Genomic variant representation in a Chlamydia population is dynamic and adaptive with dependence on in vitro and in vivo passage

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One sentence summary: Multiple variants exist in chlamydial populations and these change when a rodent host is infected.

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ABSTRACT

We have previously shown that Chlamydia muridarum has multiple genomic variants that concomitantly vary in their in vitro and in vivo phenotype. Herein, we used real-time polymerase chain reaction-based genotyping assays to query plaque-cloned isolates of C. muridarum for the frequency of eight selected polymorphisms. These strains had no history of passage in vivo since their original isolation from laboratory mice. There was significant variance in the frequency of two of the eight polymorphisms assessed with the remaining exhibiting a low rate of variance. To determine if any of these polymorphisms were more favorable for in vivo conditions, we blindly passaged non-clonal C. muridarum three times at 7-day intervals through the urogenital tract of mice. Seven of the eight polymorphisms varied in frequency following in vivo passage and four of these varied between C. muridarum strains. Selected isolates displayed variable growth rates and cytopathic effect in vitro. We conclude that multiple genotypic variants are present within the existing known C. muridarum strains and that the frequency of these variants changes upon introduction into the mouse host. These findings lend support to the concept that genotypic proportional representation in a chlamydial population is dynamic and adaptive.

Keywords: Chlamydia; genotype; polymorphism; allele; host; genome

INTRODUCTION

Chlamydia spp. are pathogens of humans and animals, infecting a wide range of tissues and anatomical sites and causing an equally wide range of disease states from host to host (Kaltenboeck 2006; Batteiger 2012). The manifestations of chlamydial infection include acute inflammatory reactions, chronic insidious disease and latent asymptomatic or ‘persistent’ infections. Variance in host response likely contributes to these disparate outcomes (Geisler et al., 2004; Natividad et al., 2005,2006, 2007,2008; Wang et al., 2005) but little is known of
how the variability of chlamydial pathogens allows for complic- ity in eliciting host responses or to enhance their chances for survival and subsequent transmission to a new host. On a global scale, the process of lateral gene transfer (LGT) allows for selec- tion of new variants from related bacterial strains circulat- ing within a host population. Evidence for LGT in Chlamydiae has been demonstrated but necessarily would only occur during mixed chlamydial infections (Gomes et al., 2006, 2007; DeMars et al., 2007; Jeffrey, Maurelli and Rockey 2012).

In addition to LGT, another more refined adaptation that could be envisioned would be the presence of spontaneous mu- tations. Like all bacteria, chlamydiae exhibit spontaneous mut- ation as a natural process. Thus, in the context of a chlamydial population represented in the inoculum during a single trans- mission event, genomic variants that arise naturally would be amplified in select hosts or anatomical sites. Niche-specific selec- tion has been demonstrated for Chlamydia pneumoniae (Gieffers et al., 2003) and several recent studies support the concept that variants likely exist within a single infectious population or inoculum. For example, Kari et al. (2008) showed that two separate isolates of C. trachomatis, serovar A, exhibited disparate in vivo and in vitro phenotypes thereby demonstrating that variances in virulence within a single serovar occurs. Subsequently, we have shown that the commonly used mouse pathogen, C. muridarum, contains at least two isolates that differ in their in vitro growth characteristics as well as their in vivo pheno- type (Ramsey et al., 2009). Two clonal genomic variants were described from these isolates but we postulated that both strains were even more heterogeneous with regard to genotypic reperto- rie. Yeruva et al. (2014) more recently confirmed this postulate when they reported that one of these strains of C. muridarum (Nigg strain) in fact contained multiple genomic variants and that these often differed phenotypically both in vitro and in vivo. This was further confirmed at a recent international meeting on chlamydial infections (Sullivan et al., 2014a,b). Similarly, a sin- gle ocular isolate from C. caviae-infected guinea pigs has been shown to contain genomic variant clones that differ in their sen- sitivity to azithromycin and exhibit disparate properties in vitro and in vivo (Binet et al., 2010). Lastly, Sturdevant et al. (2010) has shown that a single inoculum of C. trachomatis, serovar D, con- tains genomic variants that, when inoculated intravaginally into mice, display differing infection kinetics and eliciting disparate pathological outcomes.

It is important to note that in each of the aforementioned reports, the genomic variations identified were highly focused and limited in scope to small changes in a handful of genes— yet each was sufficient to significantly alter phenotype. Some of the predicted changes were in a relatively small number of non-synonymous mutations and thus resulting in alterations, deletions or insertion, of a single amino acid. In some cases, a frameshift in a gene was observed but in no case were wholesale gene deletions or substitutions observed. It cannot be discerned at this point whether these mutations were induced or amplified by cell culture passage as has been observed by some (Borges et al., 2013) or were present at the time of isolation of each of the relevant strains and preserved in culture passage; although, the concept of cell culture being an ‘evolutionary freezer’ for Chlamydiae has been proffered.

In the present study, we asked a more basic but related ques- tion with reference to specific chlamydial genes: Would the propor- tional representations of genomic variants in a chlamydial population change following passage in culture and through the natural host? To assess this possibility, we followed several previously identified single and multinucleotide polymorphisms (SNPs and MNP) and insertion/deletion (indels) in two strains of C. muridarum following in vitro and in vivo passage through the mouse host. We will demonstrate that the representation of these different genomic variants within a population is depen- dent upon whether the population is passaged in vitro or in vivo.

