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Short communication

Recovering host cell-free *Anaplasma phagocytophilum* from HL-60 cells by using rock tumbler grit in comparison to the syringe lysis method

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ABSTRACT

Anaplasma phagocytophilum (*Ap*) is a tick-transmitted obligate intracellular bacterium and the causative agent of the granulocytic anaplasmosis in various species of domestic animals and in humans. During intracellular development *Ap* transforms from a dense-cored cell form into a reticulate cell form and *vice versa*. For isolation of intracellular bacteria, a range of different purification methods is used. However, unlike other Gram-negative bacteria *Ap* is considered to be sensitive to mechanical stress and osmolarity changes. An updated semi-purification method using rock tumbler grit is introduced here to increase the outcome of bacteria and to facilitate the procedure of host cell lysis. The objective of this study was to evaluate the structural integrity and infectivity of *Ap* after lysis of the host cells using rock tumbler grit and to compare the outcome to that of the frequently used method, syringe lysis. Human promyelocytic leukemia cell lines (HL-60) were infected with *Ap* and following host cell-free bacteria were assessed by transmission electron microscopy. The outcome of the different purification methods was compared using live/dead-staining based on immunofluorescence to count the number of viable bacteria and real-time PCR to compare the amount of DNA. Subsequently the isolated bacteria were tested to infect naive cell cultures. We observed that both *Ap* dense-cored cells and reticulate cells are preserved intact after the application of rock tumbler grit. The number of viable, host cell-free bacteria was higher by factor 1.7–2.4 compared to the syringe lysis protocol. Quantitative analysis based on real-time PCR showed an increase of bacterial DNA up to 1.6–2.9 times higher using the rock tumbler grit protocol. Bacteria released from the same number of infected host cells were used for new infections. Flow cytometric analysis of the cell cultures confirmed that the number of *Ap* organisms recovered by using the rock tumbler grit protocol resulted in higher infection rates than the number of *Ap* organisms recovered by using syringe lysis protocol. Our observations indicate that the rock tumbler grit protocol can be applied as a safe, robust and convenient method to recover *Ap* compared to syringe lysis.

1. Introduction

Anaplasma phagocytophilum (Ap) is a Gram-negative, obligate intracellular bacteria and the causative agent of the granulocytic anaplasmosis (Bakken and Dumler, 2006; Chen et al., 1994). Clinical illness is documented for humans and domestic animals such as dogs, cats, horses and ruminants after transmission of the pathogen from natural hosts by ticks of the *Ixodes persulcatus complex* (Woldehiwet, 2010). According to the Center of Disease Control and Prevention (CDC), the clinical diagnosis of human granulocytic anaplasmosis (HGA) can be confirmed by PCR, serological evidence of a fourfold change in IgGspecific antibody titer or isolation in culture (Aguero-Rosenfeld, 2002; Centers for Disease Control and Prevention, 2018). For *in vitro* cultivation of *Ap* from infected blood and to maintain the bacteria for laboratory research, a human promyelocytic leukemia cell (HL60) line is routinely used (Goodman et al., 1996). During the proliferation within

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host cells, the bacteria accumulate in vacuoles, the so called "morulae" (Popov et al., 1998). These intracytoplasmic macrocolonies are detectable by light microscopy after Giemsa or Romanowsky staining (Chen et al., 1994; Rikihisa, 1991). During intracellular development two ultrastructural morphologies are observed: a smaller electron dense-cored cell (DC), which has a dense nucleoid, and a larger electron lucent reticulate cell (RC), which has a dispersed nucleoid (Troese and Carlyon, 2009). Furthermore, it is known that *Ap*, especially the fragile RC, is very sensitive to mechanical stress such as sonication, freezing, thawing, and osmolarity changes (Lin and Rikihisa, 2003). Unlike other Gram-negative bacteria, they are enveloped with a thin outer membrane that shows no sign of peptidoglycan layer or lipopolysaccharides (LPS) (Rikihisa et al., 1997) For *in vitro* applications, infection studies or transformation assays, mechanical methods are widely used for liberation of intracellular bacteria. *Anaplasma phagocytophilum* is generally released from infected host cells by different, sensitive methods including syringe lysis (Carlyon, 2005), sonication (Felsheim et al., 2006) or using a Dounce homogenizer (Lin and Rikihisa, 2003). It is challenging to lyse the infected host cells and to remove the remnant cellular debris without damaging the bacteria. A new semi-purification method using rock tumbler grit was initially introduced to improve the yield of DNA from *Ap* grown in tick cells and has also been used for *Ehrlichia chaffeensis* purification from tick cell cultures (Cheng et al., 2013). The fine-grained grit was used to lyse the host cells and filtration was applied to remove most of cellular debris and to retain a semipure bacterial preparation (majority of suspension is host cell-free bacteria and a minor portion consists cellular debris of the host cells). In a previous study the isolation of *Ap* from HL-60 cells using the rock tumbler grit method was used to establish infections in endothelial cells (Wang et al., 2015). However, it was still not known whether the use of rock tumbler grit destroyed DCs or RCs during the purification procedure. The aim of this study was to evaluate the structural integrity of *Ap* after host cell lysis using rock tumbler grit and to assess and compare the outcome to the frequently used method, the syringe lysis.

