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Human *Chlamydia pneumoniae* isolates demonstrate ability to recover infectivity following penicillin treatment whereas animal isolates do not

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Abstract

Chlamydia pneumoniae strains have recently been demonstrated to have substantially different capacities to enter and recover from IFN-γ induced persistence, depending on whether they are from human or animal host sources. Here we examined the ability of two human and two animal strains to enter and be rescued from penicillin induced persistence. The ability to form inclusions after the addition of penicillin was much reduced in the two animal isolates (koala LPCoLN, bandicoot B21) compared to the two human isolates (respiratory AR39, heart A03). The penicillin treatment resulted in a dose dependent loss of infectious progeny for all isolates, with the human strains failing to produce infectious progeny at lower doses of penicillin than the animal strains. The most remarkable finding however was the contrasting ability of the isolates to recover infectious progeny production after rescue by removal of the penicillin (at 72 h) and continued culture. The animal isolates both showed virtually no recovery from the penicillin treatment conditions. In contrast, the human isolates showed a significant ability to recovery infectivity, with the heart isolate (A03) showing the most marked recovery. Combined, these data further support the hypothesis that the ability to establish and recover from persistence appears to be enhanced in human *Chlamydia pneumoniae* strains compared to animal strains.

Introduction

Chlamydia (*C.*) *pneumoniae* is known to cause acute respiratory disease in humans including, pneumonia, pharyngitis, bronchitis, and sinusitis (Ferrari, *et al.*, 2002, Hahn, *et al.*, 2002). A range of chronic diseases have also been associated with the pathogen, including, asthma, atheroschlerosis, and Alzheimer's Disease (Hannu, *et al.*, 1999, Gerard, *et al.*, 2006, Haider, *et al.*, 2011). *C. pneumoniae* is an obligate intracellular pathogen with a unique bi-phasic developmental cycle. The developmental cycle consists of an active replicating intracellular phase which is exclusively located inside a novel vacuole termed the inclusion vacuole, and a non-replicative extracellular form (reviewed, (Adbelrahman & Belland, 2005)). The intracellular form has also been demonstrated to be capable of entering a third phase termed persistence (reviewed, (Hogan, *et al.*, 2004)). Persistence, which has been more comprehensively characterised in closely related *Chlamydia* (*C.*) *trachomatis*, is typified by extended association of the organism with the host cells in a viable but non-culturable form. The persistent form of the organism is typified by non-replicative cells that become enlarged, albeit fewer and in a smaller inclusion, and that continue to replicate the chromosome but in the absence of cytokinesis (reviewed, (Hogan, *et al.*, 2004)). The persistent phase of growth is induced by a range of conditions, including; immune stress (specifically IFN-γ mediated depletion of tryptophan), antibiotics, co-infections with viruses, and iron depletion (reviewed, (Wyrick, 2010, Schoborg, 2011)). Whilst many of these models have been conducted in *C. trachomatis*, persistence may well have been the underlying mechanism that leads to chronic disease in humans in the case of *C. pneumoniae*. There are already distinctions between the two species (*C. pneumoniae* and *C. trachomatis*), with *C. pneumoniae* more able to infect or even propagate in some host immune cells, which may facilitate systemic dissemination (Azenabor & Chaudhry, 2003, Beagley, *et al.*, 2009, Abdul-Sater, *et al.*, 2010), and evidence or isolation of *C. pneumoniae* from a range of tissue chronic disease sites has helped build the

possible association with chronic disease (Bauriedel, *et al.*, 1999, Bauriedel, *et al.*, 2000). Accordingly, it has been established that IFN-γ induction of IDO expression can induce persistence of *C. pneumoniae* in HEp-2 cells (Mehta, *et al.*, 1998), aortic smooth muscle cells (Pantoja, *et al.*, 2000, Pantoja, *et al.*, 2001), and endothelial cells (Bellmann-Weiler, *et al.*, 2010), but not in lymphocytes (Ishida, *et al.*, 2013). Additionally, it has been established that penicillin and iron limitation, can also induce persistence in HeLa cells (Klos, *et al.*, 2009). Gene expression array data supports that the *C. pneumoniae* persistence models involve continued DNA replication that is uncoupled from cytokinesis (Byrne, *et al.*, 2001). In addition to these *in vitro* persistence models, *C. pneumoniae* has been both observed and cultured from systemic chronic disease locations, including aortic or plaque tissue and brain tissue (Ramirez, 1996, Balin, *et al.*, 1998, Bauriedel, *et al.*, 1999, Bauriedel, *et al.*, 2000). Hence, *C. pneumoniae* persistence appears to be a potential factor in the chronic diseases associated with this organism.