### MATERIALS AND METHODS

#### Mice, infections and infection monitoring

Mice were purchased at 5–6 weeks of age and from the suppliers in Table 1 and allowed to acclimate in the Animal Resources Facility at Midwestern University for 10 days. Prior to urogeni- tal infection, mice were treated with medroxyprogesterone ac- etate (Greenstone LLC, Peapack, NJ, USA) at 2.5 mg subcuta- neously as has been described previously (Cotter et al., 1996). Seven days later, mice were infected with 10^7 inclusion-forming units (IFU) of C. muridarum intravaginally (Cotter et al., 1997) in 10 μl volume. In order to verify and track genital infection, cervical- vaginal swabs were collected at 4 days post-infection, at day 7, 10, 14 and every 7 days thereafter up to day 42. Swabs were placed in sucrose-phosphate-glutamic acid buffer (SPG, pH 7.2) and frozen at –80°C until isolations could be conducted. Chlamydia muridarum was isolated; isolations visualized by indirect immunofluorescence and the presence of IFU in HeLa 229 cultures determined as previously described (Ramsey et al., 1999). All mice were euthanized on day 56, necropsied and tissues were examined in situ for gross macroscopic evidence of pyosalpinx or hydrosalpinx formation (Shah et al., 2005). All animal proce- dures were approved by the Midwestern University Institutional Animal Care and Use Committee.

#### In vivo passage scheme

Serial in vivo passage was conducted by inoculating three progesterone-pretreated BALB/c mice intravaginally with stock

| Strain          | Suppliers                        | Suppliers                        | Suppliers                        | Suppliers                        |
|-----------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| BALB/c          | Harlan Sprague Dawley            | Charles River Laboratories       | Taconic Laboratories             | Harlan-Sprague-Dawley            |
| C3H/HeN         | nd                               | nd                               | nd                               | nd                               |

*Includes observations from unpublished data as well as published findings (Ramsey, DeWolfe and Salyer 2000; Shah et al., 2005; Initiaz et al., 2007; Lee et al., 2010a, b).
cell-culture-derived *C. muridarum* as described above. At day 7 post-infection, cervical-vaginal swabs were collected, pooled into a single vial containing 500 μl of ice cold SPG and 3–5 sterile glass 2 mm beads. The vial was then vortexed vigorously for 30 s. Ten microliters of the resulting suspension was then used to inoculate three additional mice (passage A). Seven days later, the process was repeated and used to inoculate three additional mice (passage B). Following another 7 days, swabs were collected and pooled as before and the resulting suspension was frozen at −80 °C (passage C). For the present study, plaque isolates from the third passage suspension were used for expansion and SNP-based genotyping assays.

**Chlamydia muridarum** growth, maintenance, clonal expansion and plaque assay

The *C. muridarum* strains used in this study were grown and maintained exactly as previously described (Ramsey et al., 2009). To derive clonal populations of *C. muridarum*, we used plaque assay in L929 mouse fibroblast monolayers grown in 6-well tissue culture plates exactly as described in Ramsey et al. (2009) and as adapted from O’Connell and Nicks (2006). Briefly, stock cultures of non-clonal *C. muridarum* strains or pooled swab samples following in vivo passage were diluted and inoculated onto confluent monolayers of L929 mouse fibroblasts in 6-well tissue culture plates in such a manner as to achieve ∼15–25 plaques per well. Following agarose-containing media overlay, the cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 4–5 days to allow plaques to form. Some plaques assays were plated separately for plaque size analysis at 4 days of incubation according to a previously published procedure (Ramsey et al., 2009). For genotyping, well-isolated plaques were selected for clonal expansion. Plaque material was aspirated using a micropipettor and placed in 100 μl of SPG and either frozen at −80 °C or diluted in media, probe sonicated briefly and inoculated directly onto 25 cm² tissue culture flasks containing confluent or near confluent monolayers of HeLa 229 cells. The flasks were rocked slowly at room temperature for 1 h in low total volume (∼1 ml) and then incubated for 1 h at 37 °C. This was followed by aspiration and replacing the inoculum with fresh media and return to 37°C. The monolayers were then monitored for inclusion formation by scanning twice daily by inverted microscopy. Inclusions were often observed by 24–36 h and the flask was harvested when inclusion density subjectively reached at least 10% of the monolayer. In any case, no flask was maintained beyond 5 days without further passage and most were harvested within 36–72 h. The 25 cm² flasks were harvested by scraping the monolayer, followed by probe sonication, and low-speed centrifugation (400 × g for 10 min at 4 °C) to clear cellular debris. The supernatant was inoculated onto a fresh monolayer of HeLa 229 in one or two 75 cm² flasks (based subjectively on inclusion density in the 25 cm² flask) as above and monitored for growth as before until at least 10% inclusion density in the monolayer was attained.

For harvest of 75 cm² flasks, the prior procedures were followed except that following low-speed centrifugation, the supernatant was centrifuged at 30 900 × g at 4 °C for 30 min and the resulting pellet resuspended in SPG and frozen at −80 °C. It should be noted that in most cases, once transferred to 75 cm² flasks, expanded plaque isolates were harvested following a single round of replication. Thus, assuming a 30–36 h time to inclusion maturation, we estimate that the data derived from most plaque-clonal isolates in these studies are reported following no less than two and no more five rounds of replication in vitro.

