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Tuning the growth conditions of *Chlamydia suis* in cell culture using high-content microscopy

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Running title:

Screening for *Chlamydia suis* in cell culture

25 **Abstract**

26 The porcine pathogen *Chlamydia suis* is widespread in pig farming. Isolation of *Chlamydia*
27 *suis* in cell culture is crucial for the generation and characterization of new isolates. However,
28 isolation of *Chlamydia suis* strains from field samples is fastidious. Therefore, we exploited
29 high-content microscopy to quantify the growth of *Chlamydia suis* strains in different cell
30 lines. We found that the cell line yielding optimal chlamydial propagation of *Chlamydia suis*
31 differs among strains, and importantly, differs from the currently used. Thus, our results call
32 for revised *C. suis* isolation and propagation methods.

33 **Keywords**

34 Chlamydia, swine, isolation, cell culture, high-content microscopy

35

Introduction

Chlamydia suis (*C. suis*) is an obligate intracellular Gram-negative bacterium, belonging to the order of the *Chlamydiaceae*. Since the pig is the only natural host currently identified, *C. suis* is generally known as a porcine pathogen. However, recently, *C. suis* isolates were detected in the eye of Nepalese villagers and Belgian pig slaughterhouse employees (De Puyseleir and others, 2014; Dean and others, 2013). Nevertheless, the knowledge on the zoonotic potential of *C. suis* is limited. The reference strain, *C. suis* S45, was isolated from feces of an asymptomatic pig in Austria in the late 1960s (Koelbl, 1969). Intestinal *C. suis* infections are assumed to be widespread but the majority is subclinical (Nietfeld and others, 1997). However, the enteric pathogenicity of the reference strain (*C. suis* S45) was demonstrated in gnotobiotic piglets (Guscetti and others, 2009). Moreover, *C. suis* infections in pigs have also been associated with conjunctivitis, pneumonia, reproductive disorder and inferior semen quality (Eggemann and others, 2000; Nietfeld and others, 1993; Rogers and Andersen, 1996; 1999; 2000; Rogers and others, 1993; Rogers and others, 1996; Schautteet and others, 2010), and several *C. suis* strains have been isolated from the intestine, conjunctiva and respiratory tract of pigs in Europe and the U.S. (Schautteet and Vanrompay, 2011). *Chlamydiaceae* are regularly isolated in cell culture. The success of isolation is influenced by the cell line, chlamydial species, sample type and storage conditions. Although more sensitive and less labour-intensive techniques have been developed for detection of chlamydial infections, isolation in culture remains crucial for the generation and characterization of new isolates, and subsequent fundamental research on bacterium-host interactions, development of new diagnostics and vaccine design. Several cell lines have been used for chlamydial isolation, with varying success depending on the chlamydial species being cultured. McCoy (mouse fibroblasts), BGM (monkey kidney cells), Vero (monkey kidney cells) and HeLa (human cervix cancer cells) cell lines are most commonly used

(Lenart and others, 2001; Rogers and Andersen, 2000; Rogers and others, 1996; Sachse and others, 2004). However, some species, such as *C. suis*, are more difficult to grow in cell culture, especially those from tissue and rectal samples (Rogers and others, 1993; Sandoz and Rockey, 2010; Wittenbrink and others, 1991). Moreover, the recovery rate can differ significantly among *C. suis* strains, which could be a reflection of the high genetic diversity observed within this species (Everett and others, 1999). In brief, knowledge on the culture of porcine *Chlamydiaceae* is limited.

Materials and Methods

Chlamydial strains

In order to improve culture conditions for *C. suis* isolates, we examined the growth characteristics of a conjunctival (H7), respiratory (R24) and intestinal (S45) *C. suis* strain in six different cell lines. The strains were propagated in McCoy cells, using standard techniques (Vanrompay and others, 1992).