2. Materials and methods

2.1. Cell cultivation and infection with Ap

A human promyelocytic cell line HL-60 (ATCC CCL-240) was obtained from the American Type Culture Collection (LGC Standards GmbH, Wesel, Germany) to propagate *Ap* in culture (Wang et al., 2015). The cells were screened for the expression of the P-selectin glycoprotein ligand-1 (PSGL-1) on the surface to verify the susceptibility for *Ap* infection. Therefore, HL60 cells were centrifuged on a glass slide and fixed with acetone for 10 min at room temperature. Following fixation, cells were incubated for 20 min with human serum to block Fcreceptors and were gently washed with phosphate-buffered saline (PBS) containing 0.5–1.0% (w/v) bovine serum albumin (BSA) (pH 7.2–7.4), afterwards. The cells were stained with a purified mouse-anti-human CD162 (PSGL-1) - antibody (eBioscience, ThermoFisher Scientific, Massachusetts, USA) and a FITCconjugated goatantimouse IgG (Molecular Probes, ThermoFisher Scientific), each 30 min at room temperature in the dark. The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and visualization was performed with a fluorescence microscope (Leica DM5000, Leica Microsystems GmbH, Wetzlar, Germany) (Wang et al., 2015). After confirmation of expression of the PSGL-1 on the cell surface, cell cultures were infected with a mCherry-transformed *Ap* strain *HGE1* (Felsheim et al., 2006). The uninfected and infected HL-60 cells were maintained in RPMI1640 medium (ThermoFisher Scientific), supplemented with 10% heat-inactivated fetal bovine serum (SigmaAldrich Chemie GmbH, Taufkirchen, Germany) and 2 mM L-glutamine (SigmaAldrich Chemie GmbH) in a humidified 5% CO_2 air atmosphere at 37 °C (Goodman et al., 1996). The culture medium was buffered with 25 mM HEPES, 0.1% $NaHCO_3$ and the pH was adjusted to 7.5 (Silaghi et al., 2011). Trypan blue (0,5%) staining was used to determine cell viability (Strober, 2015) and medium was replaced three times a week to maintain a cell density of 2 to 5 × 10^5 cells per ml in culture (Carlyon, 2005). Every two days, aliquots of *Ap*-infected HL-60 cells were cytocentrifuged onto glass slides (Microscope Slides, Henry Schein, Munich, Germany) using a table top centrifuge (Rotofix 23 A, VWR International, Pennsylvania, USA) to monitor the infected cell fraction. Cells were examined after Giemsa staining to visualize morulae and determine the infection rate, counting 100 cells per slide using a light microscope (Leica DM5000, Leica Microsystems GmbH).

2.2. Purification of Ap from infected cell cultures

Prior to bacterial purification, continuously cultured HL-60 cells infected with *Ap* were harvested when they were heavily infected (> 80%). One aliquot of the infected cell culture was transferred to a microcentrifuge tube and centrifuged at 200 x g for 5 min at 4 °C and functioned as a positive control for transmission electron microscopy (TEM). The rest of the infected cells was divided equally into two subsets and centrifuged at 2300 × *g*, 4 °C for 10 min.