**Real-time polymerase chain reaction to detect targeted SNPs, MNPs and indels**

Genomic DNA (gDNA) was extracted from a 500 μl SPG aliquot of the plaque-cloned isolates derived as described above using a commercially prepared kit according to the manufacturer’s instructions (QiAAMP DNA Mini Kit; Qiagen Inc, Valencia, CA, USA). All samples were analyzed using NanoVue Plus spectrometer (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) for final DNA concentration. The gDNA samples were diluted to a final concentration of 50 μg ml⁻¹ in DNase/RNase-free water and checked for chlamydia DNA by polymerase chain reaction (PCR) targeting the chlamydial major outer membrane protein (MOMP). We used 12.5 μl 2X Taqman® Genotyping Master Mix (Life Technologies, Grand Island, NY, USA); MOMP forward (5′-TAGCCGAGACGTAAGCTGAT-3′) and reverse (5′-ATTACATTTGCGGTCTGG-3′) primers (1 μl at 0.8 μM final concentration each); template gDNA (2 μl at 50 ng μl⁻¹), and 8.5 μl RNAase/DNAase-free water for a total volume of 25 μl per tube. The PCR parameters were 2 min at 96 °C; 40 cycles of 30 s at 96 °C, 1 min at 57 °C and 1 min at 72°C; and 5 min at 72°C. Each sample was run on a 1.5% Agarose gel to view 167 bp bands which confirmed the presence of chlamydial gDNA.

Targeted SNP, MNP and indel polymorphisms in the genome from published *C. muridarum* Nigg (GenBank database accession number NC_002620) (Read et al., 2000), Nigg2 (GenBank accession number ACOV01000000) or Weiss (GenBank accession number ACOW00000000) (Ramsey et al., 2009) strains were identified (Table 2) and analyzed for application with TaqMan® SNP Genotyping Assays (Applied Biosystems/Life Technologies, Grand Island, NY, USA). Primers were designed using the Custom TaqMan® Assay Design Tool (https://www.lifetechnologies.com/order/custom-genomic-products/tools/genotyping). The assays were designed with two allele-specific primer pairs and two probes with either 6-FAM or VIC dyes that bind the complementary allelic product utilizing the minor groove binder technology. The assay allows discrimination with probes as short as 13 bases and detection of binding is by exonuclease cleavage of the 5’ allele-specific dye label. Assays were run on 96-well PCR plates at 25 μl total volume (12.5 μl TaqMan® Genotyping Master Mix; 1.25 μl custom designed assay primer/probe constituents; and 11.25 μl gDNA) in an ABI 7300 Real-time PCR machine programed with the following conditions: 95°C for 10 min, 92°C for 15 s and 60°C for 1 min for 40 cycles. All assays were checked for validity and every 96-well plate reaction was conducted with incorporated internal positive and negative controls (gDNA containing from variants known to contain the targeted allele or not) as well as no template controls (RNAse/DNAase-free water only) to assess for contaminating gDNA.

**One-step growth rate determination for *C. muridarum***

Clonal *C. muridarum* variants were inoculated at identical multiplicity of infection (0.1), based on the concentration of IFU in 0.5 ml of minimal essential media (MEM) onto monolayers of HeLa 229 in 24-well plates. The plates were centrifuged 1 h (1100 × g) at 35–37°C followed by 1 h of incubation at 37°C and media exchange to (MEM, 10% fetal bovine serum, 3 mg ml⁻¹ added glucose and 0.5 μg ml⁻¹ cycloheximide) (Ramsey et al., 2009). At 28 h following media exchange, the 24-well plate was frozen at −80°C. Upon thawing, the contents of each well were diluted and subcultured in a 24-well plate well prepared with confluent HeLa 229 monolayers. Following identical incubation and
Table 2. Section of non-synonymous SNPs, MNPs and indels assessed for population frequency in this study.

