

## ORIGINAL ARTICLE

Differences in growth characteristics and elementary body associated cytotoxicity between *Chlamydia trachomatis* oculogenital serovars D and H and *Chlamydia muridarum*

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*J Clin Pathol* 2005;58:397–401. doi: 10.1136/jcp.2004.021543

**Aim:** In vitro growth and elementary body (EB) associated cytotoxicity of two *Chlamydia trachomatis* strains belonging to serovars D and H and *C muridarum* were compared to identify difference(s) that correlate with virulence variations between these strains in the mouse model of human female genital tract infection, and phenotypic characteristics that could explain human epidemiological data on serovar prevalence and levels of shedding during serovar D and H infection.

**Methods:** Replication cycle kinetics, inclusion characteristics, and EB associated cytotoxicity were assessed in McCoy cell monolayers using culture, light microscopy, and lactate dehydrogenase release.

**Results:** Over 72 hours, more rapid production and release of inclusion forming units (ifu) allowed *C muridarum* to initiate two replication rounds, resulting in 4–8 times more ifu/input unit of infection than with serovars D and H. Although *C muridarum* EBs were significantly more cytotoxic to McCoy cell monolayers than serovar D at moderate and high multiplicity of infection ratios (MOI), serovar H EBs were significantly more cytotoxic than *C muridarum*, even at the lowest MOI tested.

**Conclusions:** These phenotypic differences are consistent with the more invasive course and severe pathological outcome of infection in mice infected with *C muridarum*, providing an objective basis for questioning the appropriateness of *C muridarum* as a surrogate for the human biovar of *C trachomatis* in the murine model of female genital tract infection. The differences seen between the human strains could help explain human epidemiological data relating to differences in prevalence and level of shedding that occurs during infection with oculogenital serovars D and H.

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Accepted for publication  
19 September 2004

There are 19 distinct serotypes (serovars) of *Chlamydia trachomatis* that infect humans, with serovars D, Da, E, F, G, Ga, H, I, Ia, J, and K belonging to the group referred to as oculogenital serovars, which are collectively the most common sexually transmitted bacterial agents throughout the world (chlamydia website (<http://www.chlamydiae.com>)).<sup>1</sup> There is little evidence to suggest differences in virulence among oculogenital serovars, with a recent large study concluding that the severity of disease was not associated with a particular serovar or group of serovars.<sup>2</sup> However, surveys of genital isolates from around the world have consistently shown that certain serovars occur more frequently than others, and that over time this pattern has remained constant.<sup>3–9</sup> Specifically, in large surveys in the USA and Europe, serovars D and E account for approximately 50% of genital *C trachomatis* isolates, whereas serovar H is isolated from only 1–3% of culture confirmed infections. In addition, differences in the level of shedding among serovars have been noted in clinical specimens that roughly correlated with serovar prevalence.<sup>10</sup>

“Surveys of genital isolates from around the world have consistently shown that certain serovars occur more frequently than others, and that over time this pattern has remained constant”

In women, 70–80% of infections are asymptomatic and repeated infection correlates with severe upper genital tract pathology, which in decreasing order of occurrence includes pelvic inflammatory disease, ectopic pregnancy, and tubal infertility.<sup>11</sup> The factors that contribute to a particular outcome are unknown, but probably depend upon the

interplay between the host's complex response to infection and the equally complex, phenotypically variable, and environmentally responsive biphasic intracellular developmental cycle of chlamydia.

In attempts to understand the salient features of this interplay, a great deal of work has been carried out using slightly modified versions of a murine model of female genital tract infection first described by Tuffrey and Taylor-Robinson.<sup>12</sup> Until recently, most of this work has been conducted using *C muridarum*, the mouse pneumonitis agent.<sup>13</sup> However with the complete sequencing of *C muridarum* and several chlamydial strains representing human biovars of *C trachomatis*, studies are being conducted that compare the more clinically relevant phenotypes that might arise out of the variation seen at the genetic level. Noteworthy in the present context are reported differences between *C muridarum* and human oculogenital serovars in sensitivity to interferon  $\gamma$ <sup>14–15</sup> and elementary body (EB) associated cytotoxicity,<sup>16</sup> with differences in interferon  $\gamma$  having a known effect on the course and outcome of female genital tract infection in mice.<sup>17–19</sup> We used this model to determine whether the difference in prevalence among oculogenital serovars might result from differences in virulence and the observed significant differences in the duration of infection among the various serovars that loosely corresponded to the clinical prevalence of the serovars, especially for the most and least prevalent serovars—D and E versus H and I.<sup>20</sup> In addition, the invasiveness and level of bacterial shedding of the serovar D and H strains

