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Isolation of extracellular vesicles induced by *Chlamydia pneumoniae* infection

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Isolation of extracellular vesicles induced by *Chlamydia pneumoniae* infection

Abstract: *Chlamydia pneumoniae* is an obligate intracellular parasite distinguished by the increased resistance based on the unique life cycle including the switches between several morphological forms of high pathogenicity, resistance or metabolic activity. Despite the high antibody prevalence and diverse transmission ways, these days there is no optimal method of diagnostics of chronic form of chlamydial pneumonia. Here we introduce the study aimed to establish and optimize the protocol for isolating the extracellular vesicles derived from infected A549 lung epithelial cells and THP-1 monocytes, as well as for initial evaluation of vesicles amount produced by treated and non-treated cells via the total protein content and the level of CD63 exosome marker.

Keywords: *Chlamydia pneumoniae,* extracellular vesicles, community acquired pneumonia, exosome markers, infection validation

CERCS: B230 – Microbiology, bacteriology, virology, mycology

***Chlamydia pneumoniae* infektsioonist põhjustatud ekstratsellulaarsete vesiikulite eraldamine**

Lühikokkuvõte: *Chlamydia pneumoniae* on kohustuslik rakusisene parasiit, mida eristab suurenenud resistentsus, mis põhineb unikaalsel elutsüklil, sealhulgas mitme kõrge patogeensuse, resistentsuse või metaboolse aktiivsusega morfoloogilise vormi vahel. Vaatamata kõrgele antikehade levimusele ja erinevatele levikuviisidele ei ole tänapäeval optimaalset meetodit klamüüdia kopsupõletiku kroonilise vormi diagnoosimiseks. Siin tutvustame uuringut, mille eesmärk on luua ja optimeerida protokolli nakatunud A549 kopsuepiteelirakkudest ja THP-1 monotsüütidest pärinevate ekstratsellulaarsete vesiikulite isoleerimiseks, samuti töödeldud ja töötlemata rakkude poolt toodetud vesiikulite koguse esialgseks hindamiseks. valgusisaldus ja CD63 eksosoomimarkeri tase.

Võtmesõnad: *Chlamydia pneumoniae,* ekstratsellulaarsed vesiikulid, kogukonnas omandatud kopsupõletik, eksosoomimarkerid, infektsiooni kinnitamine

CERCS: B230 – mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

AB	Aberrant body
BCA	Bicinchoninic Acid Assay
CHX	cycloheximide
CPAF	Chlamydia protease-like activity factor
DNA	Deoxyribonucleic acid
dNTP	Deoxynuclotid tryphosphate
EB	Elementary body
EV	Extracellular vesicle
FBS	Fetal Bovine Serum
HSPG	Heparan sulfate proteoglycan
IPA	Infectious Progeny Assay
MOI	Multicplicity of infection
MTOC	Microtubule-organising centre
OmpA	Outer membrane protein A
PCR	Polymerase chain reaction
RB	Reticulate body
T3SS	Type III Secretion System

INTRODUCTION

Chlamydia pneumoniae is an obligate intracellular bacterial pathogen infecting respiratory tract with demonstrated antibody prevalence of 50% by age 20 and 80% by 60-70 years old, which is suspected, in chronic form, to contribute to a range of life-threatening chronic inflammatory states, including but not limited to neurological diseases (multiple sclerosis, Alzheimer disease), atherosclerosis, asthma and arthritis [1-6].

Pathogenicity of these bacteria is strongly connected to their unique life cycle, which includes the switches between morphologically and functionally distinct forms of elementary body (extracellular form adapted for surveillance in harsh environment), reticulate body (intracellular form of increased metabolic activity capable of nutrient acquision, replication, modulating the proteome and cells cycle of host cells) and aberrant body (intracellular persistent form) [7].

Taking into account this ability to differenciate between the forms of elementary (EB), reticulate (RB) and aberrant bodies (AB), remain persistent in the organism and switch back to the aqute form, connection to inflammation pathologies and community-acquired pneumonia [8], there is a need for improved diagnostics approaches bypassing the disadvantages of currently applied techniques.

Extracellular vesicles (EVs) are considered to be a newly emerging source of markers for disease diagnostics. Described as lipid-bilayer enclosed extracellular particles of size, depending on classification and origin, going up to 1 μm, extracellular vesicles are shed by (virtually) all the living cells, transferring bioactive molecules and playing a key role in intercellular communication. Moreover, qualitative and quantitative characteristics of released vesicles can be altered by numerous internal and external factors, including but not limited to oxidative stress, inflammatory processes, viral and bacterial infection, changes in microenvironment etc [9, 10]. These factors may affect the size distribution of released EV fractions, their protein, lipid and nucleic acid content, which makes them a promising tool for diagnostics and monitoring of pathological states.

Applying the research on extracellular vesicles to the mentioned purposes requires advanced approaches for EV production, isolation and purification, which allow to obtain and analyze the assets of extracellular vesicles derived from the cells of different states. In this terms it is important to optimize the existing protocols compromising between desired yield (which is generally low), purity and essential features of studies biological processes.

Pointing the stated to *C. pneumoniae* infection process, there is a need to optimize the protocol for production of EVs upon chlamydial infection. Thus, it is generally recommended to cultivate the cells using the exosome-depleted media to produce the vesicle isolates of proper

purity and minimize the impact of FBS-derived vesicles on further research [11]. At the same time, there is no currently available data on compatibility of these media with in vitro *C. pneumoniae* infection development. Additionally, the time frames for vesicles production described in most research papers are set in range of 24 to 72 hours with respectively increased yield of isolated EVs, which, according to the existing data, may overlap the life cycle of chlamydial pathogens and lead to EB released into the medium and co-isolated with target extracellular vesicles. Taking into account the overlap in size ranges of EVs and chlamydial bodies, their co-isolation may significantly decrease the sencitivity and reproducibility of EV analysis, especially when it comes to size distribution of exosome assets.

This research is dedicated to evaluation of *C. pneumoniae* infection compatibility with exosome-depleted cell culture medium and establishing the time-optimized protocol for isolation of the vesicles released by infected lung epithelium (A549) cells and monocytes (THP-1) representing the well-known sites of chlamydial infection and persistence [12] via infection validation through IPA and endpoint PCR experiments, assessing the total protein content (BCA assay) of isolated vesicles, levels of CD63 membrane-exposed exosome marker. In addition, isolates were checked for apolipoprotein B potentially co-isolated from the media for the purpose of ensuring the purity.

1. LITERATURE REVIEW

1.1 *CHLAMYDIA PNEUMONIAE*

1.1.1 Epidemiology, symptoms and diagnostics challenges

Transmitted from human to human, *C. pneumoniae* causing respiratory tract infections of pharyngitis, bronchitis and sinusitis, considered as one of the main factors of community-acquired pneumonia responsible, as of certain reports, for 1–2% of all cases of CAP in children, mainly affecting those over 3 years of age [13]. Other reports indicate the frequency between 5 and 20% in school-age children and adolescents [14]. Epidemiological studies suggest a cyclical pattern (every 4 years) of pneumonia caused by *C. pneumoniae*. In a worldwide study, the frequency of CAP due to *C. pneumoniae*, was 8% in North America, 7% in Europe, 6% in Latin America, and 5% in the Asia-Africa region [15].

Current diagnostics approaches, including cell cultures, real-time PCR targeted to detect ompA gene, 23S and 16S rRNA, and immunohistochemistry techniques are often time consuming and demanding in terms of expertise. Additionally, certain techniques a characterized by limited sensitivity (e.g. usage of McCoy and HeLa cell cultures still considered the gold standard despite sensitivity limit of 50 to 70 %) [13].

1.1.2 Life cycle and persistence

The developmental cycle of *C. pneumoniae* and other *Chlamydia* spp (fig. 1) starts with binding chlamydial EBs [16] to the membrane of host cell mediated by HSPGs, which is followed by inclusion forming and early genes transcription triggered by pre-synthesized T3SS (protein needle-like assembly on the surface of chlamydial bodies facilitating the effector injection across the host cell membrane [17]) effector. Formed inclusions are known to rapidly escape the canonical endolysosomal pathway as the inclusion membrane is modified and inclusion itself is redirected upon exposure to early protein effectors [18] (e.g. chlamydial Incs nad CPAF proteins playing essential for escaping endosomal pathway, maintaining inclusion integrity and promoting the virulence by modulating antibacterial pathways of apoptosis and complement system [16, 19, 20]).