Our own research has been interested in the relationship between animal *C. pneumoniae* and human *C. pneumoniae* strains. We have previously proposed that human *C. pneumoniae* have originally arisen from at least two zoonotic events in the past and that the animal strains appear to be ancestral to human strains (Mitchell, *et al.*, 2009, Myers, *et al.*, 2009, Mitchell, *et al.*, 2010, Mitchell, *et al.*, 2010). Importantly, we have demonstrated that animal *C. pneumoniae* strains (LPCoLN, koala source and B21, bandicoot source) have far reduced capacity to enter and recover from persistence when compared to human isolates AR39 (respiratory) and A03 (aortic) in an IFN-γ persistence model in lung epithelial cells (Chacko, *et al.*, 2014, Huston, *et al.*, 2014). Furthermore, there were also differences in the ability to enter and recover from persistence between the two human isolates themselves, that lends further support to the hypothesis that persistence *in vitro* is associated with chronic disease *in vivo* (Chacko, *et al.*, 2014, Huston, *et al.*, 2014). Here, we test the hypothesis that the same

human and animal *C. pneumoniae* strains will have distinct persistence profiles in the penicillin-G model of persistence.

Materials and Methods

Culture and analysis of chlamydial isolates

C. pneumoniae isolates from human and animal strains were cultured routinely in BEAS-2B (lung epithelial cells, ATCC) cells for these experiments for propagation and maintenance of stocks, viability assessments, and penicillin experiments. The isolates used were *C. pneumoniae* koala isolate LPCoLN (Wardrop, *et al.*, 1999), bandicoot isolate B21 (Kumar, *et al.*, 2007), human respiratory isolate (AR39) (ATCC 53592), and human aortic isolate A03 (Ramirez, 1996). BEAS-2B cells (sourced from ATCC) were cultured in RPMI 1640 culture media (Invitrogen), supplemented with 10% heat inactivated fetal calf serum, at 37°C 5% CO_2. When indicated, 50-500 U ml^{-1} of penicillin G (CSL Limited, Australia) was added (4 h PI). The assessment of viability was conducted by harvesting cell cultures in SPG buffer (218 mM sucrose, Potassium phosphate buffer pH 7.4, 4.9 mM L-glutamate) at the indicated time points, lysis by sonication (Ultrasonic Cell disrupter XL, Microson, USA), and subsequent re-culturing of serial dilutions of the harvested cultures onto monolayers of BEAS-2B cells. Monolayers were fixed and examined by microscopy at approximately 72 h PI for viability counts. Media was routinely removed at 4 h PI (hours post infection).Cultures were also routinely conducted on glass coverslips for morphological analysis.

Microscopy

Enumeration of viability was routinely conducted by culturing serial dilutions of the cultures onto fresh BEAS-2B monolayers and fixing at 72 h PI. Cultures were stained with fluorescein

isothiocyanate (FITC) labelled *Chlamydia*-specific anti-lipopolysaccharide (anti-LPS) monoclonal antibody and host cells stained with Evan's blue (Cell labs, Australia) to facilitate enumeration of inclusion forming units per ml. Coverslip cultures were washed with PBS and fixed with 100% methanol for 10 mins. Coverslips were stained with FITC labelled *Chlamydia*-specific anti-lipopolysaccharide (anti-LPS) monoclonal antibody and counterstained with Evan's blue to visualize hosts cells (Cell labs, Australia) and then mounted on slides in Prolong Gold (Invitrogen). Confocal images was obtained using Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) with a 63x oil objective. Images were prepared using the Leica suite of programs.

Statistical analysis and graph construction

Statistical testing and graph construction was conducted using GRAPHpad Prism V7.0. In each experiment a minimum of three independent experimental replicates were conducted, 20 fields of view were analysed randomly sampled from all 3 replicates (n=20, unless otherwise indicated). The fold change (FC) data are FC of the mean of the datasets and do not account for error, these are shown only to provide ease of assessment of the relative differences between the isolates and the statistical significance is indicated where appropriate on each graph.