The first subset of *Ap* infected cells was then processed by syringe lysis, using 27-G, ½-in (0.4 x 20 mm) needles after resuspension in PBS. The suspension was filled into a 5-ml Luer lock syringes and expelled through the needles. This procedure was repeated five times to lyse the HL-60 cells without damaging the bacteria. A differential centrifugation was used to separate *Ap* from host cell debris (Carlyon, 2005).

The second subset of *Ap* infected cells was processed by using rock tumbler grit (60/90 grit silicon carbide; Lortone, Inc. Mukilteo, WA, USA). The pellet was resuspended in 1.5 ml culture medium and transferred to a 2.0-ml sterile tube containing 0.2 ml grit. The cell suspension was vortexed vigorously for 30 s and subsequently kept on ice. After the grit settled to the ground the supernatant was transferred to a 5-ml Luer lock syringe and passed through a 2.0-µm pore size syringe filter (Puradisc™ 25 GD, GE Healthcare Europe GmbH, Freiburg, Germany) into a sterile 2ml microcentrifuge tube to remove the remnant HL60 debris (Borjesson, 2008). Host cellfree bacteria were finally collected by centrifugation at 11,000 x g for 5 min at 4 °C (Wang et al., 2015). After processing the samples according to the respective protocols, the preserved pellets of released bacteria were used for analysis by TEM, immunofluorescence staining and real-time PCR.

2.3. Transmission electron microscopy

Host cell-free *Ap* was obtained from infected HL-60 cells using the method of rock tumbler grit and syringe lysis as described before. Additionally, an aliquot of the original infected cell culture was used as a positive control. The cell pellets of host cell-free *Ap* and infected HL-60 cells were resuspended in 2.5% glutaraldehyde solution in Sorenson's sodium phosphate buffer and fixed for 1 h at 4 °C.

After washing cells twice with Sorenson's sodium phosphate buffer, they were postfixed in 1% osmium tetroxide for 1 h at 4 °C and dehydration was achieved in an ascending acetone series. The samples were embedded in epoxy resin and harden at 60 °C for two days. Ultrathin sections were prepared and examined with EM10 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) as described previously (Dyachenko et al., 2013).

2.4. Immunofluorescence assay

Immunofluorescence microscopy was performed to detect and count individual organisms after purification. Infected HL-60 cells were collected and divided equally into two subsets. Each subset, containing 5.0 × 10^6 highly infected cells, was purified as described before using rock tumbler grit or syringe lysis. The pellets with host cell-free *Ap* were washed with 0.9% NaCl and resuspended in 100 µl of 0.9% NaCl. 50 µl of suspension were used for Baclight Bacterial Viability staining (Syto 9

and PI stain, Molecular Probes). Finally, fluorescence microscopic images were taken and viable *Ap* (Syto 9 positive but PI negative) were counted using a Petroff-Hausser counting chamber (Goodman et al., 1999).

2.5. Quantification by real-time PCR

In order to quantify and compare the outcome of DNA of *Ap* obtained from lysed HL-60 cells using the two different semipurification protocols, a real-time PCR was performed. The infected host cell population was again divided equally into two subsets and after semipurification, DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Subsequently both methods – filtration and differential centrifugation – to remove unwanted cellular debris were compared separately. Filtration using a 2.0-µm pore size syringe filter and differential centrifugation to further clarify the lysate were applied after host cell lysis and the yield was used for DNA extraction. The DNA was diluted by 1:1000 and a real-time PCR was performed as described previously (Courtney et al., 2004). Each DNA sample was evaluated in triplicates. DNA extracted from an uninfected HL60 cell culture was used as negative control. The TaqMan probe (QuantiNova Probe PCR Kit, Qiagen) was labeled at the 5′ and 3′ ends with 6-Carboxy-Fluorescein (FAM) and Black Hole Quencher-1 (BHQ-1), respectively.A volume of 2.5 µl target DNA and a total reaction volume of 20 µl were used. For the initial activation of the QuantiNova Taq DNA Polymerase, a temperature of 95 °C was applied for 2 min, followed by 40 cycles of a 5 s denaturation step at 95 °C and by a 30 s annealing elongation step at 60 °C. PCR amplification was performed in 96-well plates (Sarstedt, Nümbrecht, Germany) using a Light Cycler 480 (Roche, Basel, Switzerland). The experiment was repeated with five infected cell cultures and infection rates ranging from 70 90%. To quantify and compare the DNA yield of both methods, an artificial standard (DNA duplex molecule) of a length of 108 bp (Metabion, Planegg, Germany) was designed. The initial concentration of the standard was 0.5 nmol/l and eight different concentrations using a 10-fold dilution series were applied to generate a standard curve. The standard was measured in triplicate for each concentration and the Ct-values were plotted against the logarithm of input amount of standard material. The standard curve and efficiency were calculated with the Light Cycler 480 Software.