Within next 24-72 hours inclusions are promoted to asynchronic EB-RB differentiation and RBs replication via binary fissions [16]. At this stage chlamydial inclusions are capable of establishing the intracellular niche by migrating to the microtubules organizing center (MTOC), alterin membrane dynamics, stabilizing itself by means of intermediate filaments dynamic scaffold, nutrient aqcuision and interaction with mitochondria, smooth EPR and peroxisomes in order to compensate the lack of certain methabolic pathways due to decreased genome compared to other bacteria. The cycle is finished with reversed RB-EB switch and release of EBs from the host cell by apoptotic or non-apoptotic way (extrusion), which was particularly shown for

Chlamydia pneumoniae. The life cycle can be reversibly arrested by stress factors including starvation and exposure to antibiotics targeting the cell wall synthesis machinery, which results in formation of Aberrant bodies responsible for chronic asymptomatic form of chlamydial infection [7].

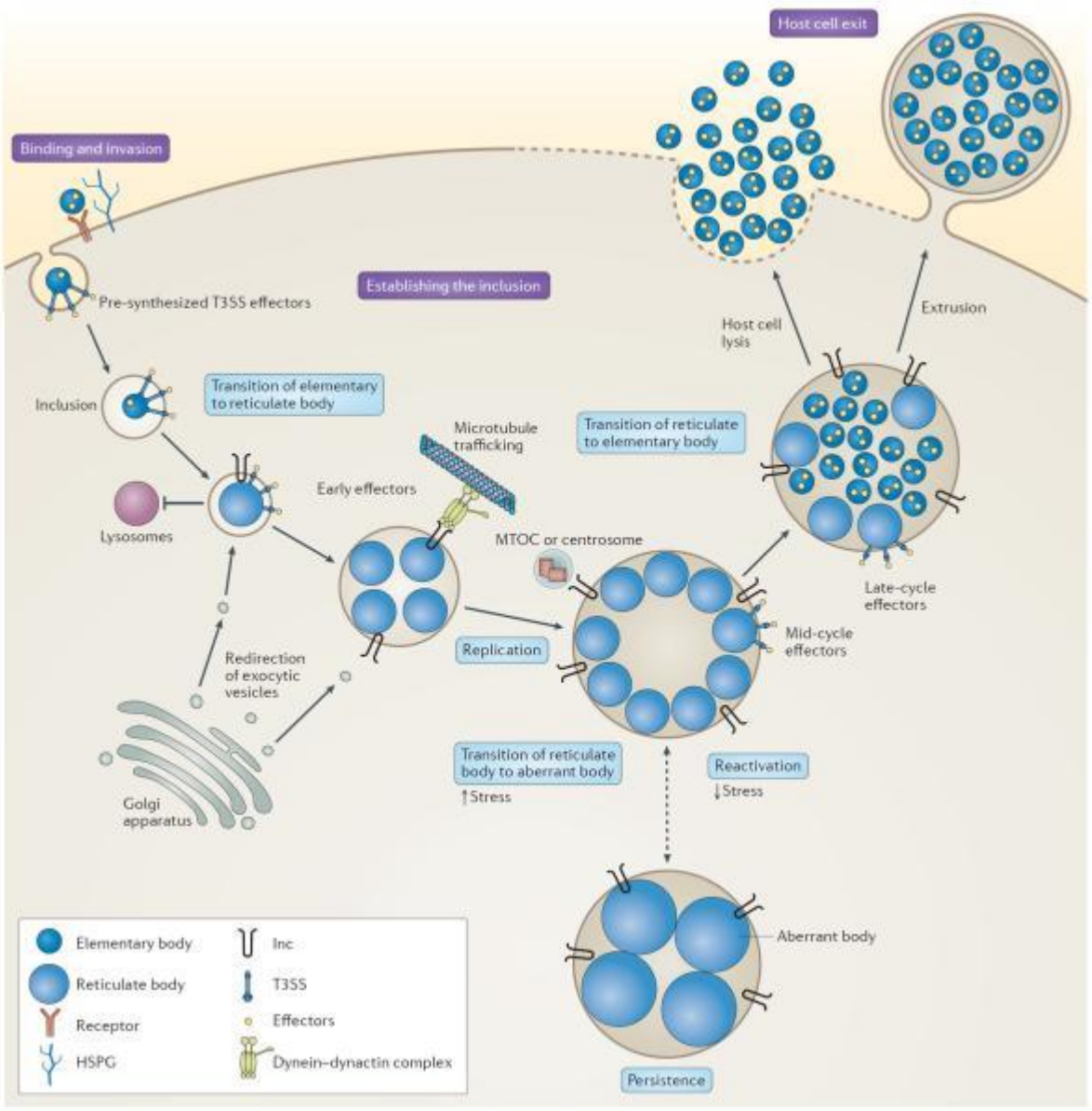

Figure 1 – The life cycle of Chlamydia spp [7]

1.2 EXTRACELLULAR VESICLES

1.2.1 Origin and classification

According to their size, content, release pathway, function and biogenesis, extracellular vesicles are conventionally divided into three main subtypes: microvesicles (MVs), exosomes and apoptotic bodies (fig. 2).

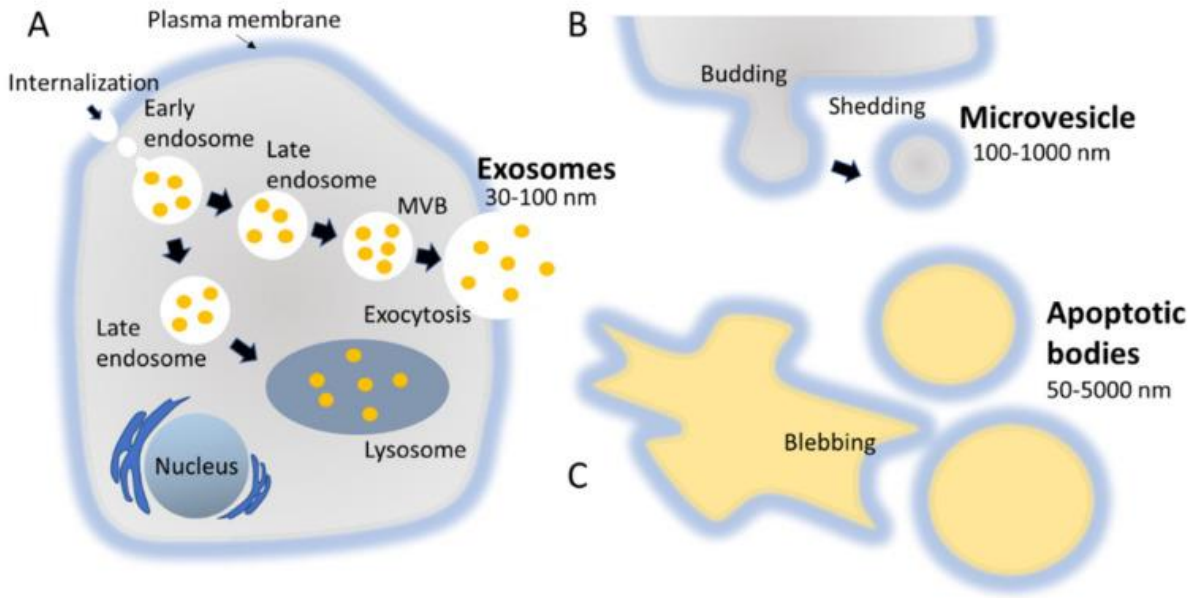

Figure 2 – Biogenesis pathways of extracellular vesicles [21]

Exosomes of 30 to 150 nm in diameter are produced through formation of multivesicular bodies (MVBs) originating from inward budding of the cell's plasma membrane. This type of vesicles is specifically involved in sorting, recycling, storage, release and transport of proteins. Exosome release or lysosome fusion is correlated to the cholesterol enrichment of exosome membrane (enriched species are likely to be released) [10].

Microvesicles are EVs formed via direct outward budding of plasma membrane. Vesicles of this type may range in size from 100 nm to 1 μm [10], significantly enriched (100-fold reported compare to cell lysates) in cytosolic proteins and function as a form of cell-cell communication being able to package and deliver active cargo molecules [22].

Apoptotic bodies are reported to range in size of 50 to 5000 nm . Unlike exosomes and microvesicles, their content can be distinguished by intact organelles, chromatin and glycosylated proteins in addition to organelle-associated protein molecules [10].

