. Phosphate buffered saline was mixed with streptomycin and added to the sputum samples and homogenized, subsequently the yolk sacs were inoculated with 1 ml homogenized samples with the aid of sterile hypodermic syringe (Dianna) and seated using candle wax to seal the opening used in inoculation. These eggs were however labeled accordingly and incubated at 37 ^{o}C in an incubator (Gamllencap, Germany) for ten days to allow the organisms to grow.

Harvesting of *Chlamydia pneumoniae*

Organisms were harvested with the aid of sterile syringes after ten (10) days and giemsa staining was conducted to determine the presence of intracellular bacteria.

The Giemsa Stain Method

A smear of the organism was made and allowed to dry in air. It was fixed in absolute methanol for 1 min. Giemsa stain was added to cover the smear and left for 1 h. Deionised water was added to the giemsa stain on the slide (such that the ratio of water of stain was 2.1). slide was allowed

to dry in the air and observed under oil immersion objectives.

Extraction and Purification

The harvested isolates containing *C. pneumoniae* in 95 differently labeled syringes were introduced into a sterile eppendof tubes to undergo differential centrifugation by using a centrifuge (spectra). Firstly, at 3,000 rpm for 5 min, the supernatants were removed. The debris left in the eppendof tubes were again centrifuged, now at 16,000 rpm for 10 min. Again, the supernatants were discarded leaving behind the sediment containing the DNA (pellet). 25 μl of proteinase k (enzyme) was added using a micropipette to aid digestion of cellular protein. 10 μl sodium dodecylsulphate (SDS) was also added. This was done to ensure extraction of pure DNA. The mixtures contained in eppendof tubes were incubated in water bath (Clifton) at 50 °C for 1 h, 30 min to activate the enzyme. 500 μl of phenol (buffered) was added to the DNA (pellet). The eppendof tubes were inverted 100 times for proper mixing. Mixture was spun carefully at 16,000 rpm for 3 min. The water phase was transferred into a new eppendof tube leaving behind the pellet.

The DNA was precipitated by addition of equal volume of cold isopropanol and tubes was kept at 4 °C for 5 min. The tubes was spun at 16,000 rpm for 3 min and DNA redesolved in 50 μl distilled water. The eppendof tubes were votex with the aid of a votex mixer in order to mix properly and make the DNA visible. 5 μl of sodium acetate (NaAc) was added to the pellet. 2x volume of cold absolute ethanol was added and mixture was kept in the freezer at 20 °C for 20 min. Mixture was again spun at 16,000 rpm for 5 min to separate it and the supernatant was discarded leaving the pure DNA behind. The DNA was rinse with 70% ethanol. The DNA (pellet) was incubated at 37 °C for 15 min in water bath to evaporate the ethanol. The DNA (pellet) was incubated at 37 °C for 15 min in a water bath to evaporate the ethanol. 500 μl of sterile water was added to dissolve the DNA. 5 μl of DNA was transferred to a new sterilized eppendof tubes. 3 μl of enzyme buffer was added to each eppendof tubes, and also 1 μl of restriction enzyme (xba1) to aid the cutting of the DNA. 11 μl of sterile water was then added to make it up to 20 μl and incubated in a water bath at 37 °C for 1 h, 10 min.

Note that :1000 μl of sterile water was added to the restriction endonuclease enzyme (xba1) to dilute it before use.

Preparation of Agarose Gel

One (1g) gram of agarose powder was measured using a salinical balance, and dissolved with 100 ml of Tris acetic acid EDTA (TAE). Mixture was boiled in a microwave (national) for 2 min. Agarose gel was formed and allowed to cool to a temperature between 50-56 °C. 30 μl ethidium bromide dye was added to the agarose gel to make the movement of the DNA visible.

Preparation of TAE

Stock provided was 50x, and IX was the TAE required then using the formula;

M1V1 = M2V2

If M1 = 50x

And required strength (TAE) M2 = 1X.

V2 = 1000ml

Making V1 the subject of formula

V1 = M2V2/M1

V1 = 1x 1000/50

V1 = 20ml.

Hence, if 20 ml is taken from stock and then diluted with 1000 ml (1L), we would have 1x TAE.

Observation of Cut *C. Pneumoniae* DNA

15 μl of DNA (mixture) from different tubes were mixed with a drop of blue dye on a foil paper, agarose gel was poured unto the electrophoresis tank and allowed to set. The TAE (IX) prepared was poured unto the main electrophoresis tank (SCIE-PLAS). The DNA mixture from different tubes (samples) were placed in compartments (wells) in a soft agarose gel and subjected to electrical current. Same was done for the maker (blue dye) which serves as the control flow of electricity from negative to positive pole for 30 min cases the DNA pieces to migrate in the gel substrate towards the positive pole. The gel was removed and placed on the ultra-violet illuminator (UV tech. Ltd.) for observation of DNA bands.

RESULTS

Out of one hundren and twenty four samples collected from hospitalised patients in the different designated centres in mid-western Nigeria and screen for *Chlamydia pneumoniae* only 95 (76.61%) were positive while 29(23.38%) were negative. The result also shows that *Chlamydia pneumoniae* infection is highest in central hospital.

DISCUSION

The greatest percentage (76.61%) of positive samples detected from 124 sample collected, only 23.38% show negative result of the presence of *Chlamydia pneumoniae*. From this study, it shows that the incidence rate of Chlamydia pneumoniae infection in the mid-western Nigeria is high. These results conform to earlier epidermiological studies done among adult population worldwide (Aldous *et al*., 1992).

1 unit of restriction endonuclease enzyme (Xba1) is able to bring about it cutting activity on the DNA of *C. pneumoniae* at an incubation temperature of 37 ^{o}C. Almost all the bunds were cut at the point with only a few that could not be cut by this enzyme, indicating that it may be due to strain variation. This study goes to reveal that Chlamydia infections may be caused by a strain still unknown.

Table 1: Showing Location of Sample Collection for the Isolation of Chlamydia Pneumoniae Within the Mid-Western

Place of Sample Collected	Numbers of Sample Collected	Positive Samples for C. *pneumoniae*	Negative Samples for C. *pneumoniae*	% of Positive Samples	% of Negative Samples
General Hospital warri	27	20	7	74.07%	23.93%
University of Benin teaching hospital (UBTH)	27	20	7	74.07%	25.03%
Central hospital, Benin city	37	30	7	81.08%	18.92%
Irua specialist teaching hospital, irua (ISTH)	33	25	8	75.7%	24.24%

Note: Total 5 of incidence of C. *pneumoniae* in this region; Total positive sample/Total number of sample collected x 100/1; and 95/124 x 100/1 = 76.61%.

CONCLUSION AND RECOMMENDATION

Chlamydia pneumoniae is a common and important intracellular bacterium implicated in upper and lower respiratory tract infections in humans. Also, *Chlamydia pneumoniae* has been associated with chronic disease such as arteriosclerosis and asthma. Since *Chlamydia pneumoniae* can cause severe clinical disease, correct diagnosis and therapy are important issues. However, since extraction and characterization of DNA of *Chlamydia pneumoniae* is time consuming and tedious, therefore adequate care should be employed for accurate result.

However, more studies is possible existence of a new strain of *Chlamydia pneumoniae*.

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