Cell cultures

The following cell lines were examined: McCoy (Mouse fibroblast cells, CRL-1696 American Type Culture Collection), Vero (African Green Monkey kidney cells, CRL-1586 American Type Culture Collection), BGM (Buffalo Green Monkey kidney cells, American Type Culture Collection), IPEC-J2 (Intestinal porcine epithelial cells) and SK-6 (Swine kidney cells, both obtained from Eric Cox, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University), and Caco-2 (Human colon adenocarcinoma cells, HTB-37 American Culture Type Collection). The cells were cultured in Eagle's minimal essential medium supplemented with 10 % calf serum, 2 mM L-glutamine (Life Technologies), 1% MEM vitamins and 0.1 mg/ml streptomycin and vancomycin. For the Caco-2 cells, this medium was additionally supplemented with 1% non-essential amino

acids. The IPEC-J2 cells were cultured in Dulbecco's modified Eagle medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 5% calf serum, 4 mM L-glutamine, 1% Insuline-Transferrine-Selenium, 0.1 mg/ml streptomycin and vancomycin, and 5 ng/ml human epidermal growth factor (hEGF).

Chlamydia culture and immunofluorescence staining

Cells were seeded in 24-well plates (Greiner) and grown to a monolayer after which they were infected with 10^4 IFU diluted in SPG (218 mM sucrose, 38 mM KH_2PO_4 , 7 mM K_2HPO_4 , 5 mM L-glutamic acid), in quadruplicate. The monolayers were centrifuged for 1 h at 1300g and 37°C. The bacteria were subsequently removed and one of two maintenance media was added, which differed from the growth medium by containing 5% fetal calf serum, 2.2 µg/ml cycloheximide and 5.5 mg/ml glucose, and were based on Iscove's modified essential medium or Minimum Essential Medium (further referred to as IMDM and MEM, respectively). At 40 h post infection (p.i.), maintenance medium was removed and monolayers were washed twice with PBS to remove unattached bacteria. The cells were fixed with methanol and stained for *Chlamydia suis* using the ImagenTM *Chlamydia* immunofluorescence staining (Oxoid, United Kingdom), as previously described (Vanrompay and others, 1992), and counterstained with DAPI (Life Technologies, Belgium).

High content microscopy and image analysis

A fully automated inverted Nikon Ti widefield fluorescence microscope (Nikon Instruments, Paris, France) was used, equipped with motorized XYZ stage, filter cube turret and shutters. Samples were magnified with a 40x Plan Fluor oil objective (numerical aperture of 1.3) and images were acquired with an Andor Ixon EM-CCD camera, yielding a pixel size of 0.276 µm/pixel. To obtain a representative sample of each condition with minimal edge effects, three separate but sufficiently central regions were chosen per slide. Per region a 5-dimensional hyperstack was recorded, consisting of 16 fields (acquired in a 4x4 mosaic), 5-7

z-slices (separated by $1\mu\text{m}$) and 3 channels (corresponding with the DAPI, Evan's Blue and FITC channels). Multidimensional images were analyzed using a dedicated macro script written in FIJI image analysis freeware (<http://fiji.sc>, (Schindelin and others, 2012)), essentially based on a pipeline described before (De Vos and others, 2010). Briefly, the analysis consists of a stepwise segmentation of the features of interest, followed by their quantification (Figure 1). Before commencing segmentation, hyperstacks are flattened by means of a maximum projection along the Z-axis. Then, presumed *Chlamydia* containing foci are segmented. To this end, the FITC channel images are convolved with a Laplacian filter to enhance the signals and automatically thresholded using the Isodata algorithm. Only spots larger than 3 pixels are taken into account for subsequent particle analysis. Nuclei are segmented with an algorithm that consists of a smoothing step (Gaussian blur), local contrast enhancement, an autothresholding step (Isodata) and a watershed procedure. Finally, cells are delineated by segmentation of the Evan's Blue channel. Cell segmentation occurs by seeking a local minimum in the intensity histogram that separates background from true signal. Optionally, cells can be separated by conditional region growing from the nuclear regions of interest. Once all regions of interest were retrieved, the following metrics were derived: mean spot area (MSA, a measure for inclusion size), mean spot number (MSN, reflecting inclusion number per cell) and mean spot occupancy per cell (CSO, ratio of inclusion and cellular surface, an estimate for the overall replication), which will be further referred to as inclusion size, inclusion number and overall replication.

Statistical Analysis

Data were subjected to a two-way ANOVA (ANalysis Of VAriance) in order to determine the effect of two independent variables (cell line, 6 levels; medium, 2 levels) on the dependent continuous variables MSA, MSN and CSO. In case two-way ANOVA indicated that there was no interaction between both factors (cell line and medium), the interaction term was

eliminated and the effects of the factors culture medium and cell line on the continuous variables were estimated. Means of all treatment groups were separated using a Tukey post hoc test. Assumptions of normality and homoscedasticity were checked and data were transformed in case of non-normality or heteroscedasticity. p-values below 0.05 were considered significant. All data were analysed using Spotfire S+ 8.2.

