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Molecular mechanisms of *Chlamydia trachomatis* resistance to antimicrobial drugs

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1. ABSTRACT

Chlamydia trachomatis (*C. trachomatis*) is a leading cause of bacterial sexually transmitted infections in developed and undeveloped countries, and therefore a global public health issue. In an era of increasing bacterial resistance to antibiotics, resistance has been an exceedingly rare phenomenon in *C. trachomatis*; however, clinical treatment failures attributed to multidrug-resistant *C. trachomatis* strains have been described on several occasions. Cell culture systems using McCoy cells and subsequent immunofluorescent staining are still the most common methodology used for antimicrobial susceptibility testing, but the presence of resistance markers should be appraised by further genetic analysis. Azithromycin resistance of *C. trachomatis* is often a result of the mutations in the peptidyl transferase region of 23S rRNA genes, tetracycline resistance is usually linked to the presence of foreign genomic islands integrated in chlamydial chromosome, whereas a predominant mechanism of fluoroquinolone resistance is a point mutation in the *gyrA* quinolone-resistance-determining region. A nucleotide substitution in *rpoB* gene is responsible for rifampin resistance, and different mechanisms have been involved in the development of resistance to aminoglycosides, lincomycin and sulphonamide/trimethoprim combinations.

2. INTRODUCTION

Chlamydia trachomatis (*C. trachomatis*) is an obligate intracellular bacterium responsible for a variety of clinical syndromes that stem from genital and ocular mucous membranes infection, primarily transmitted due to unprotected sexually intercourse. Moreover, it is a leading cause of bacterial sexually transmitted infections (STIs) in both developed and undeveloped countries. In 2015 in the United States of America (USA), and in 2014 in Europe, a total of 1 526 658 and 396 128 chlamydial infections were reported, respectively, giving an overall rate of 479 cases per 100,000 inhabitants in USA, and 187 cases per 100,000 inhabitants in Europe (1,2). It should be noted that reported rates of chlamydia infection between European countries vary considerably (*i.e.* 549/100,000 in Denmark vs. 0.1./100,000 in Romania), which reflects the differences in chlamydia testing and case finding rather than real differences in chlamydia prevalence (2).

C. trachomatis genome is the circular chromosome of a 1,042,519 base pairs (bp) which encodes minimal sets of genes needed for DNA replication, transcription and translation, and almost 900 protein-coding genes – including genes for peptidoglycan biosynthesis (3). Additionally, *C. trachomatis* carries a 7.5. kilobase (kb) plasmid that