1.2.2 Lipid, protein and nucleic acid content

Proteins found in extracellular vesicles are mostly associated with biogenesis pathways. Thus, EVs are typically enriched with different types of tatraspanins (e.g. CD9, CD63 and CD81 are listed among the most abundant and actively used as exosome markers), EGFR signal transduction protein, molecules responsible for antigen presentation (e.g. MHCI and I derived from antigen-presenting immune cells) and other transmembrane proteins specific to the cells of origin. Additionally, Notch and Wnt transcription factors normally localized in the nucleus were detected in EVs [21, 23].

Lipid composition of extracellular vesicles also tends to share the general features with the cells of origin. Nonetheless, part of the membrane lipids were found to be specifically associated with EV subtypes derived mainly from multivesicular bodies and facilitating the uptake and internalization fo vesicles. This, vesicles are typically enriched in desaturated lipids, cholesterol and sphingomyelin, ceramide and GM3 ganglioside, whereas the contect of phosphatidylcholie and diacylglycerol. In terms of membrane asymmetry of lipid composition, MVB-derived exosomes contain more phosphatidylserine facing the environment compared to cell plasma membrane [23].

Analysis of nucleic acid content shows the common enrichment with tRNAs, ribosomal 18S and 28S rRNA, as well as the presence of long and short non-coding mRNA and miRNA. Most RNA fragments encapsulated inside the vesicles do not exceed the limit of 200 nucleotides (with rare exceptions extended to 4 kb). RNA species were shown to associate with ribonucleoproteins (RNPs), high and low density lipoproteins (HDLs, LDLs) incorporated into vesicular membrane. Studies towards the DNA content of EVs generally reveal extremely low abundance of this nucleic acid type [23, 24].

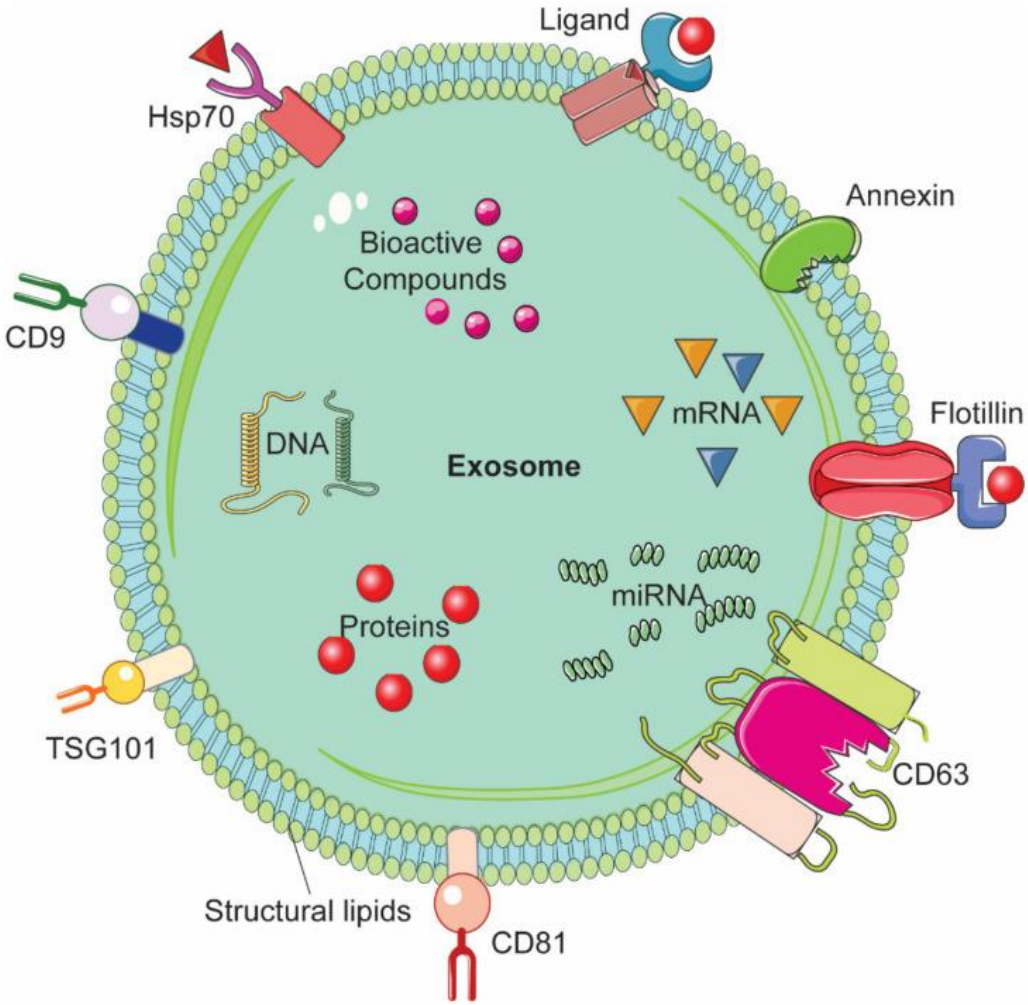

Figure 3 – Lipid, protein and nucleic acid content of EVs [21]

1.2.3 Isolation, characterization and quantification

Current set of routinely available EV isolation techniques includes the approaches based on size and immune affinity of target vesicles. The choice of some techniques over other is mostly determined by desired purity and recovery rate of isolated vesicles (fig. 4).

Isolation Technique	Recovery	Purity	Sample Volume	Time Required
Ultracentrifugation	5–25%	Low	100s of mLs	8 h
Density Gradient	Higher than UC	Similar to UC	up to 1 mL	20 h
Precipitation Kits	N/A	Low	>100 µL	Overnight
ExoChip	N/A	N/A	<400 µL	<2 h
Immunoprecipitation	>99% bead recovery	Higher than UC	up to 1 mL	Overnight
ExoSearch Chip	42–97%	Higher than UC	20 µL	40 min
Acoustic Nanofilter	>80%	High	50 µL	<30 min

Figure 4 – Comparison of exosome isolation techniques [10]

Ultracentrifugation techniques include differential ultracentrifugation and density gradient ultracentrifugation. Despite relatively low purity and recovery rates, as well as challenging upskaling and time consumption, these techniques are often preferred for little technical expertise required, affordability and no need for sample pretreatment. Other size-based techniques, including ultrafiltration, hydrostatic filtration dialysis (HFD), Flow Field-Flow Fractionation (FFFF), sequential filtration and size exclusion chromatography, are mainly distinguished by required sample volumes and recovery rates.

Immunoaffinity based techniques (ELISA and magneto-immunoprecipitation) are beneficial for their capability to capture the target exosome fractions with high specificity from the liquid phase based on the immune affinity of surface-exposed proteins. These approaches, however are challenging to apply for the clinical settings as they require the sample pretreatment

by ultracentrifugation or ultrafiltration (ELISA). At the same time, magneto-immunoprecipitation allows better preserving of exosomal proteins activity and doesn't require advanced instrumentation.

Precipitation techniques for exosome isolation can be performed using PEG or lectin. Both approaches require sample pretreatment (filtration/ultracentrifugation). Immunoaffinity techniques can be additionally combined with microfluidic approaches.

Exosomes can be analyzed based on their chemical and physical properties (fig. 5). Thus, Physical analysis methods include Tunable Resistive Pulse Sensing, Transmission Electron Microscopy, Nanoparticle Tracking Analysis and Dynamic Light Scattering. These four techniques are primarily targeted to evaluate the size distribution of obtained EV isolates by assessing zeta-potential, tracking the Brownian motion (NTA), or light scattering.

Immunodetection approaches of Western Blotting and Flow Cytometry are commonly used for compositional analysis, which can be upscaled and extended using MS techniques and microfluidic approaches.