| ORF annotation (chromosome position) | Gene description | NT change | AA change | References | Selection (reason) |
|--------------------------------------|------------------|-----------|-----------|------------|-------------------|
| TC0052 (59062)                       | Major outer membrane protein, porin (ompA) | GTT → DEL | C → DEL | Read et al. (2000), Ramsey et al. (2009), Yeruva et al. (2014) | No (non-discrimination) |
| TC0052 (59065)                       | Major outer membrane protein, porin (ompA) intergenic | C → T | S → F | Read et al. (2000), Ramsey et al. (2009) | No (non-discrimination) |
| - (126406)                           | intergenic       | INS → A | NA       | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | No (Intergenic, non-discrimination) |
| - (126416)                           | intergenic       | A → DEL | NA       | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | No (Intergenic, non-discrimination) |
| TC0124 (151212)                      | Transcription repair coupling factor (trcF) | T → DEL | FS       | Read et al. (2000) Ramsey et al. (2009) | Yes (used as control) |
| TC0138 (169449)                      | phospho-N-acetylMuramoyl-pentapeptide- transferase (mraY) | INS → TTT | DEL → F | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | Yes (variability present) |
| TC0155 (187432)                      | 3A(2A), 5A-bisphosphate nucleotidase, putative | G → A | H → Y | Read et al. (2000) Ramsey et al. (2009) Yeruva et al. (2014) | Yes (variably present) |
| TC0168 (200671)                      | Ribosomal protein L34 (RpmH) | G → C | T → R | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | No (not confirmed by PCR) |
| TC0341 (403623)                      | ABC transporter, permease protein, putative | T → DEL | FS       | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | No (non-discrimination) |
| TC0341 (403626)                      | ABC transporter, permease protein, putative | INS → C | FS       | Read et al. (2000) Ramsey et al. (2009) | No (non-discrimination) |
| TC0341 (403652)                      | ABC transporter, permease protein, putative | T → C | S → P | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | No (not confirmed by PCR) |
| TC0342 (404884)                      | ABC transporter, permease protein, putative | A → DEL | FS       | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | Nd |
| TC0408 (468932)                      | Conserved hypothetical protein | G → T | W → L | Read et al. (2000) Ramsey et al. (2009) | No (Not confirmed by PCR) |
| TC0412 (473118)                      | Conserved hypothetical protein | A → DEL | FS       | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | Yes (variably present) |
| TC0412 (473705)                      | Conserved hypothetical protein | G → DEL | FS       | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | Yes (variably present) |
| TC0708 (846475)                      | Conserved hypothetical protein | G → DEL | FS       | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | Nd |
| TC0727 (866121)                      | 60 kDa outer membrane protein (omcB) | G → T | G → C | Read et al. (2000) Ramsey et al. (2009) Yeruva et al. (2014) | Yes (variably present) |
| TC0832 (967487)                      | DNA polymerase II alpha subunit (dnaE) | A → DEL | FS       | Read et al. (2000) Ramsey et al. (2009) | Yes (variably present) |
| TC0867 (1004276)                     | Hypothetical protein | T → C | S → P | Read et al. (2000) Ramsey et al. (2009), Yeruva et al. (2014) | Yes (variably present) |

Symbols: AA, amino acid; DEL, deletion; FS, frameshift; INS, insertion; NA, not applicable; Nd, not done; NT, nucleotide.
centrifugation steps described above, the plate was incubated for an additional 28 h, media aspirated and the monolayers fixed in cold methanol for quantitative IFU determination as previously described (Ramsey et al., 2009).

Statistics

The rate of hydrosalpinx formation and the frequency of genotypes were compared between groups using chi-square analysis with Yate’s correction. One-step growth rate determination and plaque size were compared using a two-tailed t-test. Statistical significance was set at \( P \leq 0.05 \).

RESULTS

Rates of chronic disease in formerly susceptible strains of mice have declined over time following numerous in vitro passages

Hydrosalpinx (fluid-filled, distended oviduct) is a chronic manifestation of *C. muridarum* infection in the mouse and has been used extensively as a surrogate marker of infertility (Su et al., 1998; Shah et al., 2005). Certain inbred strains of mice have long been known to be more susceptible to this outcome than other strains (De La Maza et al., 1994; Shah et al., 2005). To this end, our laboratory has previously used C3H/HeN mice as a standard for susceptible strains. However, beginning in approximately 2006, we observed a drop in the rate of hydrosalpinx formation following *C. muridarum* infection in this mouse strain. At first we thought this to be either an anomaly, a change in the virulence of *C. muridarum*, or possibly that a mutation in a critical gene or genes of the host that was responsible for this manifestation. Nevertheless, the observation was repeated on several attempts ruling out the possibility of an experimental anomaly. To rule out the latter possibility (a mutation in the host), we ordered both BALB/c and C3H/HeN mice from three common commercial suppliers. Over two experiments of 10 mice in each experiment (total of 20 mice and 40 oviducts assessed), we consistently observed a highly significant reduction in the rate of hydrosalpinx (Table 1, \( P < 0.001 \), chi-square analysis with Yates correction), when compared to historical controls and a slight but statistically significant drop in the rate of hydrosalpinx in the BALB/c susceptible strain (\( P < 0.04 \)). Because a spontaneous mutation in two or even one strain of mice from three separate suppliers is highly improbable, we interpreted these results to indicate that a less virulent variant(s) had arisen in our culture stocks of *C. muridarum*. Interestingly, although not directly addressed or compared across experiments, we did not note or observe quantitative (IFU counts over time) or qualitative (numbers of animals infected over time) differences in infection course kinetics in any strain of mice (data not shown). Thus, we interpreted this observation that infectivity had not been affected but some aspect of pathogenicity was altered within our *C. muridarum* stocks.