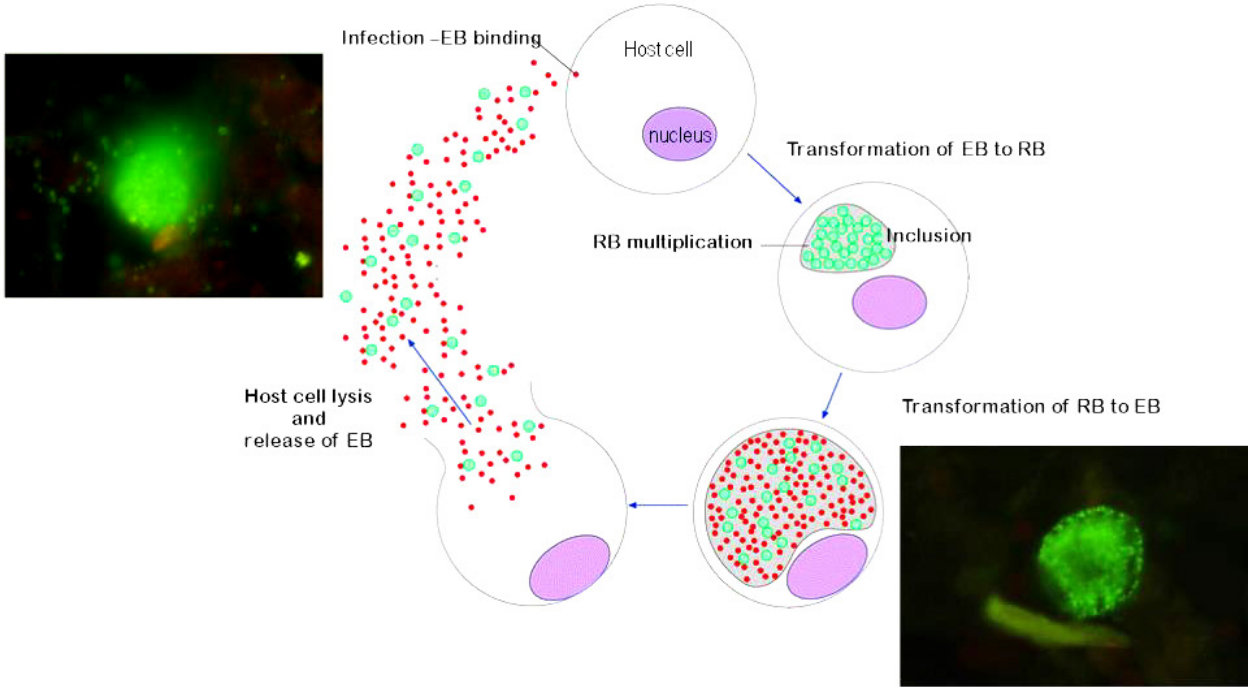

Figure 1. The life cycle of *Chlamydia trachomatis*; Photographs present *C. trachomatis* grown in McCoy cell culture and detected in different stages of infection using fluorescein-labelled monoclonal antibodies against lipopolysaccharide antigen (Pathfinder®, Bio-Rad Laboratories, France). Legend: EB = elementary body; RB= reticulate body.

encodes eight plasmid glycoproteins which are not critical for chlamydial growth *in vitro*, but play a pivotal role in chlamydial pathogenesis (4). It has been shown that recombination as a way for altering the standard set of genes is not an unusual event in *C. trachomatis*. Recombination was first revealed within *ompA* gene which encodes the major outer membrane protein, following the identification of hotspots located near genes that code for virulence factors (5,6). Recently, *C. trachomatis* recombination was generated *in vitro* under antibiotic selection (7).

C. trachomatis is characterized by its unique life cycle (Figure 1). The life cycle begins with infection of the host cell by elementary body (EB) – the infectious form characterized by spore-like cell-wall which enables *C. trachomatis* to survive outside of the host cell, and the ability to catabolize glucose, therefore providing the energy required for host cell entry and differentiation into the reticulate body (RB) (8). Upon attachment to the host cell, which is mediated by several bacterial ligands and receptors, the type III secretion system (T3SS) is injected, and ET is internalized into the inclusion (9). Transformation into RB follows, which is highly metabolically active and divides by binary fission within an inclusion that consequently grows until the entire cytoplasm is filled and the nucleus dislocated (Figure 1). A unique family of T3SS effectors termed inclusion membrane proteins are produced during establishment and maintenance of the inclusion providing its structural stability and acquisition of nutrients (9,10). After 24 to 74 hours of infection the transition of RB in EB in an asynchronous manner ensues, and finally, EB are released by host cell lysis and/or the extrusion of the inclusion (Figure 1).

Another crucial characteristic of *C. trachomatis* is its capacity to establish the persistence. This is a reversible state that occurs in unfavourable growing or stress conditions in which *C. trachomatis* remains viable but shows atypical morphology and quiescent metabolism (11). In fact, the RBs upon stress (*i.e.* depletion of nutrients such as tryptophan and iron, and treatment with gamma-interferon and penicillin) are transformed into the enlarged aberrant bodies, and in such form they may successfully survive until the stress factor is removed. Stress response in persistence is associated with lack of RB to EB transformation (12) and the lack of septum synthesis (11).

C. trachomatis causes the infection of lower and upper genital tract of both sexes, thus having the great influence on human reproductive health (13). The most common clinical manifestation is mucopurulent cervicitis in woman, and non-gonocccal urethritis in man. Untreated infection can ascend and lead to the severe complications such as pelvic inflammatory disease, ectopic pregnancy, chronic prostatitis and infertility in both sexes (14–16). The fact that complicates the diagnosis and subsequent treatment is that the most infections are asymptomatic (up to 70%

Table 1. Laboratory determined *C. trachomatis* resistance in patients with clinical treatment failure

Tested antimicrobial drug	Number of clinical isolates	Laboratory detected minimal inhibitory concentrations (MICs)	Reference
Tetracycline, Doxycycline, Erythromycin	5	≥8 μg/ml	39
Tetracycline	1	> 64 μg/ml	40
Doxycycline, Ofloxacin, Azithromycin	3	>4 μg/ml	41
Ciprofloxacin, Ofloxacin, Pefloxacin	14	4–64 μg/ml	42
Doxycycline, Azithromycin	5	4–8 μg/ml	43

in woman and 50% in man) (17,18). Such substantial rate of asymptomatic infections makes the screening programs – especially for high risk groups of population – crucial in timely diagnosis and treatment in order to prevent possible long-term consequences (19,20).