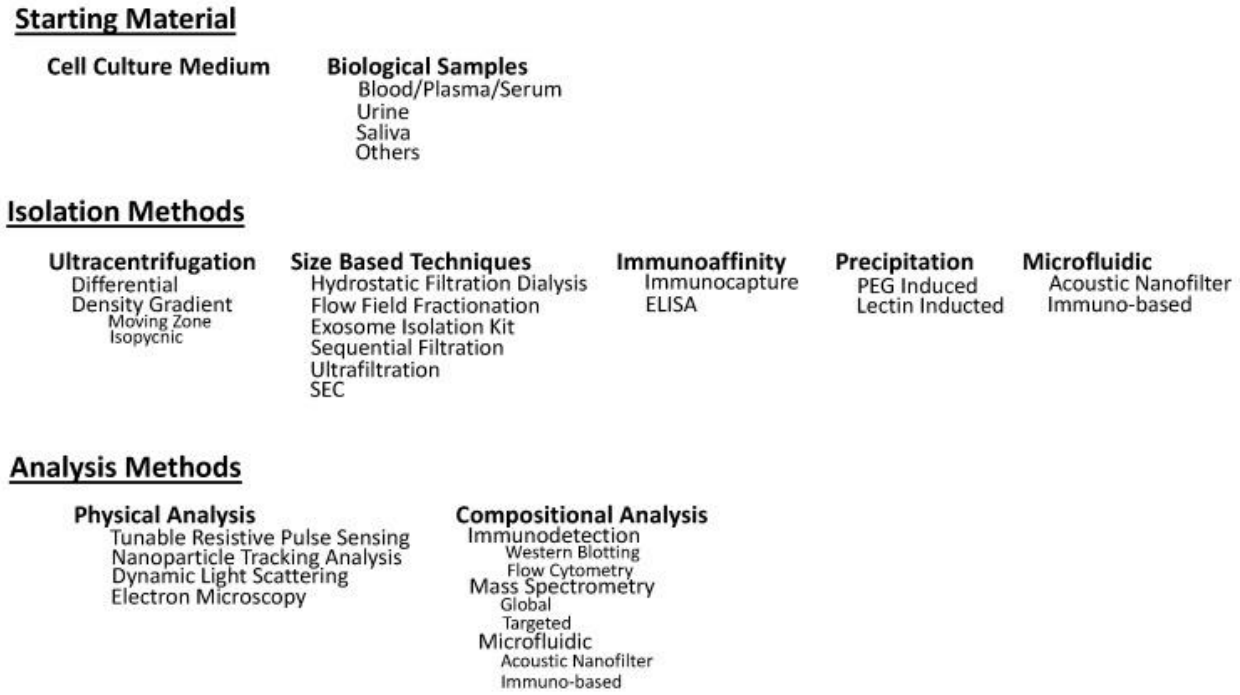

Figure 5 – Overview of EV isolation and analysis methods [10]

1.2.4 Extracellular vesicles in disease diagnostics

Collected from the main biological fluids (breast milk, amniotic and cerebrospinal fluids, blood plasma, alveolar lavage etc), extracellular vesicles have been investigated as non- or low-invasive liquid biopsy biomarkers (fig. 6) [25].

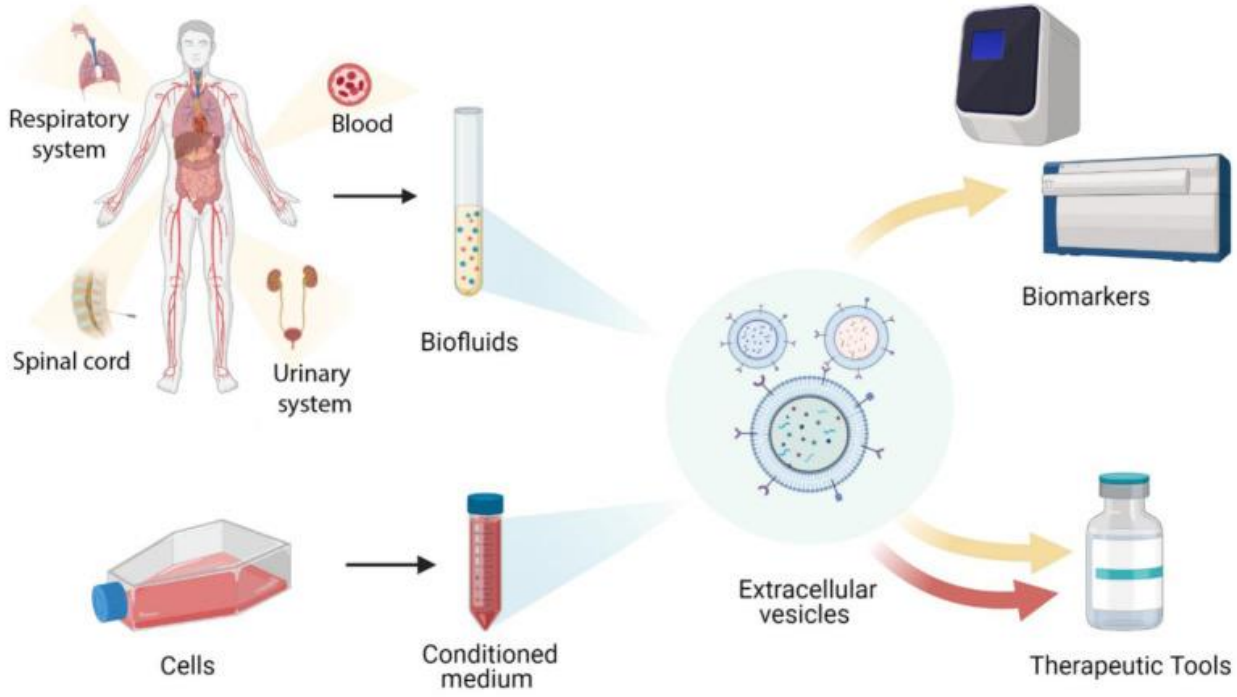

Figure 6 – Sources of extracellular vesicles for diagnostic purposes [25]

Thus, EVs circulating in urine were described as a suitable urinary fraction for prediction of post-transplantation kidney dysfunctions [26]. Saliva-derived vesicles were demonstrated to be reliable markers of chronic inflammations of oral mucosa, pancreatic and lung cancer [27-30]. Exosome isolates from CSF (cerebrospinal fluid) have been excessively investigated for upregulated biomarkers of Alzheimer's and Parkinson's disease [31]. Proteomic profiles of extracellular vesicles derived from human breast milk can be analyzed for the purpose of nutritional quality evaluation [32]. Exosomes circulating in blood are of particular interest in the context of non-invasive tumor diagnostics and assessing the treatment response [25].

2. THE AIMS OF THE THESIS

As of the given theoretical background on the scientific gaps and problems, the research was aimed to achieve the following goals:

- establish the protocol for isolation and purification of extracellular vesicles derived from adherent and suspension cells infected with *Chlamydia pneumoniae* optimizing between extended production time needed for increasing the yield of EVs and chlamydial life cycle time frames for the release of newly produced chlamydial particles, which, due to their size, may interfere with extracellular vesicles within the workflow of studies towards the size distribution;

- evaluate compatibility of exosome-depleted medium usage for EVs production with cell infection process;

- evaluate the effect of *C. pneumoniae* infection on EVs production by accessing the protein content of EB isolated.

3. EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Materials

A549 and THP-1 cell lines for the experiments were received from ATCC and cultivated using DMEM Low Gulcose (Biowest®) and 1X RPMI-1640 (Cyvita HyClone™) media respectively. Both media were supplemented with heat inactivated FBS (Biowest®, sterile filtered) and 50 mg/mL gentamicin (Gibco™) to the final concentrations of 10% and 0.02 mg/mL. Additionally, DMEM Low glucose medium included 200 mM L-glutamine (final concentration – 2 mM), whereas 50 mM mercaptoethanol was added to the RPMI-1640 mix to reach the final concentraition of 0.05 mM. For *C. pneumoniae* infection and EV production both media (referred to as exo-free) were supplemented with exosome-depleted FBS prepared by overnight ultracentrifugation at 110 000 g, 4°C (Beckman Coulter Optima™ L-80 XP Ultracentrifuge, SW 32 Ti Swinging-Bucket Rotor Package, 38.5 mL Open-Top Thinwall Ultra-Clear Tubes) followed by 0.20 μm filtration (Millex Samplicity® Filter) in order to minimize the impact of vesicles from the serum on the conducted experiments.

The set of polystyrene plasticware used in experiments was provided by ThermoFisher™ and included T175 EasYFlask™ flasks for cell culturing, infection and EV production, Nunclon™ Delta surface treated 24-well plates for IPA experiments and Nunc MicroWell™ 96-well plates as a part of BCA setup.

Monitoring the cell number at the stages of cell infection and sample collection was performed using the kit by Invitrogen™: Countess™ 3 Automated Cell Counter, Countess™ Cell Counting Chamber Slides and 0.4% Trypan Blue Stain solution.

Stocks of *C. pneumoniae* CV-6 EBs ($4.17 \cdot 10^9$ IFU/mL) for infecting the cells were previously produced at the anti-infectives research group, University of Helsinki, according to the established protocol and stored at -80°C.