Results

Figure 2 summarizes the major effects witnessed for all *Chlamydia* species studied per parameter in boxplots. Based on a two-way ANOVA, followed by Tukey HSD post hoc tests ($p < 0.05$), significantly different growth conditions were grouped and given a color-code for facile recognition of optimal growth conditions. Representative images of cells causing the most divergent *Chlamydia* growth conditions are shown in figure 3.

The inclusion size of *C. suis* H7 was significantly affected by the cell type ($p < 0.001$), but not by the culture medium. The inclusion size was significantly higher in Caco-2 cells, compared to BGM, McCoy and Vero cells. Moreover, the inclusion size in IPEC-J2 and SK-6 cells was significantly higher than in BGM and McCoy. Pairwise comparison of the groups revealed a higher number of inclusions in IPEC-J2, BGM and Vero (only MEM) cells, compared to Caco-2, McCoy and SK-6 cells. When scrutinizing the combined parameter of inclusion size and number, i.e. overall replication, the significant effect of the cell type re-emerged ($p = 0.005$). The highest overall replication was demonstrated in IPEC-J2 cells, which was significantly different from McCoy, SK-6 and Vero (only IMDM). Taking the different parameters together, *C. suis* H7 shows optimal growth in IPEC-J2, irrespective of the culture medium.

Pairwise comparison of the groups showed a significantly higher inclusion size for *C. suis* R24 in McCoy cells, when cultured with MEM, BGM and Vero cells, for both culture media,

compared to Caco-2, IPEC-J2 and SK-6 cells (Figure 2). The inclusion number was significantly higher in Vero cells, compared to all other cell lines, irrespective of the culture medium. Furthermore, a significantly higher inclusion number was demonstrated in BGM and McCoy, compared to Caco-2 and IPEC-J2. The inclusion number was significantly lower in SK-6 compared to all other cell lines. Finally, the inclusion number of *C. suis* R24 was significantly higher when cultured in IMDM medium compared to MEM, for each of the six cell lines investigated. Pairwise comparison of the groups showed a significantly higher overall replication in McCoy cells, BGM and Vero cells, for both culture media, compared to Caco-2, IPEC-J2 and SK-6 cells. Thus, the replication of *C. suis* R24 was highest in Vero cells, and significantly different from replication in Caco-2, IPEC-J2 and SK-6.

No effect of the culture medium on the inclusion size of *C. suis* S45 was shown. Moreover, no significant differences were detected among the cell lines (Figure 2). However, the inclusion number ($p<0.001$) and overall replication ($p=0.003$) was significantly lower in SK-6 and Vero cells, compared to the other cells.

Discussion

Currently, only few studies exist on the growth characterization of porcine chlamydial strains in cell culture. In the current study, inclusion size, number and occupancy were determined to estimate replication performance of the *C. suis* strains. Replication varied between the *C. suis* strains investigated, which can partly be explained by the origin of the chlamydial strains. *Chlamydia suis* strain H7 was originally isolated from the conjunctiva of a pig suffering from conjunctivitis. The overall replication of this strain was significantly higher in IPEC-J2 cells compared to McCoy and SK-6, due to a high inclusion size and a higher inclusion number. Thus, IPEC-J2 cells were more susceptible for *C. suis* H7 replication. The respiratory strain *C. suis* R24 was isolated from the nasal mucosa of a pig suffering from upper respiratory tract

disease. This strain appeared to be more adapted for replication in BGM, McCoy and Vero, which are established cell lines for propagation of *C. suis*. Unlike the other strains, the size of *C. suis* S45 inclusions was not affected by the cell line or the culture medium. However, a clear effect on inclusion number and overall replication was witnessed, in that overall SK-6 and Vero cells performed worse. This corresponds to the results obtained by Schiller *et al.* (Schiller and others, 2004), who demonstrated an elevated *C. suis* S45 inclusion number in Caco cells compared to Vero cells. Interestingly, *C. suis* S45 was isolated from the feces of a clinically healthy pig. Yet, the isolation of *C. suis* in culture can be very challenging, particularly for rectal strains (Sandoz and Rockey, 2010), which compromises further characterization. Performing isolation on intestinal cell lines, such as Caco-2 and IPEC-J2, might thus increase the recovery rate of rectal *C. suis* isolates. The divergence observed in our results might be attributed to biological differences between cell lines and chlamydial strains, changing the effectiveness of cell entry or the ability to grow in the host cell cytoplasm, leading to an altered infectivity (Knoebel and others, 1997). Indeed, the species *C. suis* is presumed to be genetically more diverse than other chlamydial species, based on DNA sequence analysis of *ompA* (Everett and others, 1999) and the extensive variation in virulence among *C. suis* strains in pigs (Bush and Everett, 2001).