Fortunately, chlamydial infection belongs to the curable STIs. Moreover, effective single-dose regimen of treatment with azithromycin exists (21). But regardless of a simple treatment regimen, STIs caused by *C. trachomatis* are continuously on the rise (1,2), which is manly attributed to the more frequent testing with improved detection systems such as nucleic acid amplification tests (NAATs) (22), but also changes in sexual behaviour and lack of education and prevention. Additionally, resistance to antibiotics of some STIs, particularly gonorrhoea and *Mycoplasma genitalium* infection, has increased rapidly in recent years, and reduced available treatment options for these infections (23,24). Such undesirable course of events with a possible increase of STIs prevalence (25) has raised concerns on antimicrobial resistance of all curable STIs. Although it seems to be very rare, *C. trachomatis* resistance exists, and may potentially also contribute to the increase of chlamydial infection. The aim of this review is to provide insight into the likelihood for appearance of *C. trachomatis* antimicrobial resistance, considering the frequency of its occurrence to date and molecular mechanisms of its development.

3. TYPES AND FREQUENCY OF ANTIMICROBIAL RESISTANCE IN HUMAN ISOLATES OF *C. TRACHOMATIS*

C. trachomatis is susceptible and treated with antibiotics that inhibit protein synthesis (tetracyclines and macrolides), and those that inhibit nucleic acid synthesis (fluoroquinolones and rifampin). Although it has been demonstrated that chlamydial persistence can be induced *in vitro* and *in vivo* when exposed to beta-lactam antibiotics (26,27), amoxicillin is still recommended as a third option in the treatment of pregnant women (28).

Clinical treatment failures rates range from 5 to 23%, depends from the population tested (29). Although, majority of cases can be explained by post-treatment reinfection or lack of treatment compliance, some of them suggest true therapeutic failure caused by other reasons – including chlamydial resistance (30). Clinical treatment failures linked to the laboratory proved chlamydial resistance are not a common event in humans. A majority of studies report excellent susceptibility of chlamydial clinical isolates in laboratory and clinical settings (31–33). Quite opposite, in animals (particularly in swine), *Chlamydia suis* (*C. suis*) resistance as the result of selective pressure of continuous exposure to the tetracycline drugs which are used as additives is common (34,35). Selection for *C. trachomatis* antimicrobial resistance has been demonstrated in laboratory settings using serial passage of *C. trachomatis* strains in subinhibitory concentrations of rifampin, fluoroquinolones, and macrolides (36–38). There are few reports, but of considerable importance, that describe chlamydial antimicrobial resistance *in vivo* (39–43). All these reports informed about limited number of clinically detected and laboratory confirmed cases of *C. trachomatis* reduced susceptibility or antimicrobial resistance (Table 1).

Basically, there are two types (or patterns) of described resistance in *Chlamydia* spp.: homotypic in which most of the organisms survive at concentrations well above the minimal inhibitory concentration (MIC), and heterotypic, a pattern in which small numbers of organisms (less than 1%) survive antimicrobial concentrations above MIC (44). All human resistant isolates showed heterotypic pattern of resistance (39–41), which has been also previously described in *Staphylococcus* spp. (45). In addition, chlamydial resistant isolates showed reduced viability (*i.e.* they could not survive long-term passage) or they lost their resistance upon passage. It is possible that heterotypic antibiotics resistance in chlamydia can be associated with aberrancy as is shown in the penicillin persistence model of *Chlamydiae* (46). Since homotypic antibiotic resistance has not yet been documented in *C. trachomatis*, Borel *et al.* hypothesise that some of the clinical treatment failure may be explained by development of heterotypic antibiotic resistance due to slower growth in certain environments or entry into a stress response in which the organisms are refractory to antibiotic treatment (47).

One explanation of *C. trachomatis* resistance as a rare phenomenon *in vivo* despite selective pressure could be its unique developmental cycle. The impermeability of EB and isolation of RB (which readily exchanges DNA) within intracellular inclusion limit genetic exchange with non-self DNA (9), making it difficult for chlamydia to acquire the foreign antibiotic resistance gene (48). The other, more plausible explanation (which has shown to play important role in macrolide resistance) is that mechanisms which confer high-level resistance in chlamydiae severely affected chlamydial infectivity and may thus limit the emergence of highly resistant clones of these important pathogens *in vivo* (38).