Further materials and equipment for sample collection and storage: Sarstedt™ Screw cap tube, 50 ml; Sarstedt™ Screw cap tube, 15 ml; 1.5 and 2.0 mL Eppendorf Safe-Lock® Tubes; 2.0 mL Eppendorf Protein LoBind® Tubes; freshly mixed RIPA buffer (150 mM NaCl, 1% NP-40 Surfact-Amps™ Detergent, 0.5% sodium deoxycholate, 0.1% SDS, 25mM 100-mM TRIS + Pierse™ Protease Inhibitor, 1 tablet per 10 mL); 1 mg/mL cycloheximide (CHX); Eppendorf™ 5810 R benchtop centrifuge; Thermo Scientific™ Fresco™ 21 Microcentrifuge.

The list of commercially available sets used for the samples collection and characterization also included GeneJET Genomic DNA Purification Kit and Micro BCA™ Protein Assay Kit by Thermo Scientific™, which were used according to the protocols provided by the manufacturer.

Characterization of samples' protein content by SDS-PAGE and Western Blot required usage of four aqueous buffers: 1X TBST (NaCl – 8.8 g/L, Tween-20 – 0.05% v/v, 1M Tris/HCl pH7.5 – 1% v/v) used as a base for blocking and antibody solutions, 1X Running and 1X Transfer buffers (Tris – 3.03 g/L, glycine – 14.4 g/L, 1% v/v of 10% SDS for Running buffer or 20% v/v of methanol for Transfer buffer), Stripping bufferglycine – 7.5 g/L, HCl_{conc} – 0.5%). The samples were tested for EV and lipid drops markers using the following antibodies: Purified Mouse Anti-Human CD63 (BD Pharmingen™), diluted 1:1000 in 1% BSA/1X TBST; Anti-h ApoB 2101 SPTN-5 (Medix Biochemica™), 1:1000; Pierce® Goat Anti-Mouse IgG, (H+L), Peroxidase conjugated, 1:10 000 (reconstituted in 2 mL of sterile mQ water for 2 hours at room temperature). Western blot experiment setup included 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 4x Laemli Buffer (Biorad™), Precision Plus Protein Dual Color Standards (Biorad™), 0.45 μm Nitrocellulose Membranes (Biorad™), SuperSignal™ West Pico PLUS Chemiluminescent Substrate and ChemiDoc MP Imaging System (Biorad™). Western Blot experiments were preceded with bicinchoninic acid assay.

Infection efficiency was evaluated by Infectious Progeny Assay and Endpoint PCR. The set of IPA materials consisted of Chlamydia LPS Monoclonal Antybody B410F (Invitrogen™), 1:100 in 0.2% BSA, Alexa FluorTM 488 goat anti-mouse IgG γ1 (Invitrogen™), 1:2000 in 0.2% BSA, 0.5% BSA blocking solution, 0.1% Evans Blue solution, Pathfinder® Mounting Media (Biorad™) and Invitrogen EVOS™ FL Imaging System.

Composition of materials for PCR analysis was kept as follows: mastermix (per reaction) – 10X ThermoScientific DreamTaq™ Buffer (10% v/v), ThermoScientific™ dNTP mix (2% v/v), ompA primers (4% v/v each; forward – 5'-TCC GCA TTG CTC AGC C-3', reverse – 5'-AAA CAA TTT TGA AGT CTG AGA A-3', 126 bp), 5U/μL DreamTaq DNA Polymerase (0.5% v/v); agarose gel – agarose (0.15 g/mL), 1X TAE buffer (TRIS, 4.84 g/L; glacial acetic acid, 0.11% v/v; 0.5M ETDA pH 8.0, 0.2%), SYBR™ Safe DNA Gel Stain, Invitrogen™ (0.01% v/v); 6X TriTrack DNA Loading Dye (Thermo Scientific™). Biorad T100™ Thermal Cycler was used for PCR amplification. PCR experiments were preceded by measuring the DNA concentration using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer.

3.1.2 Cell culture

FBS heat inactivation procedure was performed by incubating the liquid serum at 56°C for one hour. The media supplemented with inactivated FBS were then used for taking up and further passaging of frozen A549 and THP-1 cells. The protocol of thawing the cells included the steps of resuspension in 5 mL of appropriate medium pre-warmed to 37°C followed by centrifugation at 1500 rpm for 3 mins for complete elimination of DMSO, resuspension in 10 mL of fresh medium

and further incubation in 20 mL of DMEM Low Glucose of RPMI-1640 medium at 37℃ with 5% CO2 and 95% air humidity. A549 lung epithelium cells were cultivated in form of adherent cell culture, whereas THP-1 monocytes were kept in suspension. Both cell cultures were splitted in 1:8 (A549) and 1:4 (THP-1) ratio each 72 to 96 hours allowing to reach the cell numbers useable for the following experiments.

3.1.3 *C. pneumoniae* infection

The pre-requirement for infecting A549 cells was to seed the fixed number of the cells to account for the needed volume of chlamydial EBs stock for infection and ensuring the reproducibility. For this purpose $3 \cdot 10^5$ live A549 cells were incubated for 24 hours at 37℃ with 5% CO2 and 95% air humidity allowing them to adhere and reach the starting point confluency of approximately 30%. Adherent cells were then incubated for 2.5 hours with the minimum volume of exo-free DMEM Low Glucose medium (negative control) or *C. pneumoniae* CV-6 EBs inoculum (infected control) needed to cover the surface of T175 flask.

For infecting THP-1 suspension cells the protocol was modified by performing the incubation step in 50 mL falcon tubes instead of culture flasks for the purpose of increasing the probability of contacts between the cells and chlamydial bodies. Aliquots containing $2 \cdot 10^6$ live cells were mixed with the volume of chlamydial stock corresponding to the target multiplicity of infection or the same volume of non-infected exosome-depleted RMPI-1640 medium. After 2.5 hours of incubation at 37℃ the whole volume was transferred to cell culture flasks.

Both cell types were infected at MOI1 assuming the equal numbers of the live cells and chlamydial inclusion forming units. Each experiment also included the cell-free medium control incubated in parallel with infected and negative control groups. Additionally, in order to initially evaluate the dose dependency of EV production and release of chlamydial particles into the medium, A549 cells were infected at MOI0.1 and MOI0.2.

Cells and medium controls were incubated for 72 hours at 37℃ with 5% CO2 and 95% humidity, and proceeded to the sample collection.

3.1.4 Sample collection

The samples of cells and media were collected after 72 hours of incubation. 40 mL of each medium were collected at stored for up to 72 hours at 4℃. Media collected from infected and non-infected cells were aliquoted for IPA (2x 400 µL) and DNA isolation for endpoint PCR (500 µL) IPA samples were supplemented with 0.4 µL of 1 mg/mL cycloheximide and stored at -80℃.

The cells then were collected by scraping (adherent), washed and resuspended in 1X PBS. After assessing the total cell number (mixing with trypan blue in 1:1 ratio) cell suspension was

aliquoted to achieve the following cell number to be used for IPA, total protein and DNA isolation: IPA – 2 x 4 · 10^5 cells, protein isolation – 1 · 10^6 cells, DNA isolation – up to 2 · 10^6 cells. After centrifugation at 250 g for 5 minutes IPA cell samples were resuspended in 500 µL of medium-cycloheximide mix (1 µg/mL) pre-cooled to 4℃ and stored at -80℃.

Protein isolation was done by resuspending the cell pellets in 500 µL of RIPA buffer and incubation on ice for 30 minutes. The lysate was then centrifuged at 13 200 rpm 4℃ for 10 min. Collected supernatant was stored at -20℃.

The cell genomic DNA was isolated and purified according to the manufacturer guidelines [33]. Cell aliquots were pelleted down by centrifugation at 250 g for five minutes at room temperature. Obtained pellet was resuspended in 200 µL PBS, mixed with 200 µL Lysis solution, 20 µL of Proteinase K solution and incubated at 56℃ for 10 minutes. Then we incubated the mix at room temperature for 10 minutes with 20 µL RNas A solution added. Reaction was stopped by adding 400 µL of 50% ethanol solution. The whole volume was transferred to the silica-based spin column and sequentially centrifuged with 500 µL of two EtOH-resuspended wash buffers (8000 g for 1 min, 21 000 g for 3 min). Purified DNA was collected using 200 µL elution buffer (8000 g for 1 min) and stored at -20℃.

3.1.5 Isolation of extracellular vesicles

Protocol for EV isolation from the medium was modified from Kuldkepp et al, 2019 [34]. Briefly, the cell culture media depleted from FBS-derived EVs were subjected to sequential centrifugations at 4℃: at 300 g for 5 min to remove cell debris, at 2000 g for 20 min to collect the large EV fraction (referred to as 2K) and at 110 000 g for 90 min to isolate the small EV fraction (referred to as 110K). Collected pellets were washed with PBS and resuspended in 200 µL RIPA buffer in order to prevent the protein loss in storage time range. Protein isolation was performed using the protocol for cell protein isolation.

