**Optimal cultivation of Chlamydia requires testing of serum on individual species**

Malhar Desai1,2, Huirong Zhang1 and Huizhou Fan1,2*

**Abstract**

**Objective:** This report is a side product of experiments aimed at identifying serum for culturing obligate intracellular bacteria *Chlamydia trachomatis* and *C. muridarum* in mouse fibroblast L929 cells.

**Results:** Of five commercial serum samples tested, two showed optimal efficiencies at supporting growth of the human pathogen *Chlamydia trachomatis* as control fetal bovine serum, whereas two showed modest ~40% inhibitions in progeny production, and the remaining one showed a 20% inhibition. Three of the six sera poorly supported growth of the murine pathogen *Chlamydia muridarum*, resulting in 73–90% reduction in progeny formation. Most significantly, the one with the strongest (90%) *C. muridarum* inhibition activity showed optimal *C. trachomatis*-supporting efficiency. These findings indicate that in laboratories that study multiple *Chlamydia* species, serum samples should be prescreened on a species basis. Considering *Chlamydial* biology and epidemiology, it may even be necessary to perform serum tests on a serovar- or strain-basis for studying some animal chlamydiae.

**Keywords:** *Chlamydia trachomatis*, *Chlamydia muridarum*, Fetal bovine serum, Serum test

**Introduction**

Animal serum serves as a rich source of growth factors for culturing animal cells [1] and intracellular pathogens requiring animal cells as hosts (e.g., [2–5]). Fetal bovine serum (FBS), which contains a large and diverse group of growth factors, has been a popular choice. However, the high costs of FBS (due to its limited supplies) and ethical concerns associated with its production have led some researchers to pursue using other alternatives to FBS, including newborn calf serum (NBCS, defined as serum collected from calves that are less than 10 days old) and horse serum [1–5].

*Chlamydia* is a genus of bacteria that replicates only inside host eukaryotic cells [6]. *Chlamydia trachomatis* and *C. pneumoniae* are common human pathogens. Whereas *C. trachomatis* is the number one sexually transmitted pathogenic bacterium [7], *C. pneumoniae* causes communicable respiratory infections [8]. Other *Chlamydia* species known as pathogens of animals including livestock may cause severe illness in humans after their contact with infected animals [9]. *C. psittaci*, cause of psittacosis, is the best example of zoonotic *Chlamydia*. *C. muridarum* is a mouse pathogen. Although *C. muridarum* infection in humans has never been documented, it is the most widely studied animal *Chlamydia*, owing to its capacity to model human chlamydial infections in mouse [10, 11].

Like many laboratories, our laboratory studies both *C. trachomatis* and *C. muridarum*. In an event evaluating commercial serum samples, we found differential effects of the sera on the two *Chlamydia* species. These findings mandate prescreening sera for individual species in laboratories that study multiple species.
Main text

Methods

Animal serum

Samples of FBS, NBCS and ASC were supplied by American Type Culture Collection (ATCC), Atlantic Biologicals, Gemini Bio-products and Sigma Millipore. Prior to use, all serum samples as well as control FBS (Sigma Millipore) were subjected to heat inactivation (56 °C, 30 min) to eliminate adverse effects of complements on chlamydiae [12].

Evaluation of effects of serum on chlamydial growth

Mouse fibroblast L929 cells were used as host for chlamydiae. They were grown as adherent cultures using the Dulbecco modified Eagle’s medium containing 4.5 g/L glucose and 0.11 g/L sodium pyruvate (DMEM) and supplemented with 5% (vol/vol) control FBS and gentamicin (final concentration: 10 µg/ml). To evaluate the effects of serum samples on chlamydial growth, cells were seeded onto 6-well plates and cultured overnight to 90% confluency. Elementary body (EB) stocks of *C. trachomatis* L2 (strain 434/Bu) (CtL2) and *C. muridarum* (strain Nigg) (Cm) [13] were diluted in DMEM containing 5% serum samples and 1 µg/ml cycloheximide, which promotes chlamydial growth by inhibiting host cellular protein synthesis. To infect the cells, the overnight culture media were replaced with the EB-containing medium (2 ml/well). The multiplicity of infection was 0.5 inclusion-forming unit (IFU) per cell. After culture at 37 °C for 30 h (Cm) or 36 h (CtL2), the culture media were replaced with 100 µL of sucrose–phosphate–glutamate buffer, and cells were detached from the plastic surface using a Cell Lifter, and collected into a 5 ml culture tube. Tubes were then placed on ice and subject to brief sonication to release chlamydiae from cells [13]. The sonicated harvests were serially diluted in a 1:10 manner and then inoculated onto L929 monolayers grown on 96 well plates, which were fixed with cold methanol following 30 h (Cm) or 36 h (CtL2) incubation at 37 °C. Cm and CtL2 inclusions were reacted with a mouse polyclonal anti-Cm antibody [14] and a monoclonal anti-major outer membrane protein antibody (clone L2-1-5) [15], respectively, followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Sigma-Aldrich). Inclusions were scored under an Olympic IX51 fluorescence microscope.