Design and initial evaluation of real-time PCR SNP-based multilocus genotyping assays

We hypothesized that a phenotypic change had occurred within our cell-culture-derived stocks of *C. muridarum* and that this change would be reflected in the genotype of selected variants isolated from these stocks. We further hypothesized that certain genotypic variants had arisen, possibly to predominance, in our stocks. To determine the proportional representation of genotypic variants in our cell-culture-derived stocks, we utilized the commercially available customized SNP-based real-time PCR. We selected polymorphism targets based on prior results of full genome sequencing of the Nigg and Weiss strains (Ramsey et al., 2009; Yeruva et al., 2014). See Table 2. Some of the 20 non-synonymous polymorphisms previously identified were not further evaluated due to close spatial association that would likely lead to poor or no discrimination in the genotyping assays (e.g. TC_0052 and chromosomal positions 59062 and 59065 and TC_0341 at positions 403623 and 403626). Polymorphisms in the intergenic regions were also omitted from further assessment (e.g. three positions in TC_0107). We then designed our initial genotyping assays to include 12 of the remaining polymorphisms. In preliminary assessments of 24 isolates from cell-culture-derived *C. muridarum* (both Nigg and Weiss strains), some of the base changes identified previously (Ramsey et al., 2009; Yeruva et al., 2014) were constant, in that the original base at that position reported by Read et al. (2000) could not be replicated: TC_0168 (200671G>C), TC_0341 (403652T>C) and TC_0408 (468932TG>T). We interpreted this finding to either be an error in the reading of the original sequence at that position or the mutation had subsequently become fixed in our culture stocks. Thus, these were not explored further. Of the remaining targets, a single open reading frame (ORF), TC_0412, contained two polymorphisms: at 473118delA and 473118delG. Both were predicted to result in a frameshift in this gene. Because these were spaced sufficiently to prevent non-discrimination, we targeted both polymorphisms in our assays and termed these TC_0412a and TC_0412b, respectively. Interestingly, the orthologous ORF in *C. trachomatis* (CT0135) has been shown to possess similar polymorphisms associated with attenuation or virulence in the mouse model of *C. trachomatis* urogenital infection and potentially inactivating mutations arise in this gene with prolonged cell culture passage (Sturdevant et al., 2010; Borges et al., 2013; Conrad, Chen and Zhong 2014). Thus, we settled on eight final targets to assess polymorphisms in *C. muridarum*: TC_0124 (151212delT), TC_0138 (169449insT), TC_0155 (187432G>A), TC_0412a (473118delA), TC_0412b (473705delG), TC_0727 (866121G>T), TC_0832 (967487delA) and TC_0867 (1004276T>C).

Genotypic proportional representation in chlamydial populations varies by strain and following in vitro vs in vivo passage

Our initial assessment of the proportions of the selected polymorphisms involved only plaque-cloned isolates derived from our stocks of *C. muridarum* that had a >25 yr history of initial passage through embryonated hens eggs than an unknown number of passages through mammalian cell culture. The latter consisted mostly of HeLa 229 up to the point of the plaque assay and subsequently L929. A total of 55 randomly selected plaque isolates (30 of the Nigg strain and 25 from the Weiss) were minimally expanded in vitro for no less than two and no more than five passages after plaque selection and prior to genotyping. The qualified results of rPCR reactions for each isolate for each polymorphism were one signal amplification reaction, indicating the presence of a single-specific allele (e.g. 151212delT or 151212T in TC_0124); two signal amplification reaction, indicating the presence of both alleles and interpreted as a mixed population of alleles at that position (e.g. both 187432G and 1187432A in TC_0155); or no amplification reaction or ‘no call (NC)’, interpreted as neither of the targeted alleles was present at that position for that isolate. The results of independent SNP frequencies
in isolates are shown in Fig. 1 and the SNP pattern of the various isolates in Table 3.

Cell-culture-passaged Nigg and Weiss strains of C. muridarum (labeled ‘in vitro’ in Fig. 1) at six of the alleles showed no significant differences in allelic representation between the two strains. However, a significant difference was observed in the polymorphisms in two ORFs when comparing the Nigg and Weiss strains: TC_0138 (169449insTTT) and TC_0867 (1004276>C). Of note, amplification by primers targeting both allelic forms was commonly observed in TC_0138 (169449insTTT) and TC_0155 (187432G>A) and also represented a substantial minority of the reactions in the Weiss strain for TC_0867 (10024276>C). This finding could be interpreted that the populations were not sufficiently clonal from the plaque selection phase of the process or that those loci exhibit a high frequency of mutation (a ‘mutational hotspot’) that quickly mutates following in vitro expansion. The latter possibility seems to be confirmed by the recent findings of Yeruva et al., who sequenced the genome of five C. muridarum (Nigg strain) isolates and identified identical SNPs at these positions (Yeruva et al., 2014). The majority of Weiss strain isolates did not contain either of these alleles at TC_0867 (10024276>C, ‘NC’ or no call in Fig. 1), indicating a disparate polymorphism at that locus than was targeted by genotyping reactions.

What could not be determined from these assessments is if any of these polymorphisms had changed over time since the initial isolation of C. muridarum (Gordon, Freeman and Clampit 1938; Nigg 1942). This would require an original ‘wild-type’ isolate of the pathogen or at least one that had minimal passage number which, to the best of our knowledge, is not available. However, we postulated that if genotypes more favorable for in vivo conditions were present in the population represented by our cell-culture-passaged stocks, they would be amplified following in vivo passage. We tested this hypothesis by blindly passaging the same polyclonal C. muridarum stocks used to isolate plaque isolates described above for three serial passages at 7-day intervals through the lower urogenital tract of mice as described in the section ‘Materials and Methods’. Plaque isolates (38 from the Nigg strain and 16 from the Weiss strain) were then derived and minimally expanded in culture for SNP genotyping as above.