2.6. Infection of naive cells with isolated Ap

Naive cell cultures were infected with host cell-free *Ap* recovered from infected HL-60 cells using the different purification protocols. The population of *Ap* obtained by using the rock tumbler grit protocol resulted in higher infection rates than the population recovered by using the syringe lysis protocol.

In order to see whether the bacteria were still equally infectious after exposure to high mechanical stress caused by lysis of the host cells using rock tumbler grit, naive cell cultures of HL-60 cells with a concentration of 2.0×10^5 cells per ml were infected. Host cell-free *Ap* was isolated from the same number of infected cells by using the syringe lysis and rock tumbler grit method. The progress of infection was monitored microscopically by Giemsa staining (SigmaAldrich) daily. A multiplicity of infection of one was used for the initial inoculation and semi-purification respectively (MOI 1:1 refer to the number of infected cells used for purification to the number of uninfected target cell) (Borjesson, 2008).

Analysis of the cell culture based on fluorescence-activated cell sorting (FACS) was performed additionally to monitor and compare the progress of the *Ap* infection. Therefore, one aliquot of 500 µl from every infected culture and from the uninfected negative control were collected and washed twice with PBS containing 0.5–1.0% (w/v) BSA and 0.09% NaN_3. The cells were fixed for at least 30 min with 2% ice-cold paraformaldehyde (PFA) before mCherrypositive cells were detected and analyzed in the MACS Quant VYB (Miltenyi Biotec, Bergisch Gladbach, Germany) (Wang et al., 2015).