**Abbreviations:** EB, elementary body; ifu, inclusion forming units; LDH, lactate dehydrogenase; MOI, multiplicity of infection; SP, sucrose phosphate transport medium

demonstrated that serovar D was able to establish culture confirmed infection of the uterine horns significantly more frequently, in addition to shedding more during the acute phase of infection. The factors that contribute to these distinct phenotypes are unknown.

The purpose of our study was to analyse the in vitro growth characteristics and EB associated cytotoxicity of the previously studied serovar D and H strains to identify difference(s) that might explain the observed variation in the course of infection.<sup>20</sup> Extrapolating the results of these studies could provide insight into the factors that influence serovar prevalence and levels of shedding that are seen in human *C trachomatis* genital tract infection. *Chlamydia muridarum* was included in these analyses both to confirm the original observation relating to non-lipopolysaccharide EB cytotoxicity and to identify phenotypic traits that might explain the highly virulent and invasive nature of *C muridarum* infection in the mouse compared with the less virulent and invasive infection of human isolates in this model of human disease.

## MATERIAL AND METHODS

### Chlamydia strains

Polymerase chain reaction based restriction fragment length polymorphism serovar typed<sup>1</sup> and mycoplasma free *C trachomatis* strains belonging to serovars D, H, and L2 and *C muridarum* was propagated and titrated in cycloheximide treated McCoy cell monolayers using standard techniques.<sup>21</sup> EBs were purified using density gradient centrifugation,<sup>22</sup> and concentrated stock suspensions in sucrose phosphate transport medium (SP) were frozen at  $-80^{\circ}\text{C}$ .

### Growth cycle analysis

Confluent McCoy cell monolayers ( $5 \times 10^5$  cells) in 24 well tissue culture plates were inoculated with approximately 600 purified EBs. After centrifugation at  $1500 \times g$  for one hour at  $37^{\circ}\text{C}$ , monolayers were washed and incubated at  $37^{\circ}\text{C}$  in fresh medium. Immediately after and at eight, 24, 32, 48, 56, and 72 hours post-infection, culture supernatants and monolayers suspended in SP were collected separately and in duplicate. Both types of specimens were sonicated and immediately frozen at  $-80^{\circ}\text{C}$  until cultured for the presence and enumeration of inclusion forming units (ifu) in McCoy cell monolayers using iodine. A companion plate was used to verify the actual input inoculum and to observe the kinetics and microscopic appearance of iodine staining inclusions throughout a 96 hour incubation period.

### EB associated cytotoxin analysis

EB associated cytotoxicity was assessed in confluent McCoy cell monolayers using two approaches: (1) visual evaluation of the cytopathic effect with light microscopy; and (2) measurement of changes in host cell membrane integrity using a lactate dehydrogenase (LDH) release assay (cytotoxicity detection kit; Roche, Indianapolis, Indiana, USA). As a control for possible cytopathic and membrane perturbing effects resulting from particulate burden and chlamydial lipopolysaccharide, a strain of *C trachomatis* L2 was included in our analysis.<sup>16</sup> McCoy cells were grown overnight in 48 well tissue culture plates ( $2.2 \times 10^5$  cells/well), and immediately before use the culture medium was replaced with serum free medium supplemented with 1% bovine serum albumin and containing rifampin (10  $\mu\text{g/ml}$ ) and doxycycline (5  $\mu\text{g/ml}$ ), to eliminate whole serum LDH activity and to prevent bacterial metabolism during inoculation.<sup>16</sup> Purified EBs were diluted in SP and inoculated in duplicate on to McCoy cell monolayers at multiplicity of infection (MOI) ratios of 100 : 1, 25 : 1, 6.25 : 1, and 1.5 : 1. After centrifugation at  $1500 \times g$  for one hour at  $37^{\circ}\text{C}$ , the supernatants

were collected, and the monolayers were washed and incubated at  $37^{\circ}\text{C}$  in antibiotic containing serum free bovine serum albumin supplemented medium. Monolayers were inspected using standard light microscopy and subjectively scored for cytopathic effect immediately after inoculation, and when aliquots of culture medium were collected in duplicate at one, two, four, and six hours post-inoculation for the LDH assay, performed according to the manufacturer's instructions.