We found that the proportional representation of SNPs in seven of the eight assessed polymorphisms changed significantly with in vivo passage. The only polymorphism that did not change significantly with in vivo passage was TC_0138 (151212delT). This ORF encodes a putative transcription-repair coupling factor, tcrF, and the SNP is predicted to result in a frameshift mutation. Similar findings have been reported for the orthologous C. trachomatis gene, and these alterations were also shown to affect infectivity and virulence in the mouse (Sturdevant et al., 2010). We observed that the majority of isolates from cell-culture-passaged Nigg and Weiss strain C. muridarum exhibited the deletion alleles in both TC_0412a and TC_0412b. Interestingly, a single isolate out of 30 Nigg strain and none of the 25 Weiss strain isolates exhibited the parental allele at TC_0412a. However, one Weiss strain isolate exhibited an NC reaction thereby indicating that an undetermined alternative allele was present at low frequency. Following in vivo passage, there was a significant shift to predominance in the parental allele at TC_0412a in the Nigg strain (P < 0.001) isolates whereas the Weiss strain isolates all contained the deletion allele—likely indicating that the originating cell-culture-derived Weiss strain either no longer contained the parental allele or that it was exceedingly rare at the outset. In TC_0412b, again the deletion allele was in majority in the population cell-culture-passaged stocks of both Nigg and Weiss strains, but upon in vivo passage the population of variants in this allele shifted to predominance of the parental allele (P < 0.001 for both Nigg and Weiss strains).

The SNPs in TC_0412a (473118delA) and TC_0412b (473705delG) represent potentially function-disrupting frameshift mutations. Similar findings have been reported for the orthologous C. trachomatis gene, and these alterations were also shown to affect infectivity and virulence in the mouse (Sturdevant et al., 2010). We observed that the majority of isolates from cell-culture-passaged Nigg and Weiss strain C. muridarum exhibited the deletion alleles in both TC_0412a and TC_0412b. Interestingly, a single isolate out of 30 Nigg strain and none of the 25 Weiss strain isolates exhibited the parental allele at TC_0412a. However, one Weiss strain isolate exhibited an NC reaction thereby indicating that an undetermined alternative allele was present at low frequency. Following in vivo passage, there was a significant shift to predominance in the parental allele at TC_0412a in the Nigg strain (P < 0.001) isolates whereas the Weiss strain isolates all contained the deletion allele—likely indicating that the originating cell-culture-derived Weiss strain either no longer contained the parental allele or that it was exceedingly rare at the outset. In TC_0412b, again the deletion allele was in majority in the population cell-culture-passaged stocks of both Nigg and Weiss strains, but upon in vivo passage the population of variants in this allele shifted to predominance of the parental allele (P < 0.001 for both Nigg and Weiss strains).

The ORF at TC_0155 encodes a putative 3’(2’),5’-bisphosphate nucleotidase and contains a non-synonymous SNP at position (187432G>A) corresponding to amino acid change in histidine to tyrosine residues (Ramsey et al., 2009; Yeruva et al., 2014). An additional non-synonymous SNP in this ORF at position 187984C>T in a single C. muridarum variant has also been reported by Yeruva et al. (2014), but is not assessed herein. Isolates of both cell-culture-passaged Nigg and Weiss strains encoding the A allele and mixed A and G alleles were present in similar proportions (P = 0.384, black and gray bars, respectively) with a single isolate detected that contained the G allele only in each strain. Following in vivo passage, the A allele predominated in the Nigg strain isolates (P < 0.001) while all isolates of the Weiss strain had mixed population at the allele (P < 0.001).

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Note were two isolates with NC reactions at this allele in the Weiss strain isolates possessing the insTTT allele increased significantly following in vivo passage.

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Note were two isolates with NC reactions at this allele in the Weiss strain and a substantive minority containing reactions for both alleles in the Weiss strain prior to in vivo passage and in both strains following in vivo passage.
Figure 1. Proportional representation of select polymorphisms in *C. muridarum* populations changes by strain and following in vivo passage. Each panel represents the qualitative results of rtPCR genotyping reactions targeting the specific polymorphism at each ORF and chromosomal position as labeled (upper-right corner of each panel). The proportional results of the reactions are presented in the bar graphs (allele legend in upper-left corner of each panel). Symbols common to all panels: - , no base at that position in originally sequenced *C. muridarum*; Del, deletion at that position in originally sequenced *C. muridarum*; NC, hatched bars (‘no call’ reaction indicating alternative allele not targeted by rtPCR primers is present in that reaction); gray bars—mixed reaction indicating presence of both alleles. Asterisks (*) indicate significant difference (p-value specified in the text) between groups as determined by chi-square analysis with Yate’s correction. Sample size: Nigg in vitro, N = 30; Weiss in vitro, N = 25; Nigg in vivo, N = 38; Weiss in vivo, N = 16.
In vitro phenotypic changes in selected isolates