3.1.6 Endpoint PCR

DNA content of samples was quantified through absorbance measurement at 260 nm. Reaction mix of 10.25 µL mastermix, sterile mQ water and DNA isolate (ar the volume containing 50 ng DNA was subjected polymerase reaction with the following thermocycler profile: 1 cycle Initial Denaturation (95℃, 1 min); 40 cycles of Denaturation (95℃, 30 sec) – Primer Annealing (60℃, 30 sec) – Elongation (72℃, 1 min); 1 cycle of Final Elongation (72℃, 5 min); Hold at 4℃.

Mix of 10 µL PCR product and 2 µL of 6x TriTrack DNA Loading Dye was heated at 65℃ for 10 minutes, cooled down on ice and run through the gel electrophoresis at 150V for 120 minutes.

3.1.7 Infectious Progeny Assay

In order to validate the presence or absence of chlamydial inclusions in analyzed samples, A549 cells were seeded in 24-well plates on coverslips (seeding density of 400 000 living cells per well), incubated to adhere for 24 hours at 37℃ and co-incubated with 300 µL of sample (see the IPA sample collection) lysates (lysed by vortexing with 1-2 sterile glass beads added), including the lysates of infected and non-infected cells and media. To ensure the contact between A549 cell layer and chlamydial particles in the sample lysates the plates were centrifuged at 550 g for 1 hour and additionally incubated for 1 more hour at 37℃. Then we replaced the lysates with medium-CHX mix (1 µg/mL) and incubated the cells for 72 hours (for the purpose of initial screening we also performed this procedure with the incubation time decreased to 48 hours).

After 72 hour incubation the cells were fixed with methanol for 10 minutes, coverslips were blocked with 0.5% BSA aqueous solution for 15 min at room temperature, incubated in a humified chamber (37℃) in the dark with Chlamydia LPS Monoclonal Antibody (1:100 dilution in 0.2% BSA, 1 h), triple washed with PBS, incubated with secondary antibody (Goat anti-Mouse IgG1 Cross-Adsorbed, Alexa Fluor™ 488, 1:2000 dilution in 0.2% BSA, 1 h) and counterstained with Evans Blue 0.1% solution in mQ water for 20 min at room temperature in the dark.

Triple washed with water, dried coverslips were transferred to the mounting media and analyzed using a fluorescent microscope (20X magnification, 70% light intensity, GFP channel).

3.1.8 Bicinchoninic Acid Assay

Following the microplate procedure protocol from the Micro BCA Protein Assay Kit Manual [35], the protein isolates of infected (infected control) and non-infected cells (negative control), Extracellular vesicles derived from infected cell culture medium, non-infected medium and non-conditioned medium (medium control) were mixed 1:1 with the working reagent (MA:MB:MC component ratio kept at 25:24:1), incubated at 37℃ for 2 hours and subjected to the absorbance measurement at 562 nm to quantify the protein content using the BSA-based standard curve. BCA data were used for normalizing the volumes of samples (to the lowest protein content of vesicle samples) to be used for the Western Blot experiments.

3.1.9 Western Blot

Normelised volumes of protein RIPA solutions of each sample (cells and vesicles derived from infected and non-infected cells of both types) were mixed with 7.5 µL of 4x Laemli Buffer (non-reducing conditions) and mQ water to reach the volume of 30 µL, and incubated at 90℃ for 15 minutes. 28 µL mix per well were transferred to Mini-PROTEAN TGX Stain-Free Gel (4-20%)

and processed to SDS-PAGE at 60 V for 30 min and 200 V for 30 min. The gels were activated (5 minutes) using the ChemiDoc system, and the separated proteins were transferred to nitrocellulose membranes (110 V for 75 min at 4℃).

Membranes were blocked with 5%BSA for 1 hour at room temperature, incubated overnight with primary (anti-CD63 or, after stripping, anti-ApoB, 1:1000 dillution in 1% BSA, 4℃) and for 1 hour with secondary (GAM, 1:10 000, room temperature) antibodies.

Procedure was followed by incubation with the chemiluminescent substrate (5 min, RT) and developing using the ChemiDoc system.

3.2 RESULTS AND DISCUSSION

3.2.1 *C. pneumoniae* infection development

Cell counting (fig. 7) performed during sample collection revealed sustainable increase in cell number over 72 hours of infection. Given the seeding densities of A549 and THP-1 cells ($1.5 \cdot 10^4$ cells/mL and $1 \cdot 10^5$ cells/mL), it is possible to observe the average of 44-fold increase for non-infected A549 cells. Comparing these data to the number of infected cells at multiplicity of 0.1, 0.2 and 1, infection does not show any statistically significant dose dependency. At the same time, it is visible that increase in cell number for *C. pneumoniae* affected cells remains lower than corresponding increase of non-treated cells. This effect was shown for both A549 and THP-1 cells and may be explained by certain features of intracellular interactions between the host cells and Reticulate bodies of *C. pneumoniae* featured as a morphological form of increased metabolic activity. This suggestion is supported by the literature data describing ability of Chlamydia spp to scavenge the nutrients, alter the host cell transcriptome and proteome, and modulat the cell cycle of a host cell inducing early mitotic exit via bypassing the spindle assembly checkpoint. In particular, ability to mediate the cell cycle arrest in vitro was shown for *C. pneumoniae*, which is suspected to happen through binding the T3SS effector (CopN) to microtubules resulting into impaired assembling.

In terms of protocol validation and optimization, these data demonstrate that the cells are able to multiply during chlamydial infection despite chlamydial treatment itself combined with possible stress connected to the switch to exosome-depleted medium and some possible lack of quorum sensing at the given seeding densities at the initial timepoint 0 of infection process. This conclusion is additionally support by A549 cells reaching the confluence close to 100% over 72 hours for both treated and non-treated cells (fig.8).

