ELIMINATION OF MYCOPLASMA FROM CONTAMINATED CHLAMYDIA TRACHOMATIS STOCK

Ashley Harris[1], Shuaizhi-Li[1], Nicole Schleicher[1], McKenzie Herrboldt[1], Ashley Popp[2], Brandon N. Johnson[2*], Hamada A. Aboubakr[1*]

1: Cell-based Systems Team, 2: Global Genomics Center; R&D Department-Microbiologics Inc. 200 Cooper Ave. N., St. Cloud, MN-56303

* Corresponding authors: HAA (e-mail: haboubakr@microbiologics.com); BNJ (e-mail:bjohnson@microbiologics.com)

1- INTRODUCTION

- Microbiologics produces several Helix Elite™ Molecular Standards which contain inactivated *Chlamydia trachmatis* (CT) and/or *Chlamydia pneumoniae* (CP) (e. g. Catalog No. 8188, 8217, 8228, 8229, HE0034N, HE0035N, HE0045N).
- Microbiologics also provides its clients with live and inactivated lots of CT and CP in response to custom production requests. Therefore, a considerable amount of the annual revenue is generated by the production of live and inactivated Chlamydia.
- Recent research by Cell-based Systems Group (CBS) revealed that the original viable stocks of CT (serovar D and H) and CP are highly contaminated with mycoplasma as shown by real-time qPCR determination results in below.

Ct values of mycoplasma in original *C. trachomatis* serovar D stock and all live lots derived from it.

Sample	CT value
C.trachomatis Serovar D (original vial from UW)	18.4
C.trachomatis Serovar D Lot: V00061	20.6
C.trachomatis Serovar D Lot: V00066	23.7
C.trachomatis Serovar D Lot: V00180	24.0
C.trachomatis Serovar D Lot: V00255	18.9
Negative Control	Undetermined

- Mycoplasmas are considered as the simplest form of bacteria. They belong to the bacterial class Mollicutes, whose members are distinguished by their lack of a cell wall and their plasma-like form. Mycoplasma contamination can modify both host cell trafficking and the growth behavior of Chlamydia.

- Products that are contaminated with mycoplasma are not acceptable to our customers due to the industry standard that all materials are expected to be mycoplasma-free unless otherwise specified. Providing materials free from contaminants including mycoplasma is part of the Microbiologics mission to provide the highest quality biomaterials to our customers.
- Chlamydia strains that we own are difficult to be re-obtained from patients and are not easily replaced. Therefore, our aim was to eliminate Mycoplasma from our contaminated Chlamydia strains.
- There are no effective antibiotics that selectively eradicate Mycoplasma over Chlamydia. Physically separate Chlamydia from Mycoplasma by differential centrifugations are impractical due to the similar sizes of these organisms.
- Techniques to eradicate Mycoplasma from Chlamydia cultures in the literature include: (i) the treatment with detergents, (ii) isolation by plaque assay and (iii) passaging in mice under influence of the immunity.

2- AIM OF THE WORK

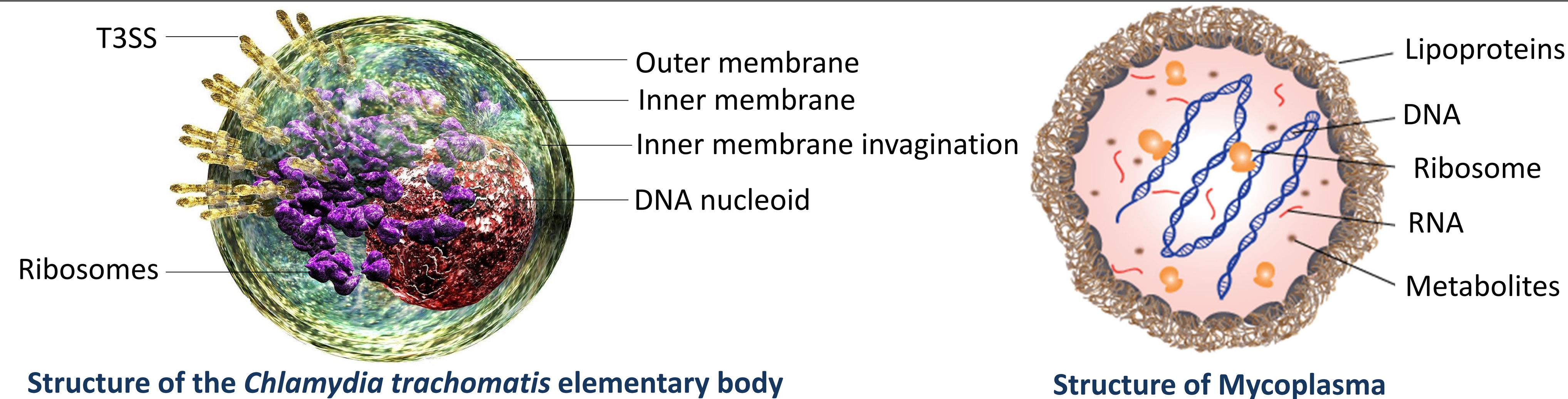
To eliminate Mycoplasma from a contaminated *C. trachomatis* stock by employing a novel stepwise approach entailing two procedures:

1. Incubation in nonionic detergent-containing solution (Triton X-100, abbreviated TX-100).
2. Isolation of single inclusion body-containing culture by limiting dilution approach.

3- SCIENTIFIC PRINCIPLE OF THE TRITON X-100 BASED METHODS

The elementary body of chlamydia is comprised of a Gram-negative cell wall structure consisting of an outer **peptidoglycan membrane** and an inner cytoplasmic membrane. In contrast, the mycoplasma cell membrane is composed of lipoproteins [two-thirds proteins and one-third lipids (glycolipids and lipoglycans)]

The difference in membrane structure makes mycoplasma more sensitive to detergent treatment. This allowed us to use TX-100 based methods to eliminate the mycoplasma from chlamydia stock.



Structure of the *Chlamydia trachomatis* elementary body

Structure of Mycoplasma

4- FRAMEWORK OF STUDY AND METHODS

1- Stock treated with TX-100

Triton X-100

A mycoplasma-contaminated stock of highly concentrated Chlamydia was treated with TX-100 (0.01%).

2- Serial dilution of treated stock

	1	2	3	4	5
A	undiluted	1:10	1:100	1:1K	1:10K
B	undiluted	1:10	1:100	1:1K	1:10K
C	undiluted	1:10	1:100	1:1K	1:10K
D	undiluted	1:10	1:100	1:1K	1:10K
E	undiluted	1:10	1:100	1:1K	1:10K
F	undiluted	1:10	1:100	1:1K	1:10K
G	undiluted	1:10	1:100	1:1K	1:10K
H	undiluted	1:10	1:100	1:1K	1:10K

Serial dilutions were prepared from TX-100 treated chlamydia. We hypothesized the wells with a single elementary body held higher probability of being mycoplasma-free.

3- Infection and propagation in Hela cells