4. MAIN METHODS OF APPRAISING ANTIMICROBIAL RESISTANCE OF CHLAMYDIAL STRAINS

Determining antimicrobial sensitivity of chlamydial strains is quite different from standard procedures in bacteriology, since it is necessary to demonstrate the ability (or inability) of *C. trachomatis* to multiply inside the cell in the presence of different concentrations of antibiotics (49). The resistant strains can be subsequently analysed with molecular techniques to ascertain potential genetic markers of resistance. Therefore systems based on cell culture with the addition of serially diluted concentrations of antibiotic represent traditional, but still the most commonly employed method of *C. trachomatis* sensitivity testing (44,50). However, there is still no universal testing methodology and the techniques that are used are time-consuming and technically challenging, which is the reason they are pursued only in highly specialized laboratories (46,49).

A plethora of cell culture types of both human and animal origin can be used for testing, although McCoy cells derived from mouse fibroblasts provide the most reliable and consistent results (49,51). HeLa (human cervical adenocarcinoma), HL (human epithelial cells) and HEp-2 (human epidermoid laryngeal carcinoma) cell cultures can also be used, whereas Vero cells and primate kidney cell line BGMK are employed less frequently (44,49,51). In the past, the detection of intracellular inclusions after appropriate incubation period in such cell culture methods was done with iodine or Giemsa (52,53), but today uniform fluorescein-labelled monoclonal antibodies are the best choice for visualization purposes (54).

Regardless of a chosen cell line, a wide range of factors can influence the antimicrobial susceptibility testing results, most notably laboratory conditions such as pH, temperature, the polarity of the infected cells, the secretion of cytokines and the general nutritional content of the medium (51,55). Accordingly, it has been shown that a medium containing a high concentration of glucose, neutral pH and a high temperature during centrifugation (*i.e.* 33–35 °C) may yield a higher number of *C. trachomatis* inclusions (56), while polarized host cells enable more efficient transport and intracellular accumulation of antimicrobial drugs (57). Also, some additional factors that play include the size of the inoculum (it should not be less than 5000 inclusion forming units per well of microtiter plate), the period between the infection and the application of an antimicrobial drug, timely removal of the antibiotic, as well as the presence (or absence) of cycloheximide that is used to slow growth of the host cells (46,51).

Still, the most important aspect for achieving reproducibility of results is to introduce standardized definitions of minimal inhibitory concentration (MIC) and minimal chlamydicidal concentration (MCC). To achieve this, it is highly recommended to introduce (and use) the transition point MIC (MIC_{TP}), defined as the concentration of drug where 90% or more of the inclusions have altered morphology and/or size (44). The MIC can then be defined as the concentration of drug that is one twofold dilution more concentrated that the MIC_{TP}, while the MCC is defined as the lowest concentration of drug at which no visible inclusions are observed after one passage from the cell culture that contains certain antimicrobial drug to the cell culture without them (44,58,59).

An alternative approach is antimicrobial susceptibility testing of *C. trachomatis* strains by reverse transcription polymerase chain reaction (RT-PCR) based on the detection of specific DnaK transcript (60). The method also requires cell culture growth, followed by a molecular detection of live bacteria in the supernatant, with the advantage of detecting chlamydia in cultures deemed negative after immunofluorescent staining. In this approach, the MIC can be defined as the lowest concentration of antibiotic that inhibits the occurrence of a 318-bp product in the form of bands on the gel stained with ethidium bromide.

The main advantage of the RT-PCR technique are corresponding (but consistently higher) MIC values when compared with cell culture method followed by immunofluorescent staining, as showed by Cross and his colleagues on the example of erythromycin and amoxicillin, which points to the improved sensitivity of this method (61). This can be explained by the adequately suppressed chlamydial growth in concentrations exceeding the MIC values measured in cell culture, but with a presence of low-level replication and the detection of RNA produced only by viable organisms (62). However, due to the questionable laboratory or clinical value of such aberrant inclusions with potential residual replication, a traditional cell culture system with a defined MIC_{TP} still represents a standard approach for antimicrobial susceptibility testing of *C. trachomatis* strains.