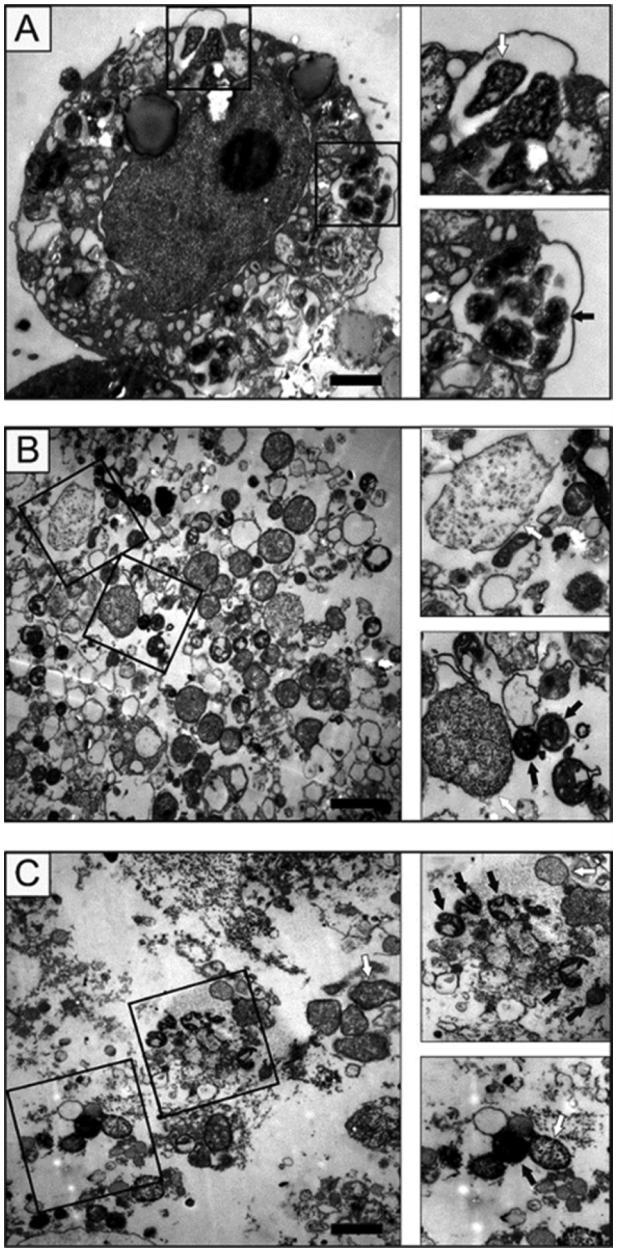

Fig. 1. Ultrastructure of Ana*plasma phagocytophilum*. **(A)** Ultrastructure of *Ap* in a HL-60 cell. Several *Ap* inclusions in a HL-60 cell showing electron-dense cell forms (DCs, black arrows) and reticulate cell forms (RCs, white arrows). **(B)** Ultrastructure of *Ap* released with the rock tumbler grit lysis procedure. Many DCs (indicated by black arrows) as well as RCs (indicated by white arrows) are visible using the rock tumbler grit lysis procedure. The area marked with solid lines and shown on the lower right contains both DC and RC forms. One lysing RC form with damaged membrane presented in the upper right box (white arrow). **(C)** Ultrastructure of *Ap* purified with syringe lysis procedure. Electron-dense (DCs, black arrows) and reticulate cells (RCs, white arrows) obtained after using syringe lysis procedure. Scale bars, 1.0 µm.

Statistical analysis was conducted using the Minitab Software version 17. (Minitab Inc., Pennsylvania, USA). Student's *t*-test was performed for paired samples at a confidence level of 95%.

3. Results

Electron microscopic images of infected HL-60 cells as well as

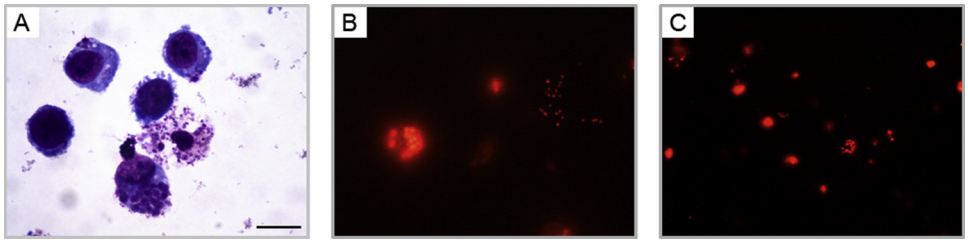

Fig. 2. Giemsa staining of *Ap* infected HL-60 cells and images of host cell-free fluorescent *Ap* organisms. *Ap* was propagated in HL-60 cells and harvested when > 80% of the cells were infected. For each experiment the infected cell culture was divided and release of the bacteria from host cell populations were carried out by the use of syringe lysis procedure and the rock tumbler grit procedure. **(A)** Representative image of Giemsa stained *Ap* infected HL-60 cells. **(B)** Representative image of fluorescent *Ap* prepared using syringe lysis. **(C)** Representative image of fluorescent *Ap* prepared using rock tumbler grit. Scale bar, 10 µm.

released *Ap* from lysed host cells using the syringe lyses and rock tumbler grit method were examined. Both stages of *Ap* development were present in the intracytoplasmic vacuoles of the infected HL-60 cells (Fig. 1A). The vacuoles were present in a variable size and with a different number of bacteria inside. No intact HL-60 cells were visible after using the rock tumbler grit or syringe lysis procedure for host cell-lysis. We detected dense-cored cells as well as reticulate cells of *Ap* after host-cell-lysis using rock tumbler grit (Fig. 1B). Densecored cells and reticulate cells were also visible after host-cell-lysis using the syringe lysis method (Fig. 1C). In both methods intact, round-coccoid DCs were seen with rippled membranes. Also the larger, pleomorphic RCs with smooth and slightly ruffled membranes were found. Most of the bacteria were undamaged however, in some cases using rock tumbler grit and syringe lysis we detected few RCs with a damaged membrane probably caused by mechanical stress.

Immunofluorescence assays showed that using the rock tumbler grit protocol resulted in a higher number of isolated *Ap* compared to the syringe lysis protocol (Fig. 2). The same number of infected cells was used for both methods of host cell lysis and isolation of the bacteria. After counting, the concentration of viable bacteria recovered by syringe lysis ranged from 3.2×10^7 to 6.08×10^7 cells per ml. The concentration of viable bacteria recovered by rock tumbler grit ranged from 7.6×10^7 cells per ml to 9.45×10^7 cells per ml. The efficiency of bacterial isolation was compared in four independent experiments. It became evident that more bacteria were recovered using the rock tumbler grit protocol with a fold change (F) of 1.7–2.4 compared to the syringe lysis method. Only viable bacteria were counted. Concentrations of *Ap* determined after purification were different at a significance level of $p = 0010$ (*t*-test for paired samples).