Results

We tested a total of 5 serum samples from commercial sources along with a control fetal bovine serum (CFBS) on the growth of *C. trachomatis* L2 (CtL2) and *C. muridarum* (Cm). Among the five serum samples, three were fetal bovine sera (i.e., FBS1-3), one was a newborn calf serum (i.e., NBCS1), and the last one was known as animal serum complex (i.e., ASC1), of which the compositions were not disclosed by the manufacturer. CFBS had been tested previously and used for culturing both Ct and Cm for more than a year in our lab prior to this work. Compared with CFBS, FBS1 or NBCS1 demonstrated similar and the most desirable effect on CtL2 growth, resulting in the highest EB yields (Fig. 1a). FBS2 showed a slight 21% decrease in EB production, which was deemed statistically insignificant ($P = 0.009$). FBS3 and ASC1 demonstrated suboptimal effects on chlamydial growth, resulting in statistically significant 41% and 40% decreases, respectively, in EB production (Fig. 1a).

Compared to CtL2, Cm displayed mostly contrasting responses to the serum products (Fig. 1b). Among all samples tested, only FBS2 showed a similar effect on Cm growth as CFBS. FBS1, which was optimal for CtL2 growth, was suboptimal for Cm, resulting in only 68% progeny EBs, compared to CFBS. Most interestingly, NBCS1, which was optimal for CtL2 growth, had the most detrimental effect on Cm growth, resulting in a 90% EB production inhibition. FBS3, which had a suboptimal effect on CtL2 growth, displayed a similarly severe adverse effect as NBCS1, leading to an 88% reduction in EB production. ASC1, which had
a suboptimal effect on CtL2 growth (40% inhibition in EB yield), exhibited a more severe adverse effect on Cm growth, giving rise to a 73% decrease.

**Discussion**

The importance of serum in culturing chlamydiae was recognized starting more than four decades ago (e.g., [2, 3]). However, previous studies reporting effects of different serum products on chlamydial growth were performed with only single species [2–5]. In this work, we have demonstrated that some animal sera have significantly different effects on Ct and Cm growth. Although we have not determined the underlying mechanism, we suspect anti-chlamydia antibodies are responsible for the observed inhibitory effects. Ct and Cm are known to infect only human and mouse, respectively. However, multiple other *Chlamydia* species, namely *C. pecorum* and *C. abortus*, *C. pneumoniae*, *C. psittaci* and *C. gallinacea* are known to infect cows [9, 16]. Thus, it should not be surprising if FBS or NBCS contains anti-chlamydia antibodies because maternal IgG is capable of entering the fetal blood stream. Although genomes of *Chlamydia* species are highly conserved, they express different polymorphic proteins on the cell surface. These proteins serve as immunodominant antigens, which induce hosts to produce neutralizing antibodies [17, 18]. Other outer membrane proteins including the major outer membrane protein are also immunodominant proteins [19]. Thus, the effects of a particular serum on Ct and Cm would be determined by antibodies with cross reactivity for the organisms (or not).

Surface-exposed domains of the major outer membrane protein are immunodominant epitopes [20, 21]. They are highly variable even within the same *Chlamydia* species [20, 21]. While our findings mandate serum testing for individual *Chlamydia* species for optimal cultivation, tests may be necessary for individual serovars of *Chlamydia* species capable of infecting cows. Perhaps, serum testing should be performed for individual strains of *Chlamydia* species without well-established serovars.

**Limitations**

We make the recommendation of serum testing on individual *Chlamydia* species on the basis of data presented in Fig. 1. However, the suggestion of serum testing for different serovars or strains within some animal chlamydial species is made without experimental evidence.

**Abbreviations**

ASC: animal serum complex; CF: control fetal bovine serum; Cm: *Chlamydia muridarum*; CtL2: *Chlamydia trachomatis* serovar L2; DMEM: Dulbecco’s Modified Eagle Medium; FBS: fetal bovine serum; IFU: inclusion forming unit; NBCS: newborn calf serum.