One other method of appraising chlamydial antimicrobial sensitivity to standard antibiotics that did not gain much prominence is using flow cytometry after culturing *C. trachomatis* in McCoy cells and staining them with fluorescein isothiocyanate (FITC) antibodies, as described by Dessus-Babus *et al.* (63). After staining the infected cells show green fluorescence, which is reflected by the right-sided peak on the histograms. Nevertheless, it is necessary to evaluate two parameters before analysing the results – the percentage of positive cells and their mean fluorescence intensity (MFI) (63). The latter is a crucial parameter when assessing antibiotic activity, while the active concentration of the antimicrobial drug is expressed as inhibitory concentration 50 (IC_{50}), which is the concentration necessary for a 50% reduction of the MFI when compared to the control without antibiotics.

Although not as sensitive as classic immunofluorescent staining after incubation in cell culture, the main advantages of cultivation with detection by flow cytometry is its specificity, reproducibility and objective interpretation. The main disadvantage is the inability to detect "heterotypic resistance" due to insufficient sensitivity of detecting low levels of infection (51,63). In addition, the method is very cumbersome and time-consuming as flow cytometry necessitates a higher inoculum of chlamydia (more specifically 100,000 IFU/mL), not to mention a high price of equipment. Therefore this method today is rarely used, and it is hard to expect any further impact in the modern era of molecular techniques.

5. RESISTANCE TO MACROLIDES – MUTATIONS IN CONSERVED REGIONS

Macrolides are a class of broad-spectrum antimicrobials of large molecular size, and the group is saliently represented by a compound azithromycin (a part of azalide subclass with a 15-membered ring) as one of the drugs of choice for the treatment of *C. trachomatis* infection (64–66). The mechanism of action of the whole class is reversible binding to the large ribosomal subunit near the peptidyl-transferase center, stopping in turn the bacterial growth due to protein synthesis inhibition (64,65). Using a specifically-designed *in vitro* model, Binet and Maurelli described a population of *C. trachomatis* serovar L2 that was eight times less sensitive to azithromycin and four time less sensitive to erythromycin due to mutations of *rplD* gene which codes for ribosomal protein L4 (38). The substitution of neutral glutamine located at the position 66 with a positively charged lysine affects the binding of chlamydial ribosomal protein L4 to the corresponding 23S rRNA molecules. Even before the aforementioned experiment, it has been known that the mutations in the conserved regions of protein L4 affect the conformational change of the 23S rRNA in domains II, III and V (67,68), leading in turn to disruption of translational activity of ribosomes and, consequently, weakened action of the antibiotic in the peptidyl transferase center.

Misyurina *et al*. described mutations A2058C i T2611C (according to *E. coli* numbering) in the peptidyl transferase region of 23S rRNA genes in clinical isolates resistant to erythromycin, azithromycin and josamycin (69). At the same time a triple mutation was found in a non-conserved region of the protein L22 (*i.e.* glutamine replacement with serine at position 52, arginine replacement with cysteine at position 65, as well as valine replacement with alanine at position 77) (69). The exact role of such amino acid replacements in the resistance of *C. trachomatis* is not yet fully elucidated, but it is assumed that they represent compensatory mutations to maintain virulence of the affected chlamydial strains.

This is corroborated by the fact that the *rplD* gene mutations are linked to *in vitro* macrolide resistance of a myriad of clinically relevant microorganisms (70–72), they are often accompanied by additional mutations of 23S rRNA or *rplV* genes (encoding ribosomal protein L22) (73,74). Wolter and his colleagues showed that the survival of *Streptococcus pneumoniae* isolates resistant to macrolides due to mutations in ribosomal protein L4 is possible primarily because of secondary mutations that compensate for the defect in the bacterial growth (75). Since the resistant strains of *C. trachomatis* in the experimental model by Binet and Maurelli showed weaker growth, formed smaller inclusions and produced fewer infectious particles in the absence of the antibiotic (38), it seems that compensatory mutations are pivotal in the development of chlamydial resistance *in vivo*.