Results

Penicillin treatment results in a loss of infectious progeny, reduced inclusion sizes and reduced infectivity for *Chlamydia pneumoniae* human and animal isolates

The impact of penicillin treatment on *C. pneumoniae* isolates was examined to investigate if animal and human isolates demonstrated different responses to this antibiotic. In this study

we chose to use penicillin-G as it has been demonstrated to induce persistence with *C. pneumoniae* CWL-029 (Klos, *et al.*, 2009). However, several of the more clinically relevant penicillins have also been demonstrated to induce *C. trachomatis* persistence at physiologically relevant doses (Kintner, *et al.*, 2014). A dose series of 0-500 U ml^{-1} of penicillin was conducted on all four isolates, as described in Materials and Methods. The ability to form infectious progeny from these cultures harvested at 72 h PI was analysed. The two human isolates showed differences in sensitivity to penicillin treatment, with A03 more sensitive with a ~2 log loss of infectious progeny after 50 U ml^{-1} (1139 IFU ml^{-1} at 50 U compared to 361265 IFU ml^{-1} in the control) penicillin and AR39 showing ~1 log less infectious units at the same dose (22073 IFU ml^{-1} at 50 U compared to 325672 IFU ml^{-1} in the control) (average titers are indicated when numbers provided). The fold change (FC) data support the increased sensitivity of A03 to penicillin treatment with 50 U ml^{-1} penicillin causing a 0.03 FC in yield compared to the untreated control, while AR39 FC of yield relative to the control was 0.06 at this penicillin dose (Fig. 1.(a, b)).

The animal isolates also showed a similar loss of infectious progeny after penicillin treatment. Even though these strains produce much higher infectious progeny compared to the human strains, treatment with 50 U ml^{-1} of penicillin resulted in 0.04 FC for LPCoLN (2065000 IFU ml^{-1} from 50 U ml^{-1} compared to 48580000 IFU ml^{-1}untreated control), and 0.004 FC for B21 (227852 IFU $ml^{-1}$with 50 U ml^{-1} treatment compared to 47870000 IFU ml^{-1} in the control) at 72 h PI (Fig. 1.(c,d)). The B21 isolate was more sensitive to penicillin than LPCoLN. All of the strains had no infectious inclusion forming units at 500 U ml^{-1} of penicillin at 72 h PI and both of the human strains also did not have detectable inclusion forming units at 200 U ml^{-1} of penicillin.

The morphological appearance of the inclusions during the penicillin treatment was analysed using confocal microscopy at 72 h PI for each of the treatment conditions (Fig. 2.).

Persistent inclusions are smaller and often contain larger cells within the inclusion, or less staining within the inclusion, when examined by confocal microscopy. The control inclusions appear as expected, with the animal isolates having much larger inclusions than the human isolates, and all inclusions under the control conditions having abundant LPS staining inside the inclusion, consistent with what we have previously reported for these isolates (Fig. 2.) (Chacko, *et al.*, 2014). The inclusion size and appearance markedly changed for the 50 U ml^{-1} penicillin conditions for all of the isolates. Substantially reduced inclusion sizes and much less intensity of LPS staining inside the inclusion were observed, suggesting altered cellular morphology within the cells.

In order to quantify the differences in inclusion size and also to measure if the reduced infectious progeny observed in Fig 1 reflects reduced inclusion size or may also be impacted by ability to maintain the inclusion after penicillin was added at 4 h PI (infectivity), the inclusion size and percent infectivity was quantified from confocal images. The inclusion sizes (Table 1) showed a marked decrease in size at 50 U ml-1 penicillin compared to the control for all isolates ($p < 0.001$) that did not further reduce in size in a dose dependent manner for any of the isolates. However the percent of infected cells did show a dose dependent decrease for all of the isolates, suggesting that inclusions did not develop or did not continue to develop in response to the penicillin treatment (Table 2).