We next sought to determine if there were detectable phenotypic variances within these isolates. One measure of in vitro phenotype that has been reported is plaque size (O’Connell et al., 2006; Kari et al., 2008; Ramsey et al., 2009; Yeruva et al., 2014). Indeed, we had begun to notice that plaque size became more uniform following in vivo passage, particularly in isolates of the Nigg strain while plaques of the Weiss isolates did not appear to vary to the same degree following in vivo passage. Fig. 2 confirms this observation. The Nigg strain plaques were highly variable following the first in vivo passage (mean area = 0.67 +/- 0.21 mm², range = 0.97 mm², N = 62), exhibiting greater size and range of plaque area when compared to the first passage of the Weiss strain (mean area = 0.39 +/- 0.14 mm², range = 0.69 mm², N = 60). At the second subsequent in vivo passage, the mean area of the Nigg strain plaques became significantly smaller and more uniform than the plaques formed following the first passage (mean area = 0.56 +/- 0.18 mm², range = 0.84 mm², N = 59, P < 0.001, two-tail t test) while the Weiss strain plaques did not vary significantly in size from the first passage. By the third passage in vivo, the Nigg strain plaques had further decreased in area (mean area = 0.39 +/- 0.14 mm², range = 0.39 mm², N = 60) although this decrease did not quite achieve statistical significance (P = 0.07). Also, by the third passage, there was no longer a statistically significant difference in the size of the Nigg and Weiss plaques. These data indicated that in vivo passage of C. muridarum induced an in vitro phenotypic change in the population of isolates within the Nigg but not the Weiss strain. It should be noted that the plaque assays and the related results were conducted simultaneously following inoculation of mice from polyclonal cell-culture-passaged stocks of C. muridarum and without knowledge of the constituent genotypic population in the originating inocula.

C. muridarum Nigg variants. While a majority of isolates of both cell-culture-passaged strains possessed the T substitution allele, after in vivo passage, there was a substantive shift to mixed expression of both the parental G and substitution T allele which achieved statistical significance with the Nigg strain (P < 0.001) but the proportional difference was smaller in the Weiss strain and was not significantly different.

The ORF annotated as TC0832 encodes the alpha subunit of DNA polymerase III (dnaE). We have previously reported the presence of an SNP (967487delA) resulting in a putative frameshift mutation in an isolate of the Weiss strain (Ramsey et al., 2009). In the present study, the majority of cell-culture-derived isolates of both Nigg and Weiss strains contained the deletion allele. In vivo passage significantly increased the presence of the parental allele in the Nigg strain (P < 0.001) but a mixture of both alleles predominated in isolates of the Weiss strain, representing a significant change from the proportions present in the cell-culture-derived isolates (P < 0.035).

Lastly, TC0867 is an ORF encoding a conserved hypothetical protein (Read et al., 2000). The Nigg strain has been reported to possess a non-synonymous SNP which is a substitution at position 1004276T>C resulting in a change from a serine to a proline (Ramsey et al., 2009). More recently, the presence of this SNP was confirmed in three of five Nigg strain isolates (Yeruva et al., 2014). Confirming these prior observations, we detected the C allele present in a majority of cell-culture-passaged Nigg isolates but that a significant shift in the proportion of alleles exhibiting this allele was observed following in vivo passage with a mix of both the C and T alleles resulting (P < 0.001). In the cell-culture-derived Weiss strain, we observed a majority of isolates expressed neither C nor T alleles (NC), with only a single isolate of 25 assessed exhibiting the T allele and 10 of 25 the C allele. Again, this changed significantly with all Weiss strain isolates derived from in vivo passage expressing the parental T allele (P < 0.001).
strains used in the present study were isolated in the et al., and in vivo phenotypic changes, we then selected four 0.004 exist as a population of multiple genotypic variants that in vivo 0.0001 vs CM005 and CM014; perbaseper 0.0001 for each using a two-tailed in vitro et al. (0.038). It should be noted that this is not the first C. caviae in vitro 0.07). shows that these same variants phenotypes (Ramsey C. muri-<br>DISCUSSION
It is now quite clear that the known laboratory-passaged C. muridarum exist as a population of multiple genotypic variants that exhibit equally diverse in vitro and in vivo phenotypes (Ramsey et al., 2009; Yeruva et al., 2014; Sullivan et al., 2014a,b). Similarly, solid evidence exists that subtle changes in the C. trachomatis and C. cauiae genome can have similar profound effects in mice and lower primates and in guinea pigs, respectively (Kari et al., 2008; Binet et al., 2010; Studervant et al., 2010). These small variations in genome have been associated with changes in growth rate, plaque size, inclusion morphology, infectivity and survivability in the host; virulence and the induction of pathological immune responses; and the differences in the rate of ascension from lower to upper genital tract. It is becoming obvious that subtle changes in the genome have more than a subtle impact on the phenotype of this pathogen. This is not surprising in that chlamydial pathogens have small genomes, likely reflecting a reductionist adaptation to the intracellular environment (Clarke 2014). Hence, few ‘dispensable’ genes remain and changes in those that remain may make profound differences.

Nonetheless, caution should be exercised in interpreting the present results. We still are not certain of the genotypic composition of most original chlamydial clinical isolates that have been sequenced, including C. muridarum. This is an important point for if we are to truly understand chlamydial pathogenesis, we should know the composition of the initial inoculum presented to a host in a natural context and whether or not this composition will determine the outcome of the inoculation event in a particular host or at a particular anatomical site. Some encouraging progress has recently been made that may shed light on this. For example, Seth-Smith et al. (2013a, b) has demonstrated that whole-genome sequences can be derived from clinical samples using a combination of immuno-magnetic separation and multiple displacement amplification). These types of studies will be important in understanding genomic variability of a chlamydial population in a single inoculum in a natural setting.