6. RESISTANCE TO TETRACYCLINES – A PIVOTAL ROLE OF GENOMIC ISLANDS AND HORIZONTAL GENE TRANSFER

Tetracyclines are a group of drugs that inhibit protein synthesis in bacteria by binding to their ribosome (with a high affinity to 30S subunit) and preventing the attachment of amino acyl-tRNA at the acceptor site (76). Doxycycline is a semisynthetic tetracycline that (alongside azithromycin) represents a first-line treatment against *C. trachomatis* (particularly for LGV strains) (65,77). However, although tetracycline usage is pervasive in human and veterinary medicine, their use has generally declined in recent decades due to the accounts of resistance in a wide array of different bacteria (47). Regarding *C. trachomatis*, mechanisms of resistance to tetracycline antibiotics have been described in detail in a closely related and highly recombinogenic species *C. suis* (34,46,78–80), and it was demonstrated

that such resistance may be transferred to clinical isolates of *C. trachomatis in vitro* (81).

Genetic characterization of resistant isolates revealed the presence of foreign genomic islands (between 6 and 13.5. kb) integrated in chlamydial chromosome (34). Each island harbors genes that encode antibiotic efflux pump (*tet(C)*) and regulatory repressor (*tetR*), a unique insertion sequence (IScs605), and up to ten additional genes involved in the replication and mobilization of the plasmid (34). Genomic islands of resistant *C. suis* that contain the gene *tet(C)* reveal 99% homology with the plasmid isolated from the Gram-negative bacterium *Aeromonas salmonicida* (*A. salmonicida*) found in fish (most notably trout and salmon) (34,82). Nevertheless, IScs605 insertion sequence was not detected in that plasmid, but in another aqueous Gram-negative bacterium – *Laribacter hongkongensis* – that is increasingly being recognized as a cause of gastroenteritis and traveler's diarrhea in humans (83). A discovery of *tet(C)* represents the first description of the horizontal transfer of antibiotic resistance genes in any obligate intracellular bacterial species (46).

Today, pig farming around the world still significantly relies on the prophylactic usage of tetracyclines, with fish being one of the commonly used sources of food (35,46). A large number of pigs in the United States is infected with resistant strains of *C. suis* (84–85), akin to the situation described in Belgium (86) and Italy (87). It is believed that the plasmid enters the digestive system of pigs inside the bacterial species *A. salmonicida* characteristic for fish; on the other hand, as insertion sequences related to IScs605 are found in a plethora of bacterial species from the genus *Helicobacter* (88), as well as in already mentioned *L. hongkongensis*, the plasmid can acquire that sequence as well while passing through the gastrointestinal tract. The entire genomic island is then physically transferred to *C. suis*, anchoring next to TTCAA sequence within *inv-like* gene (34).

The aforementioned process may be significant for *C. trachomatis* resistance development as well. In spite of the fact that the rise of resistance in this kind of natural ecosystem is very demanding, in laboratory conditions the transfer of resistance via homologous recombination between different strains can be achieved much faster and easier. Suchland and colleagues have shown that the transfer resistance markers from *C. suis* to *C. trachomatis* occurs almost routinely after co-cultivation of these two species (81). Therefore, a mere contact between the chlamydial strains resistant and sensitive to tetracyclines can enable the transfer of resistance genes and the development of resistant phenotype, which in patients treated with tetracyclines may result in the propagation and selection of such strains.

Moreover, as both *C. suis* and *C. trachomatis* are known to infect the human conjunctival tissue and rectum, this creates an ideal *in vivo* opportunity for horizontal gene transfer to *C. trachomatis* (80). Co-infections with *C. suis* and *C. trachomatis* have already been described in patients presenting with trachoma (89), and both *Helicobacter* species and *C. trachomatis* may act as cofactors in the development of chlamydial proctitis (90), creating another milieu for genetic exchange. Marti *et al.* have recently showed that, while the frequency of such recombination in the laboratory conditions is low, the transfer of resistance genes may be instigated by sub-inhibitory concentrations of tetracycline antibiotics (80). Novel research on cassette transfer will provide us with a template for figuring the mechanisms and occurrence rate of resistance gene transfer among *Chlamydia* species (most notably *C. trachomatis*) that may be present in humans at the same anatomic sites as tetracycline-resistant zoonotic strains of *C. suis*, with substantial implications for treatment and public health approaches.

7. RESISTANCE TO FLUOROQUINOLONES – POINT MUTATIONS AS A PREDOMINANT MECHANISM

Fluoroquinolones are broad-spectrum synthetic bactericidal antimicrobial agents that inhibit two bacterial enzymes of the class II topoisomerase family – DNA gyrase and DNA topoisomerase IV (91). Several different mechanisms for resistance to fluoroquinolones have been elucidated, with the mutation at the target site being the most common (91). Although clinical response of patients infected with *C. trachomatis* to fluroquinolones is superb, strains can develop resistance *in vitro* when subjected to subinhibitory concentrations of the drug (36,46,92–94).