In the next step, relative quantification was carried out by determining the yield of DNA after purification. We analyzed the Ct values and designed an artificial standard to calculate and compare the total number of copies and the fold change of both methods (Table 1). The average of Ct value of triplicate was used for data interpretation. The efficiency of the artificial standard based on a 10-fold dilution series was 94.92% (slope: -3.450). The PCR results indicate an increase of the yield of *Ap*-DNA using the rock tumbler grit protocol compared to the syringe lysis protocol. Based on the total number of copies the fold change of efficiency (F) in different experiments was F = 1.6–2.9 (rock tumbler grit *versus* syringe lysis), which describes the ratio between the yield of *Ap* DNA using the different purification protocols. Resulting Ct-values of both purification protocols were proven to be statistically different at a significance level of $p = 0.001$ (*t*-test for paired samples).

In order to see whether the bacteria were still equally infectious after exposure to high mechanical stress caused by lysis of the host cells, naive HL-60 cells were tried to be reinfected. The cell cultures were exposed to preparations of host cell-free *Ap* obtained by semi-purification with either syringe lysis or rock tumbler grit and were screened microscopically for the presence of morulae after Giemsa staining. The typical intracellular formations were initially observed on day one or day two after inoculation. Flow cytometry analysis revealed an initial infection between 3% and 11% of the HL60 cells. The infection rate increased to 100% associated with lysis of the host cell population within 5–7 days of incubation (Fig. 3). In all batches the same number of infected cells were used for the different protocols and the total amount of released bacteria were used to infect naive cell cultures. Higher infection rates in cell cultures infected with the lysate obtained using the rock tumbler grit procedure were detected 24 h after inoculation. This result was confirmed in four independent experiments. The number of infected HL-60 cells were 1.2–2.1 times higher compared to cell cultures infected with the lysate obtained using the syringe lysis protocol. It became evident that the larger number of isolated bacteria using the rock tumbler protocol lead to higher infections rates and therefore to a faster course of infection and finally lysis of the host cell population respectively. Infection rates of the HL-60 cell cultures at day one were different at a significance level of $p = 0017$ (*t*-test for paired samples).

Furthermore, we compared the efficiency of the different procedures to remove cellular debris after host cell lysis considering the outcome of DNA. Therefore, both methods including centrifugation and filtration were applied after lysis of the infected HL-60 cells by the use of rock tumbler grit and syringe lysis. We observed that using filtration results in an increase of DNA by a fold change from 1.1 up to 1.5 (data not shown).

Table 1
Relative quantification of the outcome of the different protocols: comparison of Ct values and calculation of the total number of copies based on precise standards (plasmid controls). The yield of recovered bacterial DNA by using the rock tumbler grit protocol and the syringe lysis protocol was compared in five independent experiments.

PCR No.	rock tumbler grit		syringe lysis		fold change in efficiency (F)
	Ct-Value	total copy number	Ct-Value	total copy number	
1	22.82	267.000	23.55	164.000	1,6
2	23.63	189.333	25.11	70.600	2,7
3	22.79	265.417	24.08	115.000	2,3
4	20.78	1.270.000	21.94	585.667	2,2
5	20.12	1.966.667	21.72	679.667	2,9

4. Discussion

The sensitive, intracellular bacterium *Ap* can be cultured and propagated in a human promyelocytic cell line (HL-60). To recover a host cell-free enrichment of this bacteria - these organisms are predisposed toward mechanical stress - an updated semi-purification method using rock tumbler grit was used in this study. This updated protocol was introduced to increase the number of released bacteria and to facilitate the handling. The outcome was evaluated and compared to the syringe lysis method. *Anaplasma phagocytophilum* displays unique characteristics in its physics of the cell wall. Genome sequences revealed a lack of all genes encoding lipid A and most genes for the biosynthesis of peptidoglycan (Lin and Rikihisa, 2003), which play a significant role in providing strength to the outer membrane and maintaining the structural integrity of Gram-negative bacteria (Benson, 1998; Höltje, 1998).