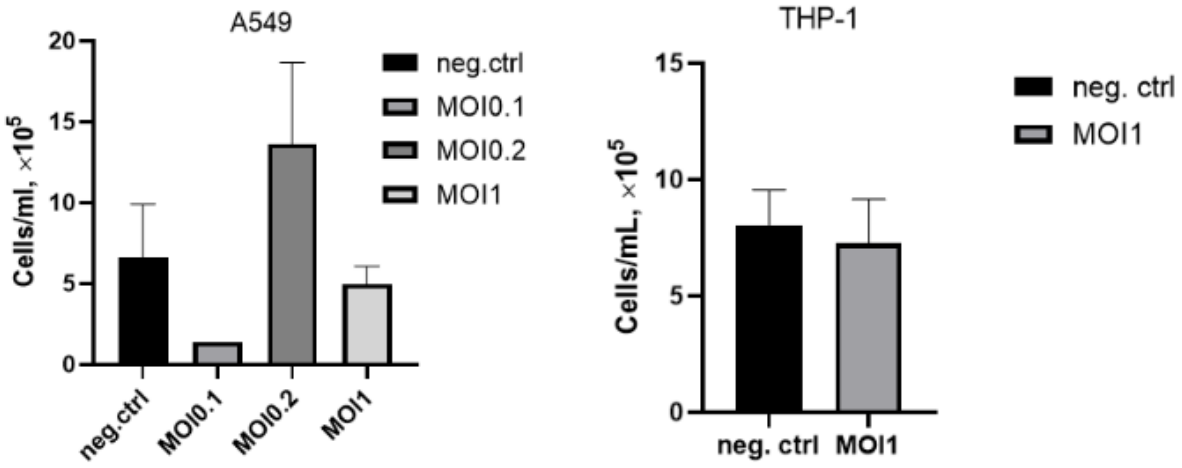

Figure 7 – Total cell number, 72 hours post-inoculation

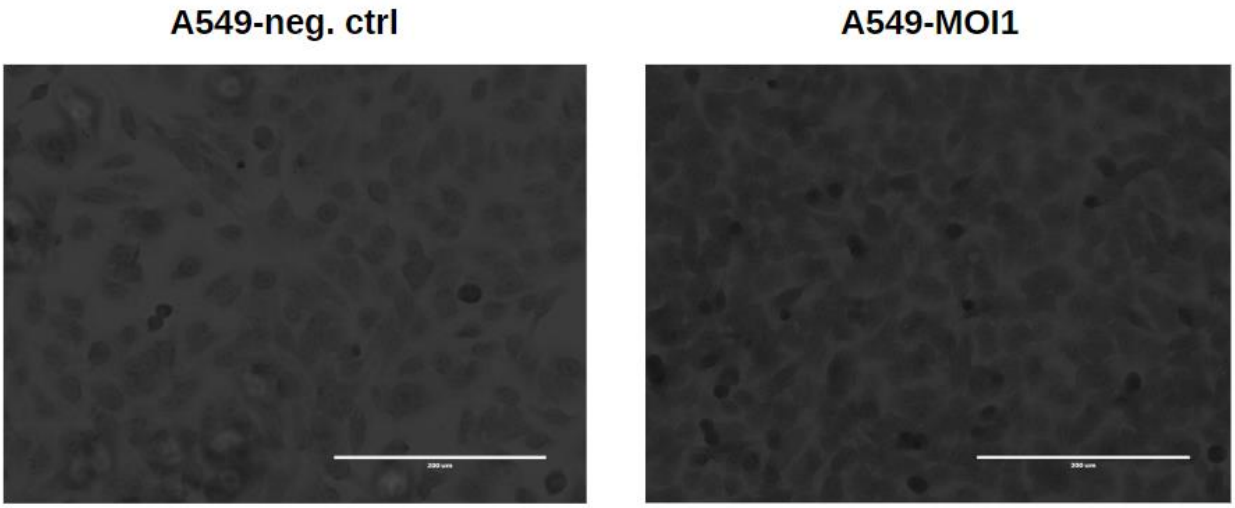

Figure 8 – A549 cells, 72 hours post-inoculation, 0.1% Evans Blue stain

Assessing the total DNA concentration in tested cells and media (fig. 9) combined with endpoint PCR and IPA results indicates the 72 hours of incubation as a suitable time point for medium collection and further processing. DNA concentration data obtained by absorbance measurement demonstrated the increase of DNA content upon chlamydial infection. Thus, DNA concentration in A549 cells increased from 48 μg/mL for non-infected cells to 54 and 50 μg/mL in case of MOI0.1 and MOI1 respectively. Even though these numbers are not enough for stating the increase to be dose-dependent, it is possible to suggest that chlamydial inclusions formed as a result of successful infection contribute to the higher amount of DNA in infected cells. The same can be stated for THP-1 cells with DNA concentrations of 28.37 and 29.57 μg/mL.

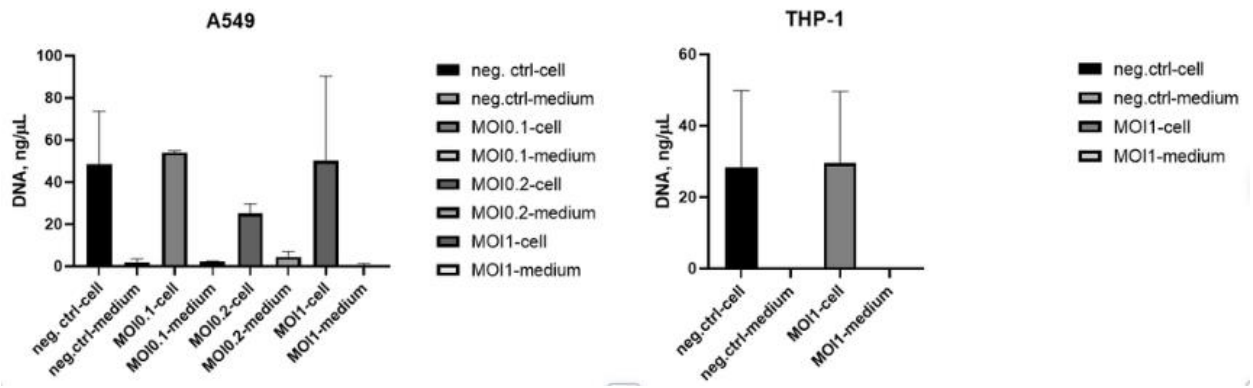

Figure 9 – Total DNA quantification, 72 hours post-inoculation

IPA assay (fig. 10) was aimed to reveal the presence or absence of morphologically distinct chlamydial inclusions in collected cells and medium. Chlamydial bodies were observed to develop only in infected cells. Being a qualitative check for infection validation and purity of non-infected samples, the results of this experiment show that *C. pneumoniae* infection developed successfully inside the cells, but the newly formed chlamydial EBs were not released into the medium over the 72 hours of incubation. Combined with PCR data, these observations allow to eliminate the challenge of possible clashes between the time frames of Chlamydia spp life cycle and the need to achieve sustainable yields of extracellular vesicles by extending the incubation time.

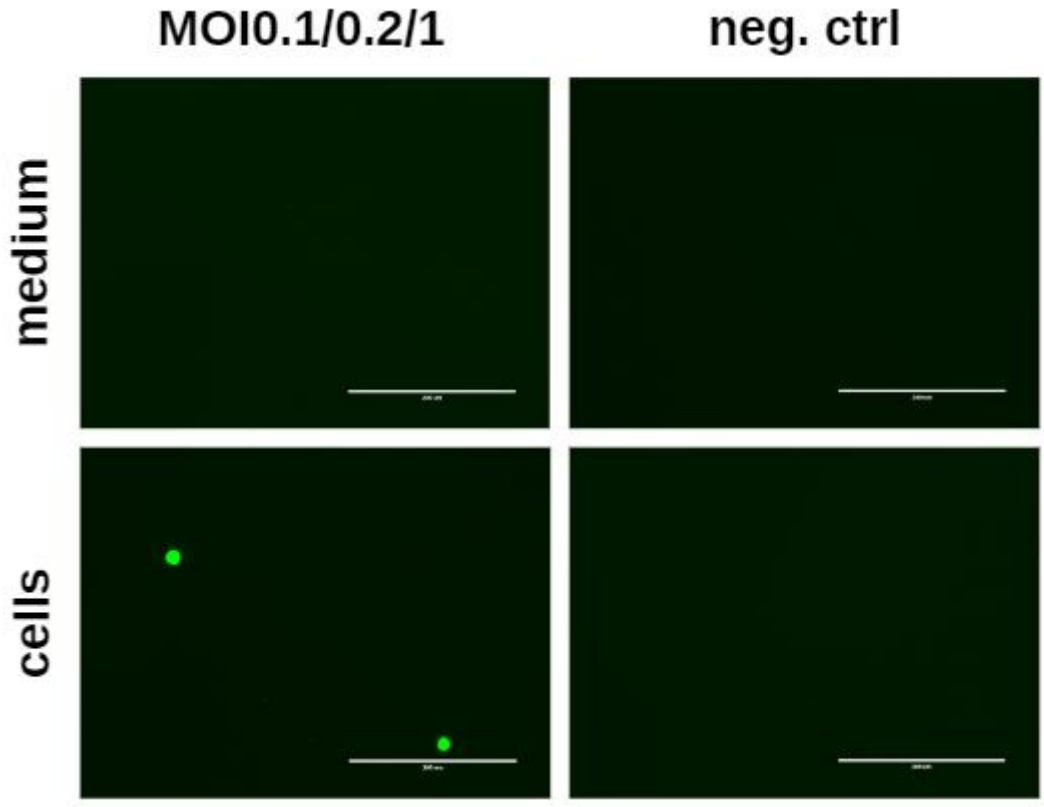

Figure 10 – Infectious Progeny Assay

It is also notable to mention that the number of visualized chlamydial inclusions did not show the specificity in terms of cell type, multiplicity of infection in range of 0.1 to 1 and incubation time (as the IPA coverslips infected for 48 and 72 hours demonstrated the similar ranges in the number of *C. pneumoniae* inclusions).

Gel electrophoresis of PCR reaction products (fig. 11) detected the presence of *C. pneumoniae* OmpA DNA fragments in both infected cells and the cell culture medium collected from these cells. Paired with the results of IPA analysis, the presence of chlamydial DNA in infected medium allows to set the hypotheses of this DNA origin. DNA fragments might be released by the host cells independently from chlamydial bodies. Alternatively, EBs might be released 72 hours after inoculation in non-infective form giving the negative results of infected medium lysates, which may be a reason for similar intensities of cells and media bands. It is also possible to suggest that some of the cells were less susceptible to *C. pneumoniae* infection, which prevented the EBs in infected media from entering the host cells.