A recent study by Borges et al. (2013) has shed light on chlamydial genomic variability over time in cell culture. Using a prototypical C. trachomatis, L2, strain L2 434/Bu, with a long history of culture in hen’s eggs and mammalian cell culture, and a recent clinical isolate of the same serovar, they followed the genomes for a year in continuous culture. They found a low mutation rate over the monitoring period (10^{-9} to 10^{-11} per base per generation). One should remember, however, that the C. muridarum strains used in the present study were isolated in the 1930s and 1940s and have been passaged countless times in cell culture and in hen’s eggs before that. Thus, these isolates pre-date the historical prototypical isolate of L2 utilized by Borges et al. by —two to three decades and untold numbers of passages (Schachter and Meyer 1969). In this sense, it is also important to note that the rate of mutation is apparently quite low in C. muridarum. Amongst the eight total isolates sequenced thus far, there is a high degree of homology and changes are largely focused to the polymorphisms described above in Table 1 (Read et al., 2000; Ramsey et al., 2009; Conrad, Chen and Zhong 2014; Yeruva et al., 2014). The evidence would suggest that the polymorphisms are mutational hotspots. Our observations could reasonably be ascribed to selection of pre-existing favorable SNPs because we did not observe the appearance of any new SNPs in the form of new NC following in vivo passage. All NC reactions were only present in the in vitro passaged isolates.
Figure 3. In vitro phenotypic variance of C. muridarum isolates. The top panel represents the results of a one-step growth rate determination of four isolates. The results shown are that of two experiments, six replicates per isolate per experiment (N = 12). The asterisk (*) in the top panel indicates that variant CM006 growth yielded significantly fewer viable EBs at the same MOI and over the same incubation when compared to the other isolates. The number sign (#) indicates that there was also a significantly greater EB yield in variant CM014 when compared to CM022. The bottom panel represents the plaque size analysis of the same variants (one experiment, N = 240). The asterisk (*) in the bottom panel indicates that variant CM005 yielded significantly smaller plaques than all other variants. The number sign (#) indicates that CM006 produced smaller plaques than CM022 (P = 0.038). The table in the bottom of the figure shows the SNP pattern for each of the four C. muridarum variants and the frequency at which they were found in vitro or in vivo.

With reference to specific polymorphisms, it is interesting that Borges et al. observed the same inactivating mutation in the putative virulence factor in C. trachomatis, serovar L2, CT135, as reported by Sturdevant et al. for serovar D (Sturdevant et al., 2010; Borges et al., 2013). Our results herein and previously confirm these findings (Ramsey et al., 2009; Yeruva et al., 2014). It seems that this gene in C. trachomatis and the orthologous C. muridarum gene TC0412 degenerate quickly in cell culture (Conrad, Chen and Zhong 2014). Our present results show that if minority variants in cell culture with intact TC0412 remain, they are amplified following in vivo passage. One should note, however, that while select variants from both in vitro and in vivo passage exhibit phenotypic differences in vitro, we have yet to determine in vivo fitness or virulence associated with the polymorphisms in any of the isolates assessed in the present study.

These studies will require sequencing the genomes of each such variant tested in order to confirm, or not, the stability of the polymorphism and whether or not the remainder of the genome has changed. Nonetheless, previous studies indicate that these polymorphisms are present in the original and parental Nigg and Weiss strains and these indeed exhibit virulence and fitness differences (Ramsey et al., 2009). Also, in a separate study, clonal genotypic variants of C. muridarum with defined genomic composition and similar SNP patterns displayed significant virulence and fitness differences (Yeruva et al., 2014). We should also point out that the use of plaque assay to derive isolates could exhibit a negative selective pressure for non-plaque forming isolates and thereby skew genotyping results. While non-plaque forming phenotype has not yet been described for C. muridarum, we cannot rule this out.
We feel that the greatest importance of the present study is the finding that selected polymorphisms are amplified or enriched following recent in vivo passage thereby implying that in vivo fitness is associated with one allele over the other. This proves that genomic representation in a chlamydial population is indeed dynamic and adaptive based on conditions presented to them. It would certainly be of interest to obtain a fresh or low passage number isolate of C. muridarum to sequence and compare to the variants we have now but this has not been forthcoming. It is safe to say that one must be careful in interpretations of data using chlamydial strains that have been passaged repeatedly in cell culture.

That selection occurs in vivo is of paramount importance. Chlamydiae are programmed for survival in the host, so the genes that are retained in vivo may be key to survival in the host environment in contrast to living in the stress-free environment of a cell culture where they are not needed and thus dispensed (Sturdevant et al., 2010; Borges et al., 2013; Conrad, Chen and Zhong 2014). However, it should also be noted that, even in vivo, the organism may be required through selection to modify its gene repertoire. One cannot forget that every host will be genetically different and every anatomic site may exhibit different pressures and this may have a significant effect on the pathologic outcome of the infection (Miyairi et al., 2007, 2011; Su et al., 2014).

Therefore, we suggest that the pathologic outcome of chlamydial infection is dependent upon both the chlamydial variant selected in vivo and the genetic makeup of the host. Likewise, the host response and physiologic parameters specifically associated with the host genotype may have a profound effect on the variants comprising the chlamydial population, and this may alter in transmission to a new genetically different host.

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