Cell culture conditions, including the passage number of the cells, and pH and composition of the culture medium, have been shown to strongly influence cell performance and chlamydial replication (Sambuy and others, 2005; Schierack and others, 2006). Schiller *et al.* (2004) reported a significant increase of *C. suis* S45 inclusion number in Vero cells when cultured with IMDM instead of MEM. Yet, we only observed a minor effect of the maintenance medium on the propagation rate of the investigated strains, which also appeared to be cell line and chlamydial strain dependent.

Our findings prove clear differences in the growth performance of divergent *C. suis* strains on the investigated cell lines. Moreover, we demonstrated further potential for isolation optimization, and identified cell lines outperforming those routinely used for chlamydial isolation. Thus, adaptation of the isolation procedure to the origin of the putative field isolate, is highly recommended to improve the recovery rate of *C. suis* isolates. The high-content approach that has been optimized in this study seamlessly lends itself for fast screening of cell lines to enhance propagation of characterized strains, or to optimize the isolation of unidentified strains.

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Author's contributions

LDP and KDP participated in the design of the study, carried out the experimental work (*C. suis* cell culture, immunofluorescence staining and microscopic analyses), performed data analysis and interpretation, and drafted the manuscript. DV was involved in the design of the study and participated in drafting and critically revising of the manuscript. WDV contributed to the design, acquisition and analysis of data, and co-wrote the manuscript. All authors read and approved the final manuscript.

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321

322 **Figure 1** - Image analysis workflow for high-content analysis of *C. suis* propagation in cell culture.

323 The block scheme depicts the major steps of the image analysis routine and the bottom panel shows

324 representative intermediate pictures. Raw hyperstacks (1) are first projected according to the

325 maximum intensity in Z (2,MP) after which individual channels (DAPI, Evan's Blue, ChLAB) are

326 separated (3, CS) for object detection. The nuclear channel (DAPI, a) is sequentially smoothed with a

327 Gaussian Blur kernel (5, GB), automatically thresholded (6, T) and touching objects are separated with

a watershed algorithm (7, WS). Separation lines between detected nuclei (d) are defined with a Voronoi tessellation (7, VT, g). The cell channel (Evan's Blue, b) is smoothed with a Gaussian Blur kernel (9, GB), thresholded (10, T, e) and individual cells (12) are detected by conditional region growing (11, RG) using the VT lines (g) as boundaries. Putative *Chlamydia* patches are detected in the third channel (ChlAB, c) by selective spot enhancement, using a Laplacian of Gaussian (13, LG), thresholding (14, T) and size filtering (15, SF, f). Once masks are defined for cells (12) and spots (16), their surface area and number are determined, to provide different readouts per image: spot number (SN), mean spot number per cell (MSN), mean spot area (MSA) and mean spot occupancy per cell (CSO).

Figure 2 - Boxplots presenting the growth characteristics of *Chlamydia suis* species H7, R24 and S45 in cell culture. The growth was characterized in six cell lines (BGM, Caco-2, IPEC-J2, McCoy, SK-6 and Vero cells) and two culture media (MEM and IMDM). The inclusion size (mean spot

number, a), number of inclusions per cell (mean inclusion number, b), and the overall replication (cellular spot occupancy, c) was determined. Based on a two-way ANOVA, followed by Tukey HSD post hoc tests ($p < 0.05$), significantly different growth conditions were grouped and given a color-code for facile recognition of optimal growth conditions (shown below each boxplot).

Figure 3 - Representative fluorescence microscopy images of *C. suis* species H7, R24 and S45 in cell culture at 40 h p.i. The images represent an overlay of DAPI (grey) and the ChlAB (red) channels. Only those cells are shown for which the strongest, divergent effects were witnessed (cfr. Fig. 2).