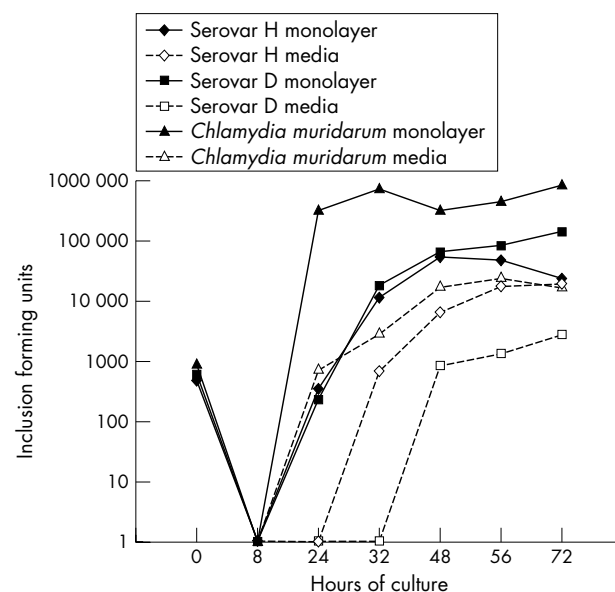
## RESULTS

### Growth cycle analysis

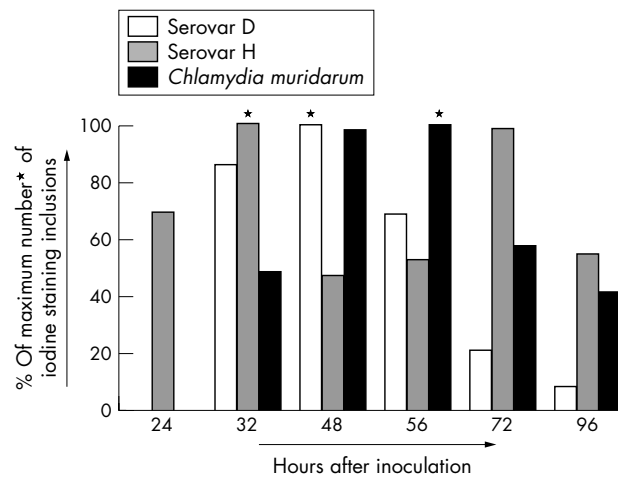
Figure 1 shows the 72 hour replication and release kinetics assessed under standard culture conditions. During this period, *C muridarum* produced significantly more infectious units in the first 24 hours than were produced during the entire period for serovars D and H, and appeared to undergo two rounds of replication, as indicated by the decrease in the number of ifu isolated from monolayers between two peaks at 32 and 72 hours. In addition, *C muridarum* infected cells released ifu sooner—24 versus 48 hours—and in four to 10 times greater numbers than serovar D. In contrast, shedding of serovar H was first seen at 32 hours and, when adjusted for input, was both more sustained and equivalent to or greater in magnitude than the numbers of ifu released from *C muridarum* infected cells, resulting in the continued reduction in monolayer associated ifu and a coincidental increase in ifu recovered in the medium between 48 and 72 hours.

Microscopic observation of the iodine stained monolayers revealed *C muridarum* inclusions by 24 hours, uniformly increasing in size between 24 and 48 hours, followed thereafter by a decrease in the number of large inclusions and the appearance of an increasing number of small iodine staining inclusions, often in clusters. This resulted in a biphasic pattern of iodine staining inclusions (fig 2), with an initial maximum of 885 at 32 hours, which was used as the assigned value of the input inoculum, and a second peak of 860 at 72 hours, with both peaks corresponding to high points of ifu recovery from the infected monolayer (fig 1).

In contrast, inclusion development was similar for serovars D and H during the first 48 hours, with small iodine staining



**Figure 1** In vitro replication and release of *Chlamydia trachomatis* serovars D and H and *C muridarum*.



**Figure 2** Kinetics of iodine staining inclusion development in McCoy cells.

inclusions being first visible at 32 hours, followed by a relatively uniform increase in size up to 48 hours. However, beginning at 48 hours and continuing throughout the incubation period, serovar H infected monolayers contained inclusions that had apparently lost inclusion membrane integrity, and appeared as more diffuse, smudge-like inclusions within the cytoplasm of the cell.