The animal *C. pneumoniae* isolates are not able to be rescued from the penicillin induced lack of infectious progeny production

The key feature of chlamydial persistence is that the loss of infectious forms can be rescued by removal of the persistence inducing agent. Therefore, we measured the ability to restore the infectivity to these cultures by removal of penicillin to determine if the growth characteristics observed here are consistent with persistence. Cultures were conducted with

penicillin treatment at 4 h PI, removal of the penicillin at 72 h PI, and continued culture until 144 h PI, to allow recovery from persistence and development of infectious progeny. For the control cultures, the penicillin was not removed. The inclusion forming units were measured from these cultures harvested at 144 h PI. The human isolates A03 and AR39 both showed considerable increases in infectious progeny recovery when 50 U ml^{-1} and 100 U ml^{-1} penicillin treated cultures were rescued. AR39 infectious yield increased by 2 log relative to the control (113 FC relative to non-rescue control) when 50 U ml^{-1} culture was rescued for 72 h, and the 100 U ml^{-1} culture, which had a complete loss of viability without removal of penicillin, had a very remarkable 3 log of infectious progeny recovery (Fig. 3.). The human strain A03 demonstrated even more marked recovery of infectious progeny, with ~2.5 log higher infectious yield with 50 U ml^{-1} culture was rescued (208 FC; 203.4 IFU ml^{-1} compared to rescue yield of 16253.7 IFU ml^{-1}); ~1.5 log higher infectious yield when the 100 U ml^{-1} culture was rescued (29 FC; 712 IFU ml^{-1} compared to rescue yield of 20649 IFU ml^{-1}) (Fig. 3.). 3 log of infectious inclusion forming units were detected after the 200 U ml^{-1} penicillin treated A03 culture was rescued (from no detectable growth in the 200 U ml^{-1} treatment), the only isolate that demonstrated any recoverability from this concentration of penicillin.

In stark contrast, both the animal isolates showed minimal (if any) ability to recover from penicillin treatment. There appeared to be minor recovery in infectious progeny for the animal LPCoLN after rescue from both 50 and 100 U ml^{-1} cultures, relative to non-rescued control (1.75 and 1.25 FC respectively or; 34889 IFU ml^{-1} rescued from 50 U ml^{-1} compared to 19937 IFU ml^{-1} and 24921 IFU ml^{-1} rescued from 100 U ml^{-1} compared to 19937 IFU ml^{-1}) (Fig. 3c.). Similarly for the B21 animal isolate, some relatively minor recovery of infectious progeny was observed from both the 50 and 100 U ml-1 penicillin rescued cultures (Fig. 3d.). Specifically, a 2.6 FC relative to the non-rescued control was detected for the 50 U ml^{-1} rescued cultures (22905 IFU ml^{-1} rescued compared to 11392 IFU ml^{-1} from 50 U ml^{-1}

continued treatment), and 7.2 FC relative to the control was observed for the 100 U ml^{-1} rescued culture. These relatively minor recoveries in infectious progeny are considerably distinct compared to both human isolates, even though there were differences between all four isolates for the initial infectious progeny loss due to penicillin treatment. However, there was a significant difference between the human isolates, with the A03 human heart isolate demonstrating the strongest ability to recover infectious progeny from penicillin treatment. Recovery was to higher yields at lower doses and detected at a higher dose (200 U ml^{-1}) that AR39 was not able to demonstrate any detectable recovery at all.

Discussion

Chlamydia has a unique ability to enter a persistent phase of growth enabling them to maintain a viable but non-infectious intracellular form for considerable lengths of time during 'stress' conditions. This persistent phase has been hypothesised to be associated with chronic disease outcomes (Hogan, *et al*., 2004, Wyrick, 2010, Schoborg, 2011). Here we have demonstrated that two animal *C. pneumoniae* strains have a significantly reduced ability to enter into and recover from persistence, with fewer inclusions detected during penicillin treatment, and also are effectively unable to recover from penicillin induced persistence, with very minor recoveries observed under any condition. In contrast, both a respiratory (AR39) and heart (A03) human strain of *C. pneumoniae* were able to enter and recover from persistence very effectively. Interestingly, the heart isolate A03, that may have a direct link with chronic cardiovascular disease, showed the most substantial capacity to recover from persistence, with higher recovery detected under all conditions and also recovery as detected from a penicillin dose (200 U ml^{-1}) that no other isolate was able to be recovered or rescued from.