Morissey *et al.* showed that initial passages of *C. trachomatis* strains with sub-inhibitory concentrations of fluoroquinolones did not affect susceptibility to this group of drugs, but after an initial lag of at least 10 passages there was a prompt development of resistance to either ofloxain or ciprofloxacin (36). Furthermore, Dessus-Babus *et al.* showed that after only four passages in the presence of ofloxacin (0.5. μg/ml) and sparfloxacin (0.0.15 μg/ml) spontaneous mutations were obtained and resistance ensued (93). Even after the first passage with subinhibitory concentrations of aforementioned antimicrobials, only a few small inclusions could be observed in the McCoy cell culture system (93).

All the available evidence from those studies suggests that the main mechanisms of *C. trachomatis* resistance to multiple derivates of fluoroquinolones is a point mutation in the *gyrA*

quinolone-resistance-determining region (QRDR), which leads to serine to isoleucine substitution at amino acid position 83 (according to the numbering pertinent to *E. coli*) in the corresponding protein (36,46,92,93). Although Yokoi *et al.* reported certain substitutions in ParC, those isolates remained susceptible to fluoroquinolones (94). One proposed explanation for ParC of *C. trachomatis* is that the alanine located at position 80 may be a reason for the lower affinity of ParC than GyrA subunit for fluoroquinolones (93), resulting in what we would call a privileged configuration. No literature data shows change of *gyrB* and *parE* QRDRs in the resistant strains when compared to the reference strains. Also, the role of other mechanisms of resistance to fluroquinoloes (such as drug efflux modification or drug permeation) may contribute to the resistance pattern (93).

8. RESISTANCE TO RIFAMPIN – NUCLEOTIDE SUBSTITUTION MECHANISM

Rifamycines and their main representative rifampin represent a group of bactericidal antibiotics which inhibit bacterial transcription by interacting with beta-subunit of bacterial DNA-dependent RNA polymerase, resulting in a potent bactericidal activity (95). Although they are the cornerstone of the tuberculosis treatment, they also show excellent activity against *C. trachomatis in vitro* (96). The favorable pharmacokinetics, high antimicrobial activity, as well as substantial cell penetration led to the belief that this class of drugs could be another addition to our antimicrobial armamentarium against chlamydial infections (37). Nevertheless, concerns about the development of resistance during treatment have discouraged the use of this group of drugs in the treatment of human chlamydial infections (97).

In a small number of studies, treatment of infections caused by *C. trachomatis* with rifampin has been found to be as effective as treatment with tetracycline (37,97). MICs that were demonstrated *in vitro* against *Chlamydia* spp. ranged from 0.0.075 to 0.0.3 µg/ml (98–100), with no signs of emerging resistance *in vivo*. Still, several *in vitro* studies showed that *C. trachomatis* can easily and swiftly develop resistance after serial passages in subinhibitory concentrations of rifamycins – both in eggs and tissue culture (100,101). In the research conducted by Kutlin and his colleagues, *C. trachomatis* developed resistance to rifampin and rifalazil within six passages; higher level resistance was noted in the case of rifampin (128–256 µg/ml), while lower level resistance was described for rifalazil (0.5.-1 µg/ml) (37).

Akin to a plethora of bacterial species that develop resistance to rifampin by nucleotide exchange in the *rpoB* gene responsible for coding beta-subunit of DNA-dependent RNA polymerase (and thus enabling bacteria to survive even high concentrations of this antimicrobial drug) (37,102–104), chlamydial species also show a preponderance of changes in the mid-portion of that gene (37,101). Therefore a substitution of only one amino acid may increase MIC of rifampin from 0.0.08 µg/ml to between 0.5. and 64 µg/ml in *C. trachomatis* belonging to D serovars, as well as between 4 and 64 µg/ml in *C. trachomatis* belonging to K serovars (46). The most common site affected with the mutation in resistant strains of *C. trachomatis* was the nucleotide at the position 471 in the gene *rpoB* (37,46,101). When this was compounded with one additional mutation, different groups of researchers have shown that the MIC may increase from 64 to 256 and 512 µg/ml in serovars D and K, respectively (101,105).