The appearance of these distorted inclusions coincided with a decrease in the number of inclusions in the monolayer (fig 2) and an increase in ifu detected in the medium (fig 1). The maximum number of iodine staining inclusions for serovar D was seen at 56 hours (610) and for serovar H at 48 hours post inoculation (460). Between 48 and 96 hours of incubation, iodine staining inclusions for serovar H decreased to 41% of maximum at 56 hours to 8.6% at 96 hours; over the same period, serovar D dropped to 78% of maximum at 72 hours to 41% at 96 hours post-inoculation.

The net effect of these different replication and release phenotypes was the production of a significantly greater number of progeny/unit input for *C muridarum* (950 : 1) compared with serovars D (235 : 1) and H (120 : 1).

**EB associated cytotoxin analysis**

Two methods were used to assess host cell cytotoxicity associated with purified EBs—direct microscopic assessment of the cytopathic effect and LDH release. Over time and in a

dose dependent manner, McCoy cells inoculated with EBs of *C muridarum* or either oculogenital serovar showed signs of toxicity, beginning with membrane perturbations at cell junctions, visible as early as one hour, and proceeding to varying levels of apparent lysis within two to four hours.

Although *C muridarum* EBs were significantly more cytotoxic to McCoy cell monolayers at moderate (25 : 1) and high (100 : 1) MOI ratios compared with serovar D, serovar H EBs were significantly more cytotoxic than *C muridarum* EBs, with an effect seen at the lowest MOI (1.5 : 1) tested, both microscopically (table 1) and in the more objective LDH release assay (table 2).

**DISCUSSION**

In an earlier study, we demonstrated a significant difference in virulence characteristics among strains representing seven oculogenital serovars of *C trachomatis* in the murine model of female genital tract infection.<sup>20</sup> In a comparison of the most (serovar D) and least (serovar H) virulent strains in this collection, the serovar D strain established a longer and more invasive infection, which was characterised by more shedding during the acute phase of infection. These findings suggested that the differences in the prevalence and shedding among serovars observed in humans might be related to differences in the ability to establish more or less durable infection, which may contribute to differences in genital tract invasiveness.

In our in vitro study, we assessed the growth characteristics and EB associated cytotoxicity of the serovar D and H strains previously characterised in the mouse, and showed that serovar H EBs were cytotoxic at an MOI of approximately 1 EB/McCoy cell, whereas a comparable level of cytotoxicity was seen at an MOI of 25 for the serovar D strain. This phenotypic difference, together with the possibly linked differences in replication and release kinetics, might help explain the differences in both the course of infection between these strains in the mouse, and the incidence and level of shedding between serovars D and H in human genital tract infections.<sup>10</sup>

Based on the expression data presented in the original description,<sup>16</sup> it can be hypothesised that the cytotoxin plays a role throughout the course of infection. It is possible that the more cytotoxic serovar H EBs might cause the death of some host cells upon contact, explaining the larger infectious dose required to establish infection in mice. During the replication cycle, newly produced cytotoxin could alter the integrity of both the inclusion and cytoplasmic membranes, causing earlier disruption of the replication cycle and premature lysis of infected cells. This would explain both the earlier and

**Table 1** Direct microscopic assessment of the cytopathic effect of chlamydia EBs on McCoy cell monolayers

Time after inoculation (hours)	Level of visual cytotoxicity seen in McCoy cell monolayers after infection with chlamydia EBs at the indicated MOI*															
	<i>Chlamydia muridarum</i>				Serovar D				Serovar H				Serovar L2			
	1.5	6.25	25	100	1.5	6.25	25	100	1.5	6.25	25	100	1.5	6.25	25	100
0	-	-	-	1	-	-	-	1	-	-	1	2	-	-	-	-
1	-	-	1	2	-	-	1	2	-	2	3	3	-	-	-	-
2	-	-	2	3	-	-	2	2	1	3	3	4	-	-	-	1
4	-	-	3	4	-	-	2	3	2	3	4	4	-	-	-	1
6	-	1	3	4	-	-	2	3	2	3	4	4	-	-	-	1

\*Confluent McCoy cell monolayers were infected at MOIs of 1.5–100 and incubated at 37°C in the presence of 10 µg/ml of rifampicin and 5 µg/ml of doxycycline. The cytopathic effect was evaluated immediately after infection and at 1, 2, 4, and 6 hours post-infection, and was subjectively scored from negative (-) to positive using a scale of 1 to 4, on the basis of the degree of loss in cell membrane integrity and apparent lysis compared with non-infected control monolayers: 4, complete disruption with apparent lysis; 3, significant perturbation, with some level of apparent lysis; 2, significant membrane perturbation; 1, slight but noticeable affect; -, same as control. Immediately after centrifugation, all monolayers inoculated at MOIs of 25 and 100 contained cells with multiple intracellular vacuoles that were not seen at lower MOIs or in uninoculated monolayers. No difference in degree of vacuolation was seen among the strains and no cytopathic score was assigned to this observation because over the course of the experiment these vacuoles disappeared in surviving cells, being completely absent in L2 inoculated cells by 4 hours. EB, elementary body; MOI, multiplicity of infection.

**Table 2** Assessment of LDH release from McCoy cell monolayers after inoculation with chlamydia EBs

Time after inoculation (hours)	% LDH release from McCoy cell monolayers after inoculation with chlamydia EBs at the indicated MOI*															
	<i>Chlamydia muridarum</i>				Serovar D				Serovar H				Serovar L2			
	1.5	6.25	25	100	1.5	6.25	25	100	1.5	6.25	25	100	1.5	6.25	25	100
0	0	0	0	3	0	0	0	3	0	0	3	12	0	0	0	1
1	0	0	2	29	0	0.6	3	14	0.4	13	72	90	0	0	0	0.4
2	0	0.4	29	93	0	0.8	6	26	5	70	83	90	0.3	0	0.1	0.7
4	0	1	72	94	0	0.7	14	48	13	77	79	84	0.7	0.3	0	1
6	1	2	89	97	0.8	1	23	65	15	80	82	83	0.7	0.6	0.6	1

\*Confluent McCoy cell monolayers were infected at MOIs of 1.5, 6.25, 25, and 100, and incubated at 37°C in the presence of 10 µg/ml of rifampicin and 5 µg/ml of doxycycline. LDH release was assayed in the supernatant immediately after inoculation and in aliquots taken at 1, 2, 4, and 6 hours post-inoculation. The per cent release was calculated using the total cell LDH activity released during the complete lysis of uninoculated monolayers as 100%. Spontaneous release from uninoculated monolayers was negligible at all time points.

EB, elementary body; LDH, lactate dehydrogenase; MOI, multiplicity of infection.

larger number of ifu released into the medium, the appearance of smudge cells, the disappearance of more cells with intact inclusions from the monolayer, and the production of less total ifu during the course of in vitro infection with serovar H. During genital tract infection, this would result in the release of less ifu, as previously described in both mice<sup>20</sup> and humans.<sup>10</sup> Ultimately, less shedding would reduce the number of ifu available to sustain infection, which might contribute to a shorter and less invasive infection, as seen in the mouse model, and a reduced level of transmission among humans, which would explain the well documented difference in the prevalence of infection with these serovars in human populations from around the world.<sup>3-9</sup>

“The ability to produce large numbers of infectious units rapidly probably contributes to the ability of *C muridarum* to ascend the genital tract and ultimately deliver an infectious challenge to the upper genital tract within the first seven days of infection, which results in the severe pathology seen during *C muridarum* infection”

Relatively little use has been made of the murine model to characterise human oculogenital serovars of *C trachomatis*, with most investigators choosing to use *C muridarum*. The historical basis for this selection, most recently expressed in a review article by Morrison and Caldwell,<sup>23</sup> rests almost solely on the ability of *C muridarum* to cause severe upper genital tract pathology and a high incidence of infertility after a single infection with as few as 100 ifu. This is in contrast to human isolates, including the serovar D and H strains used in our study, which have a limited ability to ascend from the lower genital tract with major pathological consequences after infection, even with as many as 10<sup>7</sup> ifu.<sup>20-26</sup> Originally, this important difference in virulence was attributed to adaptive evolutionary responses between *C muridarum* and its natural host, the mouse. It was later suggested that the relatively greater resistance of *C muridarum* to the infection modifying effects of interferon  $\gamma$  contributes to its virulent phenotype. However, interferon  $\gamma$  alone cannot explain either the significantly later but equivalent magnitude of shedding that occurs during the early course of infection in interferon  $\gamma$  knockout mice infected with serovar D compared with *C muridarum* or the ability of exquisitely interferon  $\gamma$  sensitive oculogenital serovars<sup>14</sup> to establish infection within the genital tract of normal mice.<sup>20</sup> Alternatively, our results demonstrate an inherent difference between these biovars that can better explain this difference. The ability of *C muridarum* to produce and release infectious units at a rate at least 2.5 times that of human oculogenital serovars, and perhaps more importantly in a 24 hour time frame, certainly has important consequences on the course and outcome of

infection, the effectiveness of innate immunity, and the type and level of acquired immune responses made to infection.

This ability to produce large numbers of infectious units rapidly probably contributes to the ability of *C muridarum* to ascend the genital tract and ultimately deliver an infectious challenge to the upper genital tract within the first seven days of infection, which results in the severe pathology seen during *C muridarum* infection.<sup>27</sup> One can speculate that in the process *C muridarum* is capable of avoiding the innate immune system during each round of replication, being ultimately confined and contained by the aggressive inflammatory and immune response that occurs either just before or coincidental with the involvement of the entire genital tract, which in this last instance results in infertility.<sup>28</sup>

Although compelling in its focus on the most severe sequelae associated with infection, *C muridarum* infection of the mouse female genital tract is not, in many of its features, analogous to human infection with oculogenital serovars of *C trachomatis*, particularly in the nature of the severe upper genital tract pathology it is meant to mimic. This infrequent and previously overestimated outcome associated with infection<sup>29</sup> is thought to be a chronic process associated with either persistent or multiple infections, in which host

### Take home messages

- Differences in elementary body associated cytotoxicity between *Chlamydia trachomatis* serovars D and H are linked with in vitro growth characteristics that can explain both the shorter and less invasive course of serovar H infection in the mouse female genital tract, and more importantly the higher prevalence and greater level of shedding that have been seen in human infections with serovar D
- Differences seen between *C muridarum* and *C trachomatis* serovars D and H, particularly the much faster in vitro replication and release kinetics, correspond to the more rapid and invasive course of mouse genital tract infection with *C muridarum* compared with human oculogenital serovars
- Taken together with previously published reports, these findings provide an objective basis to question the appropriateness of *C muridarum* as a model strain in the murine model of human female genital tract infection, and establish the model's capacity to assess the potential effects of phenotypic differences among human isolates on the course and outcome of human infection

susceptibility factors play a role, and not the result of inflammatory processes associated with a single acute disease episode. In contrast, infection of the mouse with human strains mimics in many ways both the course and outcome of infection in most women—that is, an asymptomatic and self-limiting infection that only rarely results in severe upper genital tract sequelae. The results presented in our study, together with other reports describing differences in potentially virulence determining phenotypes between *C muridarum* and human isolates, again raise the issue of the appropriateness of *C muridarum* as a model agent in the murine model of female genital tract infection.<sup>30</sup> The issue is made more compelling given that the collection of phenotypes that define *C muridarum* may not have a single counterpart in the diverse collection of serovars that comprise the human oculogenital biovar of *C trachomatis*. Systematic in vitro and in vivo investigation of the phenotypic variation among human disease causing strains could provide results with translational value that will help direct the development of a vaccine and/or other intervention strategies effective against *C trachomatis* genital tract infections.

In conclusion, the results of our study provide a strain specific phenotype based explanation for the profound differences in the course and outcome of female genital tract infection in mice infected with strains belonging to the human oculogenital biovar of *C trachomatis* and *C muridarum*, raising the issue of the appropriateness of using *C muridarum* as a surrogate for *C trachomatis* in this model of human disease. More importantly, the human epidemiological data relating to differences in the prevalence and level of shedding that occurs during infection with oculogenital serovars D and H can be understood in the context of defined phenotypic characteristics.

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## Differences in growth characteristics and elementary body associated cytotoxicity between *Chlamydia trachomatis* oculogenital serovars D and H and *Chlamydia muridarum*

J M Lyons, J I Ito, Jr, A S Peña, et al.

*J Clin Pathol* 2005 58: 397-401

doi: 10.1136/jcp.2004.021543

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