These observations are consistent with our recent report examining the IFN-γ induced persistence model (tryptophan depletion) and recovery for these same isolates. During the IFN-γ model, the animal isolates also had notable reductions in the precent of infected cells (inclusion numbers declined) (likely indicating death) than the human isolates, and also were much less able to recover infectious progeny compared to the human isolates (Chacko, *et al.*, 2014). In spite of these remarkable differences in growth and persistence the genome sequences between the *C. pneumoniae* strains are relatively highly conserved. However, upon analysis of the published genome sequences there are non-synonymous SNPs between human and animal isolates (LPCoLN and AR39) in the penicillin binding proteins and related proteins that might be relevant to these findings (Read, *et al.*, 2000, Mitchell, *et al.*, 2010, Mitchell, *et al.*, 2010). PBP3 has three non-synonymous SNPs, one of which introduces a premature stop codon making the protein five residues shorter in LPCoLN (Read, *et al.*, 2000, Mitchell, *et al.*, 2010, Mitchell, *et al.*, 2010). PBP2 has seven non-synonymous SNPs in LPCoLN compared to AR39. MreC has one non-synonymous SNP in LPCoLN compared to the AR39 sequence (Read, *et al.*, 2000, Mitchell, *et al.*, 2010, Mitchell, *et al.*, 2010). However, in all instances the SNPs are not in core protein motifs and are not predicted to alter the secondary structure, so there are no candidates that could be unequivocally used to explain the data we present here.

Combined, these data lead to two important conclusions about *C. pneumoniae* persistence. Firstly, that whilst the chlamydial adaptive responses to enter and recover from persistence must have some similarities between the different persistence inducing conditions, clearly there are also distinctions, as the lack of recovery observed here is much more marked for the animal isolates compared to our previous IFN-γ data (Chacko, *et al.*, 2014). Secondly, for *C. pneumoniae* the ability to enter and recover from adaptation appears to be a human strain specific feature, and this appears to have been enhanced/ or further selected for in strains that

have the ability to disseminate and be associated with chronic disease tissue locations. This supports our hypothesis that the disease modes associated with animal and human *C. pneumoniae* persistence are directly linked with the fundamental biological traits around ability to persist or not in these different strains (Huston, *et al.*, 2014).

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Tables:

Table 1. Inclusion sizes for human and animal *C. pneumoniae* strains during penicillin exposure at 72 h PI

	No PEN	50* PEN	100 PEN	200 PEN	500 PEN
Human AR39	9.19 ± 1.74	2.91 ± 0.94#	2.88 ± 1.00#	2.90 ± 0.94#	2.43 ± 0.34#
Human AO3	7.99 ± 1.39	2.86 ± 0.98#	2.85 ± 0.99#	2.68 ± 0.68#	2.49 ± 0.64#
Koala LPCoLN	21.21 ± 4.58	6.21 ± 1.39#	6.08 ± 1.16#	6.12 ± 1.03#	6.23 ± 1.02#
Bandicoot B21	24.86 ± 5.00	5.97 ± 1.13#	5.90 ± 1.21#	5.73 ± 1.20#	5.94 1.18#

*Penicillin U ml^{-1}

Inclusion sizes from each condition are represented as mean ± SD and are measured from independent coverslips n=20. Statistics was conducted using Student's two-tailed *t*-test compared to the control culture (No penicillin treatment) (# represents *** $p < 0.001$).

Table 2. The percentage of cell infected for each *C. pneumoniae* strain during penicillin exposure at 72 h PI.

	No PEN	50* PEN	100 PEN	200 PEN	500 PEN
Human AR39	6.37 ± 1.77	4.21 ± 1.76#	3.75 ± 1.86#	1.85 ± 1.00#	0.79 ± 0.32#
Human AO3	4.21 ± 1.76	2.27 ± 1.10#	2.12 ± 0.94#	2.30 ± 1.32#	1.01 ± 0.67#
Koala LPCoLN	8.64 ± 2.61	6.90 ± 2.23ns	4.38 ± 2.20#	2.83 ± 1.25#	0.83 ± 0.31#
Bandicoot B21	7.45 ± 2.84	4.38 ± 2.20#	2.83 ± 1.25#	2.30 ± 1.32#	0.80 0.31#

*Penicillin U ml^{-1}

The percentage of cell infected from each condition are represented as mean ± SD and are measured from independent coverslips n=20. Statistics was conducted using Student's two-tailed *t*-test compared to the control culture (No penicillin treatment) (# represents *** $p < 0.001$, ns (non-significant) $p > 0.05$).