On the other hand, in the research of Kutlin and his colleagues, strains resistant to rifalazil (as evidenced by BU-434/L2 strain) were characterized by a mutation at the beginning rather than at the middle of the *rpoB* gene (at codon 136 to be more precise) (37). This rare mutation is not a novel discovery, as Lisitsyn *et al.* found it in the *rpoB* gene of *E. coli* resistant to rifampin a few decades ago (106). On the other hand, in that same BU-434/L2 strain that same group of authors observed high-level phenotypic resistance to rifampin without any evident genetic alterations in the *rpoB* gene (37), which brings into play other potential mechanisms of rifampin resistance described in other bacteria (such as ribosylation, phosphorylation and glucosylation enzymes of the activity of efflux pumps) (37). Thus far, no resistance has been found in *C. trachomatis* strains propagated in the addition of the rifamycin derivatives (namely 3-azinomethyl-rifamycin and rifabutin) under identical *in vitro* conditions (37).

9. RESISTANCE MECHANISMS TO OTHER ANTIMICROBIAL DRUGS

Aminoglycoside antibiotics are a family of compounds with an aminocyclitol nucleus (streptamine, streptidine or 2-deoxystreptamine) linked to amino sugars via glycosidic bonds (107). They interact with the 30S ribosomal subunit, interfering in turn with the initiation of genetic material translation (107). Since this family exhibits poor penetration into the mammalian cells, MIC values for *C. trachomatis* are quite high (approximately 1 mg/ml) (46); therefore, these drugs are not used in routine clinical conditions, albeit certain research endeavors were pursued using kasugamycin and spectinomycin to see whether aminoglycoside-resistant strains of *C. trachomatis* would develop (46). This was indeed shown for kasugamycin, where resistant *C. trachomatis* strains carried a two-nucleotide insertion in *ksgA* gene that encodes a protein responsible for post-transcriptional

methylation of adenosine residues in the ribosome (108). Conversely, spectinomycin-resistant *C. trachomatis* strains have hitherto not been generated, most likely as a result of (safeguarding) dual rRNA and dual drug target sites (46,109).

Lincomycin is a bacteriostatic agent that is widely used in clinical practice, stimulating dissociation of peptidyl-tRNA from ribosomes (110). It has not been used nor widely research in regards to *C. trachomatis*, although there has been one report of lincomycin-resistant *C. trachomatis* strains generated *in vitro* after the infected cells were grown and passaged in subinhibitory concentrations of antibiotic (46,110). Characteristic for these mutant strains were mutations in both 23S ribosomal RNA genes that corresponded to the same sites in *E. coli* with comparable resistance response (110).

A combination of trimethoprim and a sulfonamide can successfully interfere with and block the synthesis of folic acid which is pivotal for bacterial growth (111). Sulfonamides competitively inhibit the incorporation of para-aminobenzoic acid into folic acid, preventing in turn the synthesis of folic acid, whereas trimethoprim binds to and inhibits dihydrofolate reductase, also decreasing folic acid synthesis by preventing the formation of tetrahydrofolic acid (111). One paper described stable trimethoprim-resistant strains of *C. trachomatis* after *in vitro* culturing in subinhibitory concentrations of the antimicrobial drug (110) – these were in very low frequencies (less than 5×10^{-10}) as a result of mutations in the *folA* gene (coding for dihydrofolate reductase) (112).

10. CONCLUSION

Antimicrobial resistance arises as the result of the perpetual evolutionary struggle between hosts and pathogens, and as we aimed to demonstrate, various genetic changes represent the main mechanism in pathogenic bacteria (113). In *C. trachomatis* such events may involve mutations in conserved regions of 23S rRNA genes, horizontal gene transfer, nucleotide substitution and a myriad of other significant mechanisms. For one small, intracellular bacterial species, it is a rather rich repertoire of mechanisms responsible for developing resistance to antimicrobial agents (although its occurrence is still mostly confined to the *in vitro* setting). In order to ensure long-term and effective management of all infections caused by *C. trachomatis* we should be adequately prepared for the possibility of further development of clinically significant antibiotic resistance, as well as refine our approaches to livestock management, with an end-goal of preventing the rise of antibiotic resistance. As researchers have already shown, the use of antibiotic-resistant strains in research settings may lead to improved understanding of *C. trachomatis* recombination *in vitro* and the genetic underlying different phenotypic traits and growth characteristics of chlamydial strains (114). Consequently, this will open the doors for the use of evolutionary solutions in the development of new drugs and compounds.

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