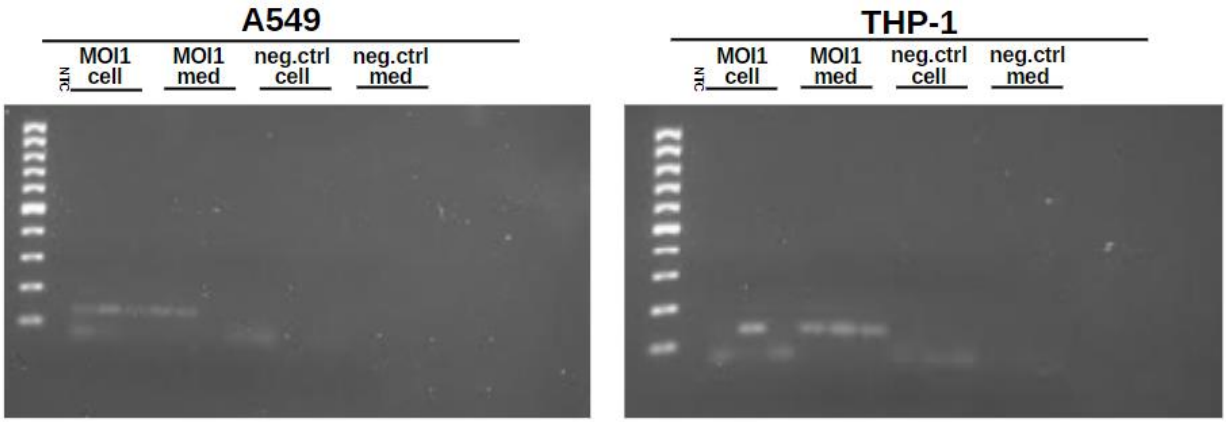

Figure 11 – OmpA amplification product, 126 bp

3.2.2 EV isolates

Total protein content of EV isolates (fig. 12) exceeded 20 μg/mL for A549 cells and 30 μg/mL in case of vesicles produced by THP-1 monocytes. Protein content of the pellets from cell-free medium controls was observed to be equal or even exceed the protein level of exosome isolates from conditioned media. Exceeding level of proteins in medium control was observed for small EV fractions, remaining equal in case of 2K controls.

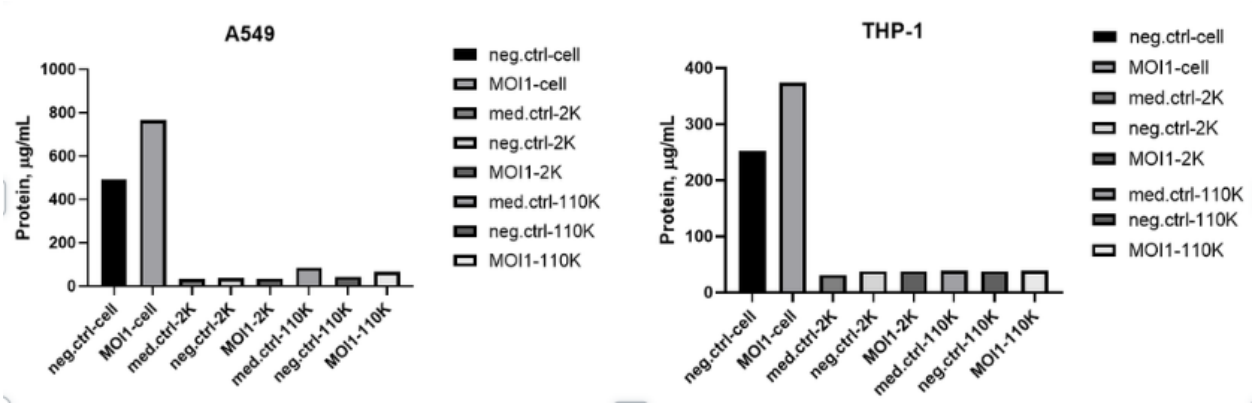

Figure 12 – Total protein content of EV isolates

Presence of exosome markers in these protein isolates was validated by Western Blot search for CD63 and ApolipoproteinB in order to mitigate the risk of co-isolation FBS-derived lipid droplets from the medium (fig. 13). Protein isolates of large and small EV fractions of A549 and THP-1 derived media revealed positive for CD63 exosome marker, whereas this protein was not detected in non-conditioned medium control samples. All the samples appeared negative for apolipoprotein B, which indicates proposed protocols of FBS exosome depletion and isolation of cell-derived vesicles to be optimal for isolating the vesicles of a given size range as well as further EV analyzing and investigations.

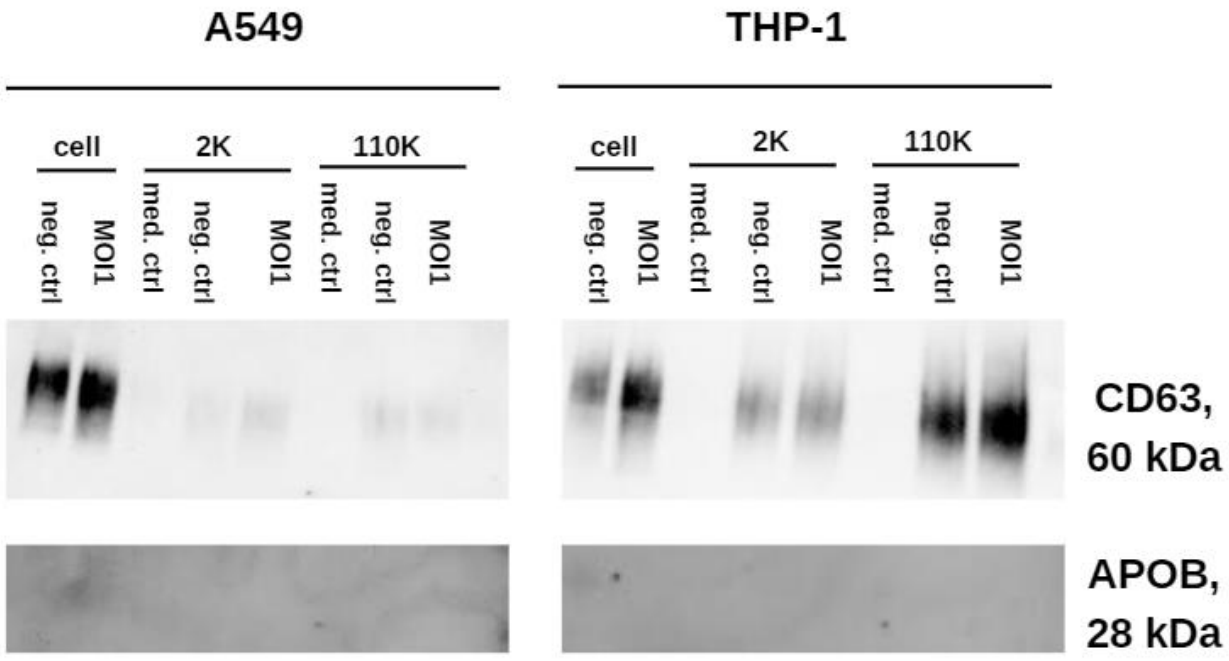

Figure 13 – Detection of exosome protein markers

3.2.3 *C. pneumoniae*-induced extracellular vesicles

Analyzing intensity of CD63 protein bands obtained through Western Blor membrane development, we were able to observe the increase in protein content of extracellular vesicles derived from infected cell culture medium. This change in protein levels is more distinguishable for monocyte-derived EV pellets and supports the hypothesis of EVs production induced upon chlamydial infection mediated by altered pathological states of the host cells, included but not limited to immune response modulation and oxidative stress, which were previously shown to alter the number and composition of extracellular vesicles elevating the levels of exosome markers [36].

The mentioned suggestions may be checked by Western Blot search for a set of several EV markers and assessing their levels upon increased multiplicity of infection.

SUMMARY

Conducted studies resulted into the protocol for transition field between the research on microbiological features of Chlamydia pneumoniae infection process and the research on extracellular vesicles molecular biology for the purpose of diagnostics and pathophysiological role studies.

We investigated infection process to be fully compatible with optimized conditions for EV production in terms of exosome-free medium switch stress, risk of impaired quorum sensing upon decreased seeding densities and exosome characterization possible interferes with the products of chlamydia pneumoniae life cycle and development.

Established procedures of media preparation and EV isolation allowed to obtain the suitable amounts of extracellular vesicles of distinguished fractions and purity allowing further characterization of their basic and altered protein, lipid and nucleic acid content, uptake and size-number distribution. Tested vesicle assets derived from infected and non-infected monocytes and epithelial cells revealed the altered levels of exosome markers suitable for future studies

Established protocols and achieved experimental results set bases for further research extended to broader range of cell types and infection multiplicity representing the in vivo features of pathological process and allowing the further studies towards evaluating the prevalence and characteristics of extracellular vesicles induced by *C. pneumoniae* infection relevant for the purpose of diagnostics and therapy.

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