Figure legends

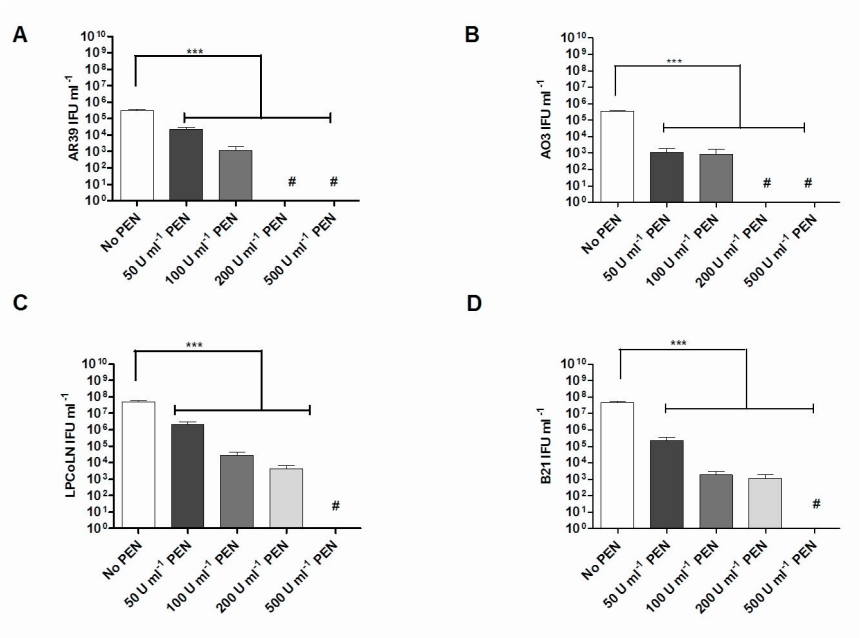

Fig. 1. Penicillin exposure reduces the infectious progeny production in both human and animal *C. pneumoniae* isolates. The figure shows the infectious progeny of *C. pneumoniae* isolates infectious yield (IFU ml^{-1}, y axis) from penicillin exposure. The different penicillin treatments are indicated on the x axis. # indicates that no inclusions were detected. The graphs show; (a) *C. pneumoniae* AR39; (b) *C. pneumoniae* A03; (c) *C. pneumoniae* LPCoLN, and (d) *C. pneumoniae* B21. Statistics were conducted using Students's two- tailed *t* –test relative to the untreated controls (Data are mean ± SEM with n = 20; $^{***}P < 0.001$). All the pen treated cultures were statistically significant ($^{***}p < 0.001$) compared to the controls for each isolate.