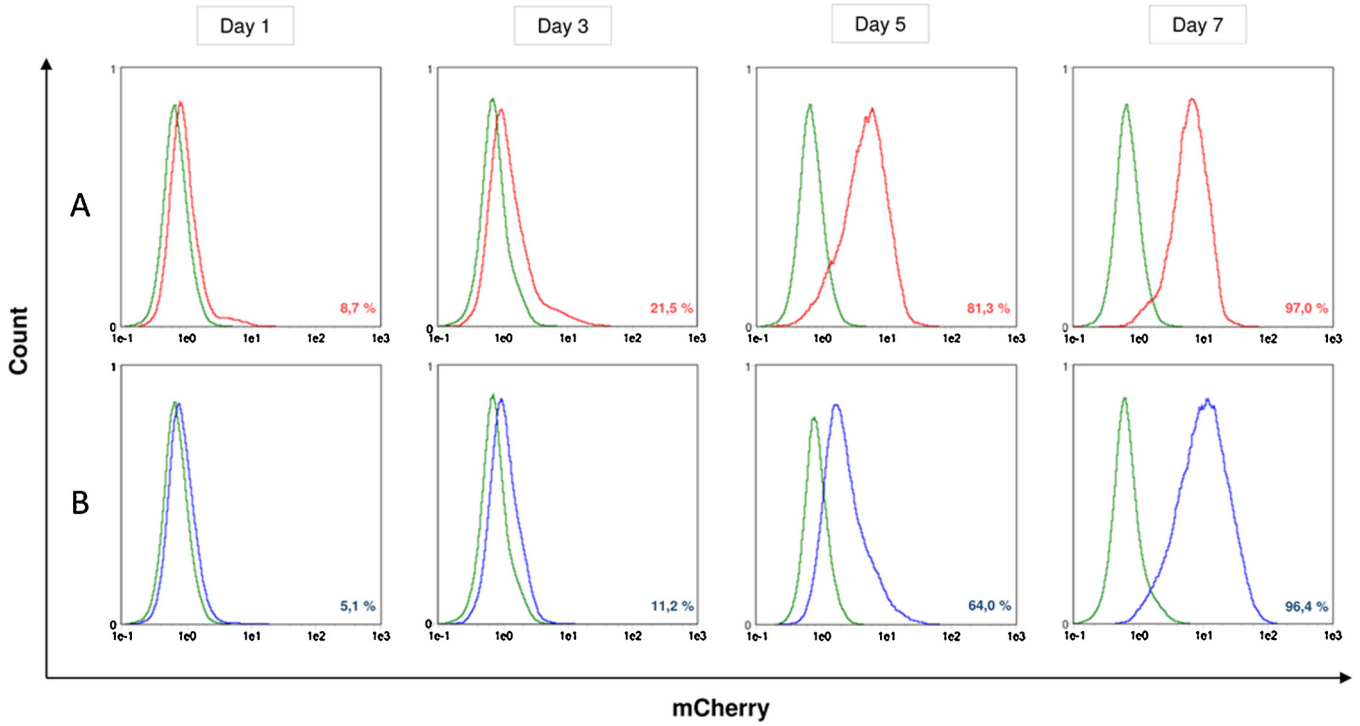

Fig. 3. Flow cytometric analysis of HL-60 cell cultures after infection with host cell-free *Ap*. Naive cell cultures were infected with *Ap* released from a defined number of HL-60 cells (MOI 1:1) using the rock tumbler grit protocol **(A)** and the syringe lysis protocol **(B)**. Cell cultures were screened for mCherry-positive cells on day 1, 3, 5 and 7 post inoculation. In the first days after exposure the fraction of infected cells was higher when the rock tumbler grit protocol was applied. After logarithmic expansion of *Ap* organisms in HL-60 cells, infection rates of the HL-60 cell cultures increased continuously and cultures became completely infected. blue/red panels: infected cell population; green panel: uninfected cell population (negative control) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Unlike the situation seen with other obligatory vacuolar pathogens that exhibit biphasic development, such as *Chlamydia pneumonia* (Abdelrahman and Belland, 2005) or *Coxiella burnetii* (Mccaul And Williams, 1981), *Anaplasma* spp. are extremely susceptible to mechanical stress and osmolarity changes due to the lack of a peptidoglycan layer or synthesized lipopolysaccharides (Ristic and Kreiser, 1984; Severo et al., 2012). The release of *C. burnetii* and *Ch. pneumonia* from infected cells is achieved by a freeze-thaw technique or by the use of deionized water (Henning and Sting, 1999; Thiele, 1992). These methods induce a strong impact on the host's cell walls and result in a subsequent breakdown of the cell wall integrity. In contrast, to avoid damage to the shape of the bacterial cell, different, less damaging methods are used for the isolation of *Ap* such as syringe lysis (Borjesson, 2008; Carlyon, 2005) or rock tumbler grit (Wang et al., 2015). Troese and Carlyon (2009) could show using TEM that a host cell-free population of DC and RC organism was recovered following syringe lysis, which was also confirmed in following studies (Huang et al., 2010a, 2010b). In the same way, we demonstrated for the first time that both *Ap* DCs and RCs were recovered with a sustained physical integrity by the usage of rock tumbler grit. However, we clearly observed and confirmed that *Ap* are sensitive to mechanical stress as indicated by the presence of some RCs that appeared to undergo lysis as suggested by a damaged outer membrane. Moreover it is reported that high mechanical stress such as sonication causes the lysis of the fragile RC form and only the DC form is received (Felsheim et al., 2006).

The count of viable bacteria with immunofluorescence after isolation showed a higher number of *Ap* using the rock tumbler grit protocol compared to syringe lysis protocol. A similar increase of the outcome was confirmed by PCR to compare the amount of bacterial DNA recovered after purification (Table 1). The variance of the Ct value ranges between the individual experiments are due to minute variations in starting cell concentrations and infection rates. We conclude that the increase is due to