Published in final edited form as:

Future Microbiol. 2010 September ; 5(9): 1427–1442. doi:10.2217/fmb.10.96.

Antibiotic resistance in Chlamydiae

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Abstract

There are few documented reports of antibiotic resistance in *Chlamydia* and no examples of natural and stable antibiotic resistance in strains collected from humans. While there are several reports of clinical isolates exhibiting resistance to antibiotics, these strains either lost their resistance phenotype *in vitro*, or lost viability altogether. Differences in procedures for chlamydial culture in the laboratory, low recovery rates of clinical isolates and the unknown significance of heterotypic resistance observed in culture may interfere with the recognition and interpretation of antibiotic resistance. Although antibiotic resistance has not emerged in chlamydiae pathogenic to humans, several lines of evidence suggest they are capable of expressing significant resistant phenotypes. The adept ability of chlamydiae to evolve to antibiotic resistance *in vitro* is demonstrated by contemporary examples of mutagenesis, recombination and genetic transformation. The isolation of tetracycline-resistant *Chlamydia suis* strains from pigs also emphasizes their adaptive ability to acquire antibiotic resistance genes when exposed to significant selective pressure.

Keywords

antibiotic resistance; *Chlamydia*; heterotypic resistance; persistence; phenotypic resistance; sexually transmitted infection; trachoma recombination; transformation

Chlamydiae are a successful group of obligate intracellular pathogens that cause serious diseases in a wide range of hosts (Box 1). Chlamydial infection of cells is initiated by an infectious, but metabolically inactive, elementary body (EB) that subsequently differentiates into a metabolically active, but noninfectious, reticulate body (RB). All chlamydial development occurs within a membrane bound vacuole termed the inclusion (Figure 1A & D). Replication by *Chlamydia trachomatis* RBs is synchronous until approximately 18–24 h post-infection, at which point dedifferentiation to infectious EBs can first be observed [1]. During infection, a subset of host-derived vesicles are trafficked to the inclusion, where Chlamydiae direct the modification of the inclusion membrane through secretion of proteins that facilitate vacuolar modification and manipulate host signaling pathways, via interactions with other chlamydial or host cell proteins [2–4]. Additional chlamydial proteins are secreted into the host cytosol, where they affect immune recognition and intracellular survival of the pathogen [4–7]. Most chlamydial developmental cycles are complete in 40–

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Financial & competing interests disclosure

Daniel Rockey receives grant support from the NIH and the US Department of Defense (MT/DTRA). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

72 h when, in most cases, the host cell lyses and infectious progeny are released from the cell. Although chlamydia have a highly reduced genome of approximately 1 Mb [8], the inability to introduce gene-specific DNA or culture the organism in the absence of host cells imposes constraints on the experimental techniques available to study basic chlamydial biology. As a result, genome sequencing and comparative genomics are of primary importance in developing a clearer understanding of chlamydial biology.

Box 1

Diseases caused by Chlamydiae

Ocular

It is estimated that 40 million individuals worldwide have active trachoma caused by singular or mixed infections of *Chlamydia trachomatis*, *Chlamydia pneumoniae* and *Chlamydia psittaci* [24,72,117]. An additional 8.2 million have trichiasis and 1.3 million are blind as a result of ocular infections caused by chlamydia. Particular strains of *C. trachomatis* that cause trachoma are hyperendemic to regions of sub-Saharan Africa, the Middle East, Asia and parts of South and Central America; however, the distribution and involvement of *C. pneumoniae* and *C. psittaci* strains in active trachoma cases around the world is currently unknown [202]. Transmission occurs through both direct and indirect contact, and roughly 25% of all individuals infected are children under the age of 10 years. However, serious disease and blindness is found in older individuals caused by cumulative scarification left by untreated infections [24].

Genital

Sexually transmitted infections caused by *C. trachomatis* are the most prevalent bacterial cause of sexually transmitted infections worldwide, and around 92 million men and women are estimated to be infected [202]. The majority of infections are asymptomatic in both men and women, but if left untreated can result in a variety of pathologies, including urethritis, cervicitis, salpingitis, pelvic inflammatory disease, ectopic pregnancy and infertility [118].

Respiratory

10% of community-acquired pneumonias are attributable to *C. pneumoniae* infection, and 50–80% of adults demonstrate antibody titers to the pathogen. Most primary infections are not serious, but secondary pathologies are associated with *C. pneumoniae* infections and they are a risk factor in the development of atherosclerosis, asthma, chronic bronchitis, chronic obstructive pulmonary disease and Alzheimer's disease. The contribution of *C. pneumoniae* in these secondary sequelae can be controversial and challenging to assess because of the ubiquity of the pathogen [119–122].

Arthritis

Both respiratory and genital chlamydial pathogens are implicated in long-term infections that are resistant to antibiotic eradication. *C. pneumoniae* can disseminate via macrophages to deeper lung, arterial and joint tissues. *C. trachomatis* can infect mobile monocytes, which may carry the bacteria to joint tissues where they reside in an aberrant intracellular state and induce inflammation. Some studies have found a 50–80% prevalence of *C. trachomatis* DNA in patients with reactive arthritis [25].

Zoonotic respiratory infections

Psittacosis outbreaks caused by *C. psittaci* were documented as early as 1879 and continue through to today, where they largely affect individuals that routinely contact psittacines (parrot-like birds), pigeons, turkeys, ducks and geese. Inhalation of

contaminated aerosols from urine, feces or other secretions of birds represents the primary route of transmission. Untreated *C. psittaci* infections of the lung can involve severe respiratory distress and systemic organ involvement, leading to serious disease and death. In 2001, 11 reported cases were documented in the USA, although it is thought that many cases go unnoticed or undiagnosed [123].