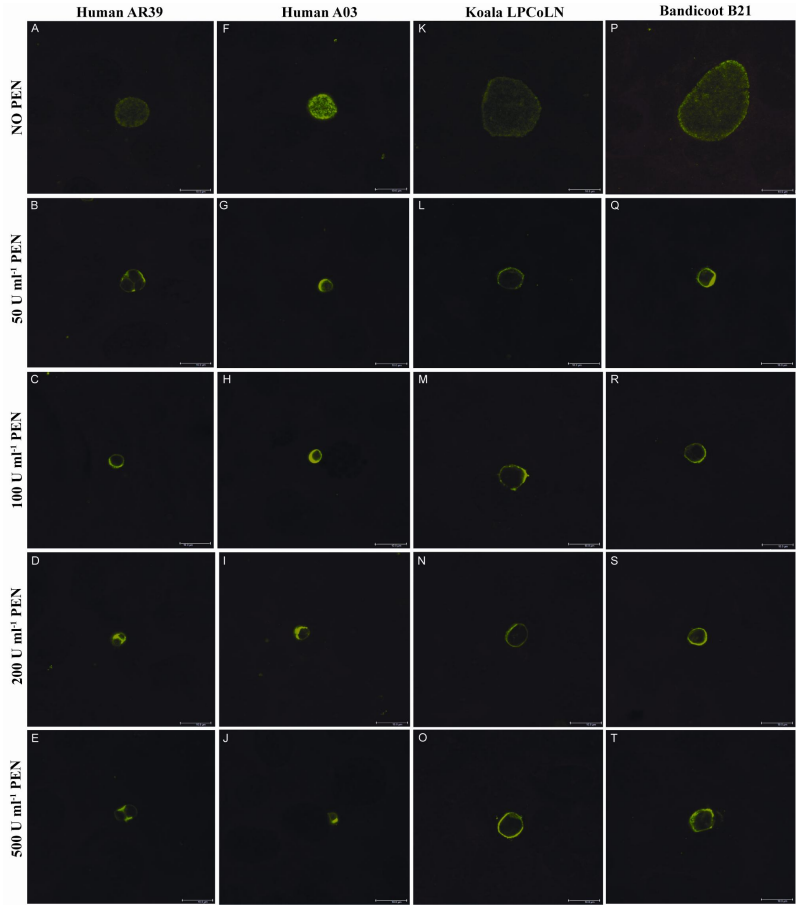

Fig. 2. Penicillin exposure alters chlamydial morphology in both human and animal *C. pneumoniae* isolates. BEAS-2B cells were plated on glass coverslips and were infected at an MOI of 0.1 with *C. pneumoniae* isolates. Chlamydial inclusions are shown in green and host cells are shown red. (A-E) Representative confocal images of human AR39 *C. pneumoniae* cultures from control, 50, 100, 200 and 500 U ml^{-1} penicillin at 72 h PI. (F-J) Representative confocal images of human A03 *C. pneumoniae* cultures from control, 50, 100, 200 and 500 U ml^{-1} penicillin at 72 h PI. (K-O) Representative confocal images of animal LPCoLN *C. pneumoniae* cultures from control, 50, 100, 200 and 500 U ml^{-1} penicillin at 72 h PI. (P-T) Representative confocal images of animal B21 *C. pneumoniae* cultures from control, 50, 100, 200 and 500 U ml^{-1} penicillin at 72 h PI. The scale bar in the bottom right of each image represents 10μm.

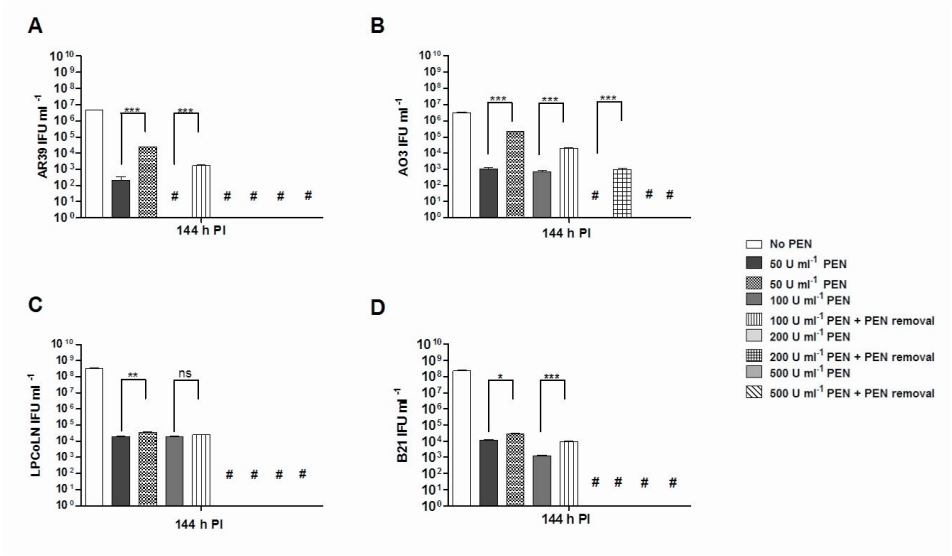

Fig. 3. Inclusion forming units at 144 h PI for each isolate that has been 'rescued' (i.e. penicillin removed at 72 h PI) and controls that were not rescued. The graphs show the inclusion forming units infectious yield (IFU ml^{-1}) (y axis) for each isolate at 144 h PI; (a) *C. pneumoniae* AR39; (b) *C. pneumoniae* A03; (c) *C. pneumoniae* LPCoLN; and (d) *C. pneumoniae* B21. The treatment conditions are indicated in the image to the right of the graphs. Statistics were conducted using Students's two- tailed *t* –test (Data are mean ± SEM with n = 20).