an effective host cell lysis with only few losses of bacteria during the procedure. Furthermore, we observed that a higher yield of bacterial DNA could be achieved using filtration compared to centrifugation to remove cellular debris. For generating *Ap* preparations that are more highly purified from contaminating host cell components, density gradient centrifugation can be used, which separates bacteria and host cellular debris into different layers (Carlyon, 2005). *Ap* population isolated from host cells by lysis was used to infect naive cell cultures in order to verify their infectivity. In the first days after exposure, we detected higher rates of infected HL-60 cells using the suspension of host cell-free bacteria recovered by the rock tumbler grit protocol compared to syringe lysis protocol. Although using the same MOI 1:1 for all of our experiments, we observed a lenient variation of the initial infection rate in target cell cultures 24 h after incubation. The fraction of infected cells was determined by Giemsa staining. However, it is not possible to differentiate and count the separate intracellular forms of *Ap* under light microscopy. It has been shown that only DCs are infectious for HL-60 cells, while RCs are considered to be in the genome replication stage of *Ap* development that is not infectious for HL-60 cells (Troese and Carlyon, 2009). Thus, we conclude that the diverse bacterial load was likely due to the varying numbers of infectious DCs present in different batches of HL-60 cells cultures, which were used for semi-purification.Besides the human promyelocytic cell line, tick cell lines such as IDE8 or ISE6 are also used for isolation and propagation for *in vitro* culture of *Ap*. Host cell-free *E. chaffeensis* organisms were successfully isolated from tick cells by the usage of rock tumbler grit (Wang et al., 2017). It stands to reason that this convenient method may be used to release *Ap* from tick cell cultures, since both bacterial organisms display very similar characteristics in terms of intracellular development and cell envelope structure (Lin and Rikihisa, 2003; Rikihisa, 2010). However, in this study *Ap* was not isolated from tick derived cell lines using rock tumbler grit and this has to be proven in

further experiments.

We conclude that the application of rock tumbler grit is an effective method to lyse the infected host cells and following filtration is appropriate to retain *Ap* and remove remnant HL-60 debris. We showed that more bacteria could be received, compared to the syringe lysis protocol and that the product can be used for subsequent enrichment of DCs and RCs respectively. The updated protocol facilitates the procedure for host cell lysis and semi-purification and it is a faster and easier method to recover host cell-free *Ap* from HL-60 cells compared to the syringe lysis protocol. In consequence, saving time for preparation of the samples is achieved and a minor instrument setting is required.

Declarations of interest

None.

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