**References**

1. Yao T, Asayama Y. Animal-cell culture media: history, characteristics, and current issues. Reprod Med Biol. 2017;16(2):99–117.
2. Allan I, Pearce JH. Serum modulation of cell susceptibility to chlamydial infection. FEMS Microbiol Lett. 1977;14:211–3.
3. Karayiannis P, Hobson D. The role of calf serum in the growth of *Chlamydia trachomatis* in McCoy cell cultures. Microbiology. 1981;122(1):47–54.
4. LaScola L Jr, Baldigo SM. Infectivity of *Chlamydia trachomatis* in tissue culture with newborn calf serum. J Clin Microbiol. 1982;15(5):951–3.
5. Levy NJ, Benes S, McCormack WM. Growth of host cells and *Chlamydia trachomatis* in medium containing serum from 16-week-old calves. J Clin Microbiol. 1983;17(1):68–71.
6. Borel N, Greub G. International Committee on Systematics of Prokaryotes (ICSP) Subcommittee on the taxonomy of *Chlamydiae*. Minutes of the closed meeting, 5 July 2018, Woudschoten, Zeist, The Netherlands. Int J Syst Evol Microbiol. 2019;69(8):2606–8.
7. WHO: Global prevalence and incidence of selected curable sexually transmitted infections: Overview and estimates. 2001.
8. Kalayoglu MV, Libby P, Byrne GI. *Chlamydia pneumoniae* as an emerging risk factor in cardiovascular disease. JAMA. 2002;288(21):2724–31.
9. Cheong HC, Lee CYQ, Cheok YY, Tan GM, Looi CY, Wong WF. Chlamydiae: diseases in primary hosts and zoonosis. Microorganisms. 2019;7(5):146.
10. Zhong G. *Chlamydia* spreading from the genital tract to the gastrointestinal tract—a two-hit hypothesis. Trends Microbiol. 2018;26(7):611–23.
11. de la Maza L, Pal S, Khamesipour A, Peterson E. Intravaginal inoculation of mice with the *Chlamydia trachomatis* mouse pneumonitis biovar results in infertility. Infect Immun. 1994;62(5):2094–7.
12. Yang Z, Conrad T, Zhou Z, Chen J, Dutow P, Klos A, Zhong G. Complement factor C5 but not C3 contributes significantly to hydrosalpinx development in mice infected with *Chlamydia muridarum*. Infect Immun. 2014;82(8):3154–63.

13. Bao X, Gyffe A, Studevant GL, Gong Z, Xu S, Caldwell HD, Elofsson M, Fan H. Benzylidene acylhydrazides inhibit chlamydial growth in a type III secretion- and iron chelation-independent manner. J Bacteriol. 2014;196(16):3989–3001.

14. Zhang H, Kunadia A, Lin Y, Fondell JD, Seidel D, Fan H. Identification of a strong and specific antichlamydial N-acylhydrazone. PLoS ONE. 2017;12(10):e0185783.

15. Zhang YX, Stewart SJ, Caldwell HD. Protective monoclonal antibodies to *Chlamydia trachomatis* serovar- and serogroup-specific major outer membrane protein determinants. Infect Immun. 1989;57(2):636–8.

16. Li J, Guo W, Kaltenboeck B, Sachse K, Yang Y, Lu G, Zhang J, Luan L, You J, Huang K, et al. *Chlamydia pecorum* is the endemic intestinal species in cattle while *C. gallinacea, C. psittaci* and *C. pneumoniae* associate with sporadic systemic infection. Vet Microbiol. 2016;193:93–9.

17. Crane DD, Carlson JH, Fischer ER, Bavoil P, Hsia RC, Tan C, Kuo CC, Caldwell HD. *Chlamydia trachomatis* polymorphic membrane protein D is a species-common pan-neutralizing antigen. Proc Natl Acad Sci U S A. 2006;103(8):1894–9.

18. Tan C, Hsia RC, Shou H, Haggerty CL, Ness RB, Gaydos CA, Dean D, Sculock AM, Wilson DP, Bavoil PM. *Chlamydia trachomatis*—infected patients display variable antibody profiles against the nine-member polymorphic membrane protein family. Infect Immun. 2009;77(8):3218–26.

19. Caldwell HD, Perry LJ. Neutralization of *Chlamydia trachomatis* infectivity with antibodies to the major outer membrane protein. Infect Immun. 1982;38(2):745–54.

20. Zhang YX, Stewart S, Joseph T, Taylor HR, Caldwell HD. Protective monoclonal antibodies recognize epitopes located on the major outer membrane protein of *Chlamydia trachomatis*. J Immunol. 1987;138(2):575–81.

21. Zhong G, Berry J, Brunham RC. Antibody recognition of a neutralization epitope on the major outer membrane protein of *Chlamydia trachomatis*. Infect Immun. 1994;62(5):1576–83.

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