Veterinary

A variety of *Chlamydia* infect different animal species, causing a variety of diseases in many organ systems. Serious chlamydial disease is found in sheep and other ruminants, swine, marsupials, cats, birds and fish. Specific chlamydial species or strains are generally limited to the different animal host species [124].

Although Chlamydiae share many similarities with other Gram-negative bacteria, they constitute a distinct phylogenetic and genetic lineage. Their genomes are marked by a high degree of genetic conservation and very limited evidence of horizontally acquired foreign DNA. Chlamydiae can evolve *in vitro* resistance to antibiotic stressors through the accumulation of point mutations, and these resistance properties can be circulated among strains via horizontal gene transfer and homologous recombination [9–21]. Despite this ability to evolve in the laboratory, stable genetic antibiotic resistance in clinical settings has yet to be documented.

Complications associated with the treatment of chlamydial infections

The primary frontline antichlamydial antibiotics, tetracyclines (TETs) and azithromycin (AZM), are highly effective in the treatment of uncomplicated chlamydial infections [22]. However, accumulating data suggest that a break in the normal chlamydial developmental cycle can result in persistence and long-term infection that is refractory to antibiotic therapy. An understanding of this phenomenon is far from complete. Although 50% of genital *C. trachomatis* infections resolve spontaneously within 1 year of testing [23], a further understanding of long-term infections is important, because it is hypothesized that persistence can cause a cascade of potentially serious inflammatory-induced sequelae, such as pelvic inflammatory disease, infertility, blindness, arthritis, asthma and atherosclerosis [24–28].

Because Chlamydiae are widely distributed and often have high prevalence in human populations, these organisms are often present with other bacterial, viral and parasitic organisms [29–31]. For example, 15–60% of individuals with *Neisseria gonorrhea* genital tract infections are also infected with *C. trachomatis*, and concurrent infections with both *Treponema pallidum* and *C. trachomatis* also occur [29,32]. These co-infections have historically led to therapy complications. For example, β -lactam antibiotics have historically been the recommended drugs for both *T. pallidum* and *N. gonorrhea* [32–34], but treatment of chlamydial infections with these antibiotics induces chlamydia to become persistent. This persistence may exacerbate disease in the genital tract and lead to treatment failure and long-term complications (see later) [33,34]. For these and other reasons, carefully evaluated broad spectrum antibiotic therapies for bacterial genital tract infections are recommended, and this has been the case for many years [32]. While significant antibiotic resistance is emerging in *N. gonorrhea* and *T. pallidum*, stable antibiotic resistance remains undetected in human chlamydial isolates, despite significant selective pressures. This lack of chlamydial antimicrobial resistance in clinical settings reinforces the relative resistance of Chlamydiae to alterations of genome structure, a subject that remains a significant barrier of progress for chlamydial research scientists.

Persistence *in vitro* & *in vivo*

In vitro or *in vivo* evidence of chlamydial persistence can be demonstrated in all chlamydia species, and can be routinely induced in the laboratory when infected cells are exposed to β -lactam antibiotics, IFN- γ or are deprived of iron supplements or amino acids [35,36]. Persistent or 'aberrant' RBs continue to synthesize proteins and replicate DNA, but they halt cell division. The resulting inclusions contain small numbers of very large aberrant RBs, and yield a prolonged infection caused by viable but nonculturable chlamydia (Figure 1B & E). Removal of the stressor results in septum formation, RB division and differentiation to EBs [36]. Failure to respond to antibiotic treatment can follow establishment of chlamydial persistence *in vitro*, and it may be challenging *in vivo* to differentiate persistence from potential cases of antibiotic resistance. Although uncomplicated infections are quite responsive to antibiotics, unresolved genital, ocular and respiratory infections that fail to respond to antibiotic treatment are extensively documented [36–38]. It is possible that this is a function of poor therapeutic control of aberrant, persistent Chlamydiae in patients.

Both *in vitro* and *in vivo* evidence of penicillin treatment show that a dramatic change in the bacterial cell structure can suspend the developmental lifecycle and trigger a persistent state.

Several studies have identified possible ways that antibiotic therapy in clinical settings or long-term infection might lead to phenotypic resistance to antibiotics that are normally very effective in both *C. trachomatis* and *Chlamydia (Chlamydophila) pneumoniae* [19,32,39–41]. Examples include a study showing that persistent chlamydia became phenotypically resistant to AZM clearance after initial exposure to penicillin [42], and work showing that the macrolide erythromycin blocked EB to RB differentiation if added prior to infection, but caused RBs to enlarge and blocked differentiation to EBs when the antibiotic was added 18 or 24 h postinoculation [43].

The presence of chlamydial RNA and DNA in culture-negative patients showing evidence of chronic chlamydial disease provides support for some form of persistence in clinical settings [44–47]. Atypical RBs were found in cases of reactive arthritis (Reiter's syndrome) and in chronic prostatitis cases caused by *C. trachomatis* after antibiotic treatment [25,48]. Morphologically aberrant RBs in macrophages from an aortic valve sample from chronic *C. pneumoniae* infection have been identified [49]. These *in vivo* reports, along with *in vitro* experimental data, establish possible mechanisms for clinical treatment failures in chlamydial infections that might lead to erroneous conclusions regarding the antibiotic resistance of a clinical isolate.

Heterotypic resistance in Chlamydiae

There are only a few reports describing the isolation of antibiotic-resistant *C. trachomatis* strains from patients [50–55]. Although 11 of the 15 reportedly resistant isolates were associated with clinical treatment failure, all of the isolates screened displayed characteristics of 'heterotypic resistance', a form of phenotypic resistance in which a small proportion of an infecting microbial species is capable of expressing resistance at any one time. This phenomenon has also been described in *Staphylococcus* spp. [56,57], and parallel observations of similar phenotypic resistant states can be referred to in the literature as drug indifference, persistence, tolerance and, in some cases, as properties of biofilms [58,59]. It is possible that these descriptors of bacterial interactions with antibiotics can be associated with chlamydial aberrancy and phenotypic antibiotic resistance in Chlamydiae. For example, tolerance is often specific to antibiotics that affect cell wall synthesis, as is shown in the penicillin persistence model of Chlamydiae [58,59].

In each case of clinical resistance reported, only a small portion of the population (<1–10%) expressed resistance, and those that did also displayed altered inclusion morphology. In addition, the isolates could not survive long-term passage (in the presence or absence of antibiotics) or lost their resistance upon passage. In some cases, heterotypic resistance was observed when a large inoculum was infected on to cells, but a smaller inoculum was not resistant under the same conditions [50,60]. Many of these characteristics suggest that a form of phenotypic resistance is responsible for the sustained presence of small populations of clinical strains of *C. trachomatis* under antibiotic stress and may be an adaptive behavior that influences the survival of bacteria within communities rather than stable genetic resistance mechanisms employed by singular cells.

A distinct characteristic of chlamydial growth is the asynchronous differentiation of RBs to EBs that begins relatively early and continues throughout the developmental cycle. A midstage inclusion will harbor actively dividing RBs as well as nondividing EBs. It is plausible that multistage development is an evolved trait that can ensure the survival of a subset of the population regardless of the timing of antibiotic or metabolic stress. AZM, clarithromycin, levofloxacin and ofloxacin approach 100% inhibition in synchronized assays, but when used in a continuous model of *C. pneumoniae* infection, none of these antibiotics eliminated the organism, even in the presence of concentrations greater than four-times their minimum inhibitory concentrations (MICs) [39,40,61]. A continuous model may more accurately reflect *in vivo* infections because inclusions of varying developmental stages will be present at any given time. The standard MIC assay synchronizes the infection and applies antibiotics within 1–2 h post infection, long before EB differentiation can be observed. Perhaps chlamydia are most vulnerable in the log-phase of growth prior to EB differentiation, and are capable of expressing phenotypic resistance when both replicating and nonreplicating forms are present. This principle is corroborated by other studies, in particular one in which ciprofloxacin and ofloxacin failed to eradicate *C. trachomatis* in infected cells and induced persistence when antibiotics were applied to established infections (2–3 days post infection) [41,43]. Although it is assumed that the inclusion is a nutrient-rich environment, it is unknown whether adequate nutrient levels can support replication and sustain active metabolism, or whether toxic byproducts accumulate, particularly in the late stages of the developmental cycle when several hundred bacteria occupy a single inclusion. These factors may also contribute to the onset of phenotypic or heterotypic resistance observed both *in vivo* and *in vitro*.

It is challenging to distinguish persistence from issues of treatment compliance, re-infection of treated patients and actual antibiotic resistance in Chlamydiae. It remains even more challenging to assess the relevance of heterotypic resistance when it is observed in strains isolated from patients with clinical treatment failure. In the absence of true genetic differences, it is challenging to find a way to study antibiotic resistance that arises only under certain conditions in approximately 1% of the population and which often does not appear to manifest itself following expansion of the bacteria.

Challenges to accurate & reproducible surveillance of antibiotic resistance

All antimicrobial susceptibility assays in the chlamydial system involve isolating and expanding clinical isolates and then culturing chlamydial progeny in cells with media containing different dilutions of antibiotics. There remains no universal testing methodology for these assays, and the techniques themselves are technically challenging and time-consuming [60]. Many different cell lines and techniques are used in different diagnostic laboratories, which presents significant challenges in monitoring and evaluating potential emergence of antibiotic resistance. The following can all influence the outcome of an antibiotic susceptibility analysis: cell line; passage number of both host cells and

Chlamydiae; multiplicity of chlamydial infection; developmental stage when antibiotic is added to the infected cells; and presence or absence of cycloheximide (used to slow growth of the host cells) [60]. Additional attributes of chlamydial growth as well as cellular uptake of antibiotics by host cells can vary substantially in different models of polarized and nonpolarized cells [42,62–65]. For example, different cell lines permit differential growth of chlamydia strains when exposed to the same concentration of AZM. The relevance of this observation is highlighted in a report of AZM-resistant isolates from patients with relapsing infections that were characterized using a cell line permissive to chlamydial growth in the presence of inhibitory concentrations of AZM [55,66]. These observations highlight the challenges that arise when small differences in methodological approaches can further complicate the interpretation of *in vitro* resistance and its association with clinical relevance.

Clinical isolates can be extremely fastidious and often have much slower growth rates, increased potential for cytotoxicity or persistence, or can be present in very low numbers relative to the reference strains that are used as controls in MIC/minimum chlamydiacidal concentration assays. Although specific percentages vary, there is a significant fraction of nucleic acid amplification test (NAAT)-positive cases that are not detected by culture. Culture recovery rate is particularly low from rectal samples and is modestly better from urine, cervical, oropharyngeal and other sites [67–69]. Despite the marginal sensitivity of culture-based diagnostics, their specificity approaches 100%. By contrast, even though the sensitivity of NAATs is 20–30% better than culture and other non-NAATs, the occurrence of false positives and a lack of reproducibility between different NAATs has led the US Centers for Disease Control and Prevention to recommend confirmatory testing in certain cases [70,71]. Additionally, molecular-based diagnostics are limited by the inherent bias in our understanding of particular strains, species or even ecology of infectious organisms implicated in disease pathologies. This is particularly true in regards to the etiology of *Chlamydia* ocular infections, where it was recently shown that many individuals may carry mixed infections with *C. trachomatis*, *C. pneumoniae* and/or *C. psittaci*. These Chlamydiae are not routinely screened for, and have likely been missed using the routine species-specific NAATs [72]. Each of these issues highlights the challenges associated with the diagnosis of chlamydial infections. Although NAATs are expensive, culture is certainly more so. Culture-based methods have also become a less attractive tool because of sensitivity issues, and the time and technical expertise required for their completion. This means many laboratories are not positioned to perform routine culture and are thus ill-equipped to conduct routine antibiotic screening of chlamydial isolates. This may hinder timely and accurate assessment of antibiotic resistance of clinical chlamydial isolates, even if such isolates are present in patients.

Chlamydial resistance to individual antibiotic classes

Chlamydiae are known to acquire resistance through mutations to six major classes of antibiotics. Both naturally acquired and laboratory-generated resistance found in selected chlamydial strains have facilitated the study of conserved biological pathways, such as peptidoglycan synthesis, folate synthesis and methionine synthesis, which cannot be approached directly in the chlamydial system [17,73,74]. The ability to generate resistant mutants has supported new experimental methods that facilitate recombination and transformation in or between Chlamydiae *in vitro* (Table 1). The following sections will describe resistance phenotypes that are stably expressed by Chlamydiae in cell culture systems.

Tetracyclines

Tetracyclines block bacterial protein synthesis by preventing aminoacyl tRNAs from interacting with ribosomes. TETs are widely used in both human and veterinary medicine

because of their relatively low cost, broad spectrum of activity and excellent tissue distribution. Gram-negative, Gram-positive, atypical bacteria (*Chlamydia*, *Mycoplasma*, *Rickettsia*) and even some protozoa respond to therapeutic doses of TET. TET and its derivatives are often well-absorbed, have low toxicity and are relatively inexpensive [75]. Since their initial discovery several decades ago, a large percentage of the total volume of antibiotics used in veterinary medicine for both therapy and growth promotion were TET [76,77,201]. In many bacterial systems, TET resistance is quite common [78]. Resistance to TET was first discovered in a *Shigella dysenteriae* isolate in 1953, only 7 years after the initial discovery of the drug [79]. As of 2005, 38 genes that encode TET efflux pumps, ribosomal protection proteins or inactivating enzymes were known [80]. Several of the resistance genes encode proteins that function against a broad range of TET derivatives. Many of the factors associated with chlamydial ecology in the veterinary system led to speculation that this might be the first antibiotic in which Chlamydiae would show clinically relevant stable antimicrobial resistance, and this speculation has proved true. In the mid-1990s, the first stably resistant *Chlamydia suis* strains were isolated from diseased and normal pigs in the Midwestern USA [81]. Eight independent strains were identified, and each exhibited high level resistance to TET (Figure 1D & F). Six of eight strains also exhibited a stable, but currently uninvestigated, resistance to sulfadiazine. Unlike previous reports of TET resistance in chlamydial strains, these strains were passaged up to 15 times in antibiotic-free media, and survived in media containing antibiotics without showing obvious signs of morphological abnormalities [82]. Genetic characterization of the isolates revealed the presence of foreign genomic islands (ranging in size from 6 to 13.5 kb) that had integrated into the chlamydial chromosome [83]. Each island carries genes encoding a TET efflux pump and a regulatory repressor (*tet(C)* and *tetR*, respectively), a unique insertion sequence (IScs605) plus three to ten additional genes involved in plasmid replication and mobilization. This TET resistance allele is identical to the *tet(C)* gene in the cloning vector pSC101 and a wide range of other vectors. In 2008, a report identified 14 additional *C. suis* strains collected in Italy that shared 100% nucleotide identity with the *tet(C)* gene from the original US strains [84]. Of the 14 strains, 12 of these isolates grew in the presence of TET, whereas two could not. It is not clear why these two strains, which did contain the resistance gene, were unable to grow in the presence of TET.

The *C. suis tet(C)* islands share more than 99% identity to sections of the resistance plasmid pRAS3.2 isolated from *Aeromonas salmonicida* [83], a Gram-negative pathogen of salmon and trout that grows poorly and becomes avirulent at mammalian body temperatures [85]. However, this plasmid lacked the IScs605 sequences. More recently, a TET-resistant element isolated from another aquatic associated Gram-negative bacteria, *Laribacter hongkongensis*, shared 100% nucleotide identity to IScs605 of *C. suis* [86]. *L. hongkongensis* is an emerging cause of community-acquired gastroenteritis and travelers' diarrhea in humans. The significance of either *Aeromonas* or *Laribacter* to the acquisition by *C. suis* of the *tet(C)* island is not clear. The discovery of the *tet(C)* islands represents the first identification of antibiotic resistance acquired through horizontal gene transfer in any obligate intracellular bacteria. Developing hypotheses to explain the acquisition of *tet(C)* islands isolated by *C. suis* is challenging and currently relegated to scientifically supported speculation. However, as more data accumulate, clues surface that may start to piece it together. Two of the genes identified in the islands were part of a novel insertion element (IScs605) related to other insertion sequence elements found in *Helicobacter* spp. IScs605 mediated site-specific transposition and integration in a heterologous system where the transposed DNA localized adjacent to a conserved pentameric sequence (5'-TTCAA) in 36 of 38 sequenced clones. Each island integrated next to a TTCAA sequence within the *inv* homolog of *C. suis* [87]. These data, from both the transposition assays in a heterologous system, and the sequence specificity surrounding the integration site of each island, suggest the insertion sequence element mediated transposition into each of the TET-resistant *C. suis*

genomes. Prior to this discovery, no other insertion sequence had been identified in Chlamydiae. From the work on *C. suis* and the singular *L. hongkongensis* isolate, there are now two sources that link an aquatic organism to the islands found in *C. suis*. *L. hongkongensis* is the only known organism to harbor the IScs605 insertion element, while the plasmid in *A. salmonicida* is the only species that shares identity with the remaining sequences of the *tet*(C) island. The feeding and rearing practices in the pig industry that rely heavily upon the prophylactic delivery of TET and the use of fish as a significant feed source may have promoted the ideal environment for the acquisition of DNA by chlamydia that commonly infect the porcine intestinal epithelia [Andersen AA, Pers. Comm.]. Many questions remain regarding how the *tet*(C) island was delivered to bacteria that grow within vacuoles inside cells, and how the island was incorporated into the *C. suis* genome. No conjugative machinery or competence genes have been identified in the genomes of *Chlamydia* spp. and the absence of a practical genetic system renders these questions very challenging.

Rifamycins

Rifamycins, represented in most studies by rifampin (RIF), are bactericidal antibiotics that specifically interact with the β -subunit of RNA polymerase to inhibit bacterial transcription. These are not primary drugs of choice for treating chlamydial infections, although they do possess strong *in vitro* activity and are a therapeutic option in the treatment of clinical infections. Rapid emergence of resistance *in vitro* has been demonstrated in *C. trachomatis*, *C. pneumoniae*, *C. caviae*, *C. psittaci*, *C. suis* and *C. muridarum* after exposure to subinhibitory concentrations of drug [10–12,14,19,20,88]. Amino acid substitutions in the RNA polymerase (RNAP) β -subunit decrease the binding capacity of RNAP to RIF, which allows bacterial survival even under high concentrations of drug. Many bacterial species develop resistance through nucleotide changes in the RNAP β -subunit gene, *rpoB*. Similar to these bacteria, RIF-resistant Chlamydiae carry a variety of conserved and unique nucleotide changes in the central region of *rpoB*. A singular amino acid substitution leads to low-level resistance, but the acquisition of an additional substitution increases the MIC several fold. Single mutations increased the MIC from 0.008 $\mu\text{g/ml}$ to between 0.5 and 64 $\mu\text{g/ml}$ in *C. trachomatis* serovar D, and to between 4 and 64 $\mu\text{g/ml}$ in serovar K. The nucleotide at position 471 of *rpoB* (*Escherichia coli* position 526) was the most common site mutated in resistant clones of *C. trachomatis* serovars D and K. When this nucleotide change was found in combination with one additional mutation, the MIC increased from 64 to 512 $\mu\text{g/ml}$ for a serovar D isolate, and from 64 to 256 $\mu\text{g/ml}$ for a serovar K isolate [19,88].

Work by Kutlin *et al.* [20] and Rothstein *et al.* [89] led to the development of RIF-resistant *C. pneumoniae* strains, but increases in resistance were modest and often took repeated passage for success. In most cases, resistance was associated with mutations in *rpoB*; however, of the two *C. pneumoniae* strains evaluated, only one strain (TW-183) developed resistance and carried the *rpoB* mutations [20]. Rifalazil (RZL), a semisynthetic rifamycin derivative, has high efficacy against *C. trachomatis* infections in clinical trials and is effective *in vitro* against *C. pneumoniae*. Both *C. trachomatis* and *C. pneumoniae* strain TW-183 develop resistance to RZL when passaged in subinhibitory concentrations of the drug and acquire mutations in *rpoB*; however, *C. pneumoniae* strain CWL-029 did not develop such resistance [20]. As seen with RIF, strains of *C. pneumoniae* can be selected for resistance to low concentrations of RZL and require more passages to develop resistance [20]. Interestingly, RZL maintains activity against both RIF-resistant *C. trachomatis* and *C. pneumoniae* mutants [88,90]. Although clinical resistance to rifamycins in chlamydia has not been documented, the ability of these organisms to quickly accumulate mutations *in vitro* raises concern about the use of these drugs in treating infections.

Fluoroquinolones

Fluoroquinolones are bactericidal antibiotics that inhibit DNA gyrase and DNA topoisomerase IV [91]. *C. trachomatis*, *C. muridarum* and *C. suis* can each develop quinolone resistance *in vitro* when exposed to subinhibitory concentrations of antibiotic [10–12,18,21,92]. After only four passages in 0.5 µg/ml of ofloxacin, the *C. trachomatis* MIC increased from 1 to 64 µg/ml. A similar result was achieved after four passages in the presence of 0.015 µg/ml of sparflaxacin. Two additional studies identified similar mutations associated with passage of *C. trachomatis* in the presence of quinolones, but the number of passages required to select for resistant mutants varied between four and 24 [18,21]. Quinolone-resistant strains were resistant to multiple derivatives and carried the same point mutation in the quinolone-resistance determining region of *gyrA*. Although, attempts to generate fluoroquinolone-resistant *C. pneumoniae* were unsuccessful for one group [21], a different group was able to cultivate moxifloxacin-resistant *C. pneumoniae* that carried an amino acid substitution at the same nucleotide position of *gyrA* as other fluoroquinolone-resistant *C. trachomatis* isolates [93].

There is also evidence for natural quinolone resistance, via mutations in the quinolone-resistance determining region of *gyrA*, in *C. muridarum* and the distantly related Chlamydiae-like bacteria, including *Parachlamydia acanthamoebae*, *Neochlamydia hartmannellae*, *Simkania negevensis* and *Waddlia chondrophila*. Some of this latter group have been associated with respiratory disease in humans, and this natural resistance is important to note as quinolones are often prescribed for the treatment of generalized respiratory disease [94–96].

Aminoglycosides

Aminoglycosides interfere with translation initiation by interacting with the 30S ribosome. These antibiotics have poor penetration into mammalian cells, leading to MIC values for Chlamydiae that are extremely high (~1 mg/ml). Kasugamycin (KSM) and spectinomycin (SPC) are antibiotics used to generate aminoglycoside-resistant chlamydial strains in the laboratory. Passage of infected cells in concentrations greater than the MIC led to selection for *C. psittaci* 6BC at a frequency of approximately 2.3×10^{-5} . Resistant strains carried mutations in the 16S rRNA gene at the KSM binding site [9,17], and resistance was present against all tested aminoglycosides.

Chlamydia trachomatis strains resistant to KSM were selected for using culture in sub-inhibitory concentrations of the antibiotic. Strains of *C. trachomatis* that were resistant to KSM did not have a mutation in the 16S rRNA, but did carry a two-nucleotide insertion in *ksgA*, which encodes a protein (KsgA) that is responsible for post-transcriptional methylation of ribosomal adenosine residues in other bacteria. The resistant *C. psittaci* strain was stable and grew comparable to wild-type strains. By contrast, the *C. trachomatis* KSM mutant was severely impaired for growth and was sensitive to high concentrations of antibiotic [9,17].

Similar to KSM, *in vitro*-generated and naturally occurring resistance to SPC is associated with mutations in the 16S rRNA gene. Exposure to subinhibitory concentrations of SPC selected for stable resistance in *C. psittaci* 6BC, and resistant mutants were recovered at a frequency of 1×10^{-6} . Four different SPC-resistant mutants carried unique 16S mutations and varied in their fitness in competition assays with wild-type *C. psittaci*. One out of four of the mutations had no significant fitness cost to the bacteria; however, the other three mutations at adjacent nucleotides reduced bacterial fitness significantly. Some of these mutant genes conferred resistance to SPC in *E. coli*. The mutations identified in these

studies were used to create an electroporation vector that was important in demonstrating the first stable transformation via electroporation of any Chlamydiae [9].

Spectinomycin-resistant *C. trachomatis* L2 mutants have not yet been generated. The inability to produce these mutants is likely due to the duplicity of rRNA operons and drug target sites [9,14,15]. For antibiotics that target ribosomal machinery, a single mutation in an organism encoding more than one rRNA operon is typically recessive, and the frequency at which resistant mutants can be recovered correlates with the number of ribosomal operons encoded in the genome. *C. trachomatis* encodes two nearly identical copies of the operon, whereas *C. psittaci* 6BC only encodes one. Simultaneous complementary mutations in two rRNA operons would arise at very low frequencies *in vitro*, possibly explaining why aminoglycoside-resistant strains containing mutations in 16S rRNA genes were not recovered in *C. trachomatis*.

Sulfonamides & trimethoprim

Sulfonamide (SFM) and trimethoprim antibiotics interfere with bacterial folate synthesis, which is critical for DNA synthesis, repair and methylation. Stable trimethoprim-resistant mutants were reported to arise at very low frequencies ($<5 \times 10^{-10}$) in *C. trachomatis* cultured *in vitro* in subinhibitory concentrations of the antibiotic [11]. *C. trachomatis* L2, *C. psittaci* 6BC and *C. suis* (with the exception of some TET-resistant isolates) are all sensitive to SFM, while *C. pneumoniae* and all other tested strains of *C. psittaci* are naturally resistant. SFM resistance in other bacteria can be conferred through horizontal acquisition of mobile elements, but can also arise from mutations in the folate synthesis genes targeted by the drug. Specific insertions, repeats and point mutations in the *folP* gene (dihydropteroate synthase) can confer stable resistance to sulfa drugs, while mutations in the *folA* gene (dihydrofolate reductase) can confer resistance to trimethoprim [97]. Iclaprim is a new dihydrofolate reductase inhibitor currently in development; however, this antibiotic maintains activity against both *C. trachomatis* and *C. pneumoniae* *in vitro* [98].

Macrolides

Azithromycin is a bacterial protein synthesis inhibitor and front-line drug for the treatment of chlamydia infections. High-level resistance to AZM was selected for in *C. psittaci* 6BC and *C. caviae* GPIC, while a *C. trachomatis* L2 strain was selected for in lower concentrations of AZM [13,16]. Cultivation of resistance was unsuccessful in *C. pneumoniae* clinical isolates with elevated MICs to AZM [37,99]. AZM-resistant *C. psittaci* strains were also resistant to other macrolides as well as a lincosamide, which share similar 23S rRNA target sites. Resistant strains were stable and survived passage in the presence and absence of these drugs. Similar to observations of resistance to KSM, the AZM resistant strains were isolated after exposure to inhibitory concentrations of AZM, while the modestly resistant (AZM tolerant) *C. trachomatis* strain was isolated only after exposure to subinhibitory concentrations of antibiotic. The AZM-tolerant *C. trachomatis* strain harbored a mutation in *rplD* that encodes the ribosomal protein L4.

Although some antibiotic-resistant mutations resulted in no overall effect on the physiology of the bacteria, *in vitro* AZM resistance imposes a competitive defect. The resistant *C. psittaci* strains were delayed in their differentiation from EB to RB compared with wild-type strains, and also had a slower doubling rate, produced significantly smaller plaques and were outcompeted in the absence of selection by the wild-type parent strain. The drug-tolerant *C. trachomatis* strain did not grow well in the absence of antibiotics, formed smaller plaques and produced fewer infectious particles than wild-type parent strains. The *C. caviae* AZM resistant strains carried mutations in the 23S rRNA of their single rRNA operon, produced

fewer infectious particles *in vitro* and were less fit *in vivo*, compared with the wild-type strain [13].

Lincosamides

Lincomycin is a bacteriostatic protein synthesis inhibitor that causes premature dissociation of peptidyl-tRNA from the ribosome [100]. There is a single report of *in vitro*-generated lincomycin-resistant *C. trachomatis* mutants. These mutants were recovered at very low frequencies ($<5 \times 10^{-10}$) by growing and passaging infected cells in subinhibitory concentrations of antibiotic. The resistant mutants carried mutations in both 23S rRNA genes, corresponding to sites in *E. coli* that conferred similar resistance [11].

Utility of antibiotic resistance in chlamydial genetics, recombination & transformation

Recombination

Extensive comparative analyses of DNA sequence data (including a broad range of clinical and laboratory isolates) support the conclusion that Chlamydiae are highly recombinogenic [46,84,101–115]. Discordant rates of genetic mutation between polymorphic loci and the rest of the genome are linked to evidence supporting genetic recombination as a source of genetic variation and genome maintenance and repair. This conclusion is challenged by the fact that chlamydial genomes are highly syntenous and encode only a very few extrachromosomal elements or genomic islands [115]. In the absence of host-free growth or a genetic system, experiments addressing recombination or genetic exchange have been very difficult. Recent research by different laboratories are making progress in this area. Studies by Demars and colleagues used laboratory-generated antibiotic-resistant strains to develop an artificial system that screened for *in vitro* lateral gene transfer and recombination [10,11]. Co-infecting two resistant parental *C. trachomatis* isolates facilitated the selection of doubly resistant progeny strains with the antibiotic-resistant genetic markers from each parental strain. In one experiment, an ofloxacin (OF)-resistant *C. trachomatis* L1 strain harboring a mutation in *gyrA* (T249->G) and a RIF-resistant *C. trachomatis* D/UW-3/CX strain harboring a mutation in *rpoB* (C1400->T) were co-infected and grown in the presence of RIF and OF. A RIF- and OF-resistant *C. trachomatis* D strain was isolated that carried both the *gyrA* (T249->G) and the *rpoB* (C1400->T) mutations. Quantitative evidence supported lateral gene transfer, as opposed to spontaneous mutation, as the source of resistant phenotypes in the selected strains. Antibiotic resistance was stable in recombinants grown in the presence and absence of antibiotic selection. Sequencing of highly polymorphic loci (*ompA*, *murA*, *pmpC*, *trpA*, *incA*, *ribF* and *recF*), in addition to the mutated genes (*rpoB* and *gyrA*), was used to coarsely map several cloned recombinant genomes to estimate the length and composition of the transferred DNA. Remarkably, the authors provide evidence that large fractions of the genome were exchanged; between 123 and 790 kb was estimated to have transferred to the recipient. Although it is likely that very large fragments of the chromosome were exchanged in these crosses, it is difficult to genuinely determine the recipient and the donor strain in these experiments.

Work in our laboratory confirmed the fundamental discoveries of Demars *et al.* and demonstrated lateral gene transfer between several different *Chlamydia* spp. [12]. Studies of Suchland *et al.* used the horizontally acquired TET-resistant marker from *C. suis* R19 as a primary tool of selection, and mapped recombination sites in cloned recombinant progeny using genome sequencing. These studies showed routine transfer of TET resistance from *C. suis* to any of several *C. trachomatis* strains, as well as the mouse-tropic *C. muridarum*. Transfer of TET resistance involved the insertion of between 40 and 100 kb of *C. suis* DNA into recipient strains. Progeny from primary crosses were used as donors in subsequent

crosses, and recent clinical strains readily acquired TET resistance via recombination. Some recombinants are marked by substantial genome rearrangements and/or genetic mosaicism, while other recombined sequences are relatively short products from classical double-crossover recombination events. Additional *in vitro*-generated interstrain recombinants made from parental strains carrying OF or RIF resistance harbor a unique genetic patchwork of parental DNA across the entire genome [116]. The recombinant genomes include the expected antibiotic resistance genes from parental strains, but somewhat unexpectedly, regions unrelated to the loci that conferred antibiotic resistance used for selection. The three Chlamydiae used in these experiments (*C. suis*, *C. trachomatis* and *C. muridarum*) are unique in their ability to form fusogenic inclusions when occupying the same cell, and it was initially hypothesized that sharing the same vacuole might be a requisite for the exchange of DNA. However, extensive work [Rockey DD, Unpublished Data] by our laboratory has shown that nonfusogenic strains of *C. trachomatis* that lack IncA, an important protein involved in homotypic inclusion fusion, still recombine *in vitro* when subjected to the same selection parameters as IncA-positive, fusogenic strains. New studies using *in vitro* transformation and classical genetic techniques are beginning to tease out genetic regions specific to unique phenotypes and growth characteristics of different clinical and laboratory strains [116]. These techniques are currently being used by our group, and others, to serve as a rudimentary genetic system until more direct methods are developed.

Although these results were initially surprising and controversial, it is now acknowledged that multiple antibiotic-resistance genes can be readily recombined between *Chlamydia* spp. It is likely that recombination occurs naturally, and therefore, clinical resistance might spread rapidly in patients, following an initial, perhaps rate-limiting, introduction of an exogenous resistance gene into the chlamydial population.

Transformation

The first successful transformation via electroporation in chlamydia was recently published and highlights again the importance and utility of antibiotic resistance in the study of chlamydial genetics [9]. These individuals developed and characterized several antibiotic-resistant strains and associated mutation frequencies, using SPC and KSM as the selecting antibiotics. Several plasmids were produced that carried a *C. psittaci* 16S rRNA gene with mutations that conferred resistance to both antibiotics. These constructs were amplified in a methylase-deficient *E. coli* strain and electroporated into *C. psittaci*. The chlamydia were infected onto host cells, grown in the presence of selecting antibiotics and transformants were plaque purified. Transformants were stable and could survive several passages in the presence and absence of antibiotics, and sequencing of progeny 16S rRNA confirmed that resistance was derived from the electroporated plasmid DNA. These authors used homologous sequences ranging from approximately 1.5–8 kb, and estimated that regions of recombination were between 0.4 and 1 kb.

Although the transformation frequency using electroporation is low compared with the frequency of doubly resistant strains recovered from recombination (10^{-6} vs 10^{-3}), the work of Binet and Maurelli has provided an important proof of concept for introduction of foreign DNA into chlamydia. This technology is currently limited to altering only a single locus in selected chlamydial strains, and is not a generally applicable method for introducing or inactivating genes in these bacteria. Translation of these techniques into standard methods to introduce or inactivate chlamydial genes remains a significant challenge in this system.

Conclusion

Although there is no genetic evidence of antibiotic resistance leading to treatment failures in humans, the *C. suis* strains resistant to TET and the *in vitro* results with the other described

antibiotics indicate that clinicians should be vigilant for the possibility in the future. Current culture and diagnostic methods may not be sufficient to detect emerging antibiotic-resistant strains due to these persistent states, the low recovery rate in culture from various infection sites, potential instability of resistant isolates *in vitro*, or the unknown significance of heterotypic resistance in treatment failure. Little is known about the heterotypic-resistant phenotype observed in the MIC assays, and whether or not it has biological relevance to *in vivo* conditions or can be correlated with cases of treatment failure. Although there is currently no evidence for heritable antibiotic resistance in human clinical settings, results discussed in this review indicate that several antibiotic-resistance genotypes can be generated and transferred to most *C. trachomatis*, *C. suis* or *C. muridarum* isolates, and that chlamydial antibiotic resistance will be an important laboratory tool for researchers.

Future perspective

The characterization of TET-resistant *C. suis* isolates, spontaneous mutants and recombination strains demonstrate that resistance can emerge and disseminate amongst chlamydia, and in some cases with relative ease. Major biological barriers have probably prevented the acquisition of DNA or mutations that promote antibiotic resistance in clinical and veterinary isolates. If resistance genes were to enter the chlamydial population from other species, we expect that this resistance could spread rapidly within species via recombination.

The use of antibiotic-resistant strains in chlamydial research has led to significant understanding of chlamydial recombination *in vitro* and the resistant strains will likely continue to serve as an essential tool to further our understanding of chlamydial genetics. By utilizing differently resistant strains with specific phenotypes, unique inter- and intrastrain crossing may generate chimeric chlamydial strains that display targeted phenotypes, while carrying a unique and unnatural set of genetic markers. These approaches may be useful for correlating unidentified genes with known chlamydial phenotypes, and for exploring the role of individual chlamydial genes in infection and disease.

Acknowledgments

Sara Weeks and Bob Suchland are gratefully acknowledged for their critical review of the manuscript. We wish to dedicate this work to our recently deceased colleague Walter E Stamm. Dr Stamm was a wonderful individual who contributed in so many ways to our personal and professional lives, and we miss him dearly.

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Figure 1. Fluorescence microscopy of *Chlamydia trachomatis* L2/434Bu- or TET-resistant *Chlamydia suis* R19-infected McCoy cells fixed with methanol 40 h postinfection

Infected cells were cultured in the presence of either 10 µg/ml ampicillin, 1 µg/ml tetracycline or were not treated with antibiotic. *C. trachomatis* is labeled with antibodies to major outer membrane protein (red) and *C. suis* is labeled with antibodies to lipopolysaccharide (green). DNA is labeled blue with DAPI, which primarily labels the host cell nuclei and DNA within aberrant reticulate bodies. The scale bar in the bottom right panel indicates 10 µm for all panels.

Table 1

Distribution of *in vitro* and natural antibiotic resistance amongst the different strains of Chlamydiae.

Antibiotic group	Antibiotics	Mechanism of action	<i>C. trachomatis</i>	<i>C. muridarum</i>	<i>C. suis</i>	<i>Cp. pneumoniae</i>	<i>Cp. psittaci</i>	<i>Cp. caviae</i>	<i>Chlamydia-like</i>
Macrolides	Azithromycin	Protein synthesis	S	-	-	-	S	S	-
Tetracyclines		Protein synthesis	R	R	H, R	-	-	-	-
β-lactams		Peptidoglycan synthesis	N	N	N	N	N	N	-
Rifamycins	Rifampin(N) Rifalazil (Z)	RNA polymerase	S _{NZ} , R _N	S _N , R _N	S _N , R _N	S _{NZ}	S _N	S _N	-
Fluoroquinolones	Ofloxacin (O) Sparfloxacin (S) Moxifloxacin (M)	DNA gyrase	S _{OS} , R _O	S _O , R _O	S _O , R _O	S _M	-	-	N
Sulfonamides		Folate synthesis	R	-	U	N	N [†]	-	-
Trimethoprim		Folate synthesis	S, R			-	N	-	
Lincosamides		Protein synthesis	S, R	-	-	-	-	-	-
Aminoglycosides	Kasugamycin (K) Spectinomycin (S)	Protein synthesis	S _k	-	-	-	S _{KS} , R _{KS} S _{KS} , R _{KS}	-	-
Fosfomycin		Peptidoglycan synthesis	N	-	-	-	-	-	-

Strains of Chlamydiae are listed that have acquired or natural antibiotic resistance to the listed classes or to a specific antibiotic within each class.

[†] With the exception of *Cp. psittaci* 6BC.

-: Resistance is unknown or unreported; H: Strains that have acquired resistance through horizontal transfer of a plasmid; N: Strains that demonstrate natural or physiological resistance; R: Strains that have acquired resistance through *in vitro* recombination or electroporation; S: Strains that have acquired resistance through *in vitro* selection in culture with antibiotics; U: Strains that have been isolated with stable resistance, but the mechanism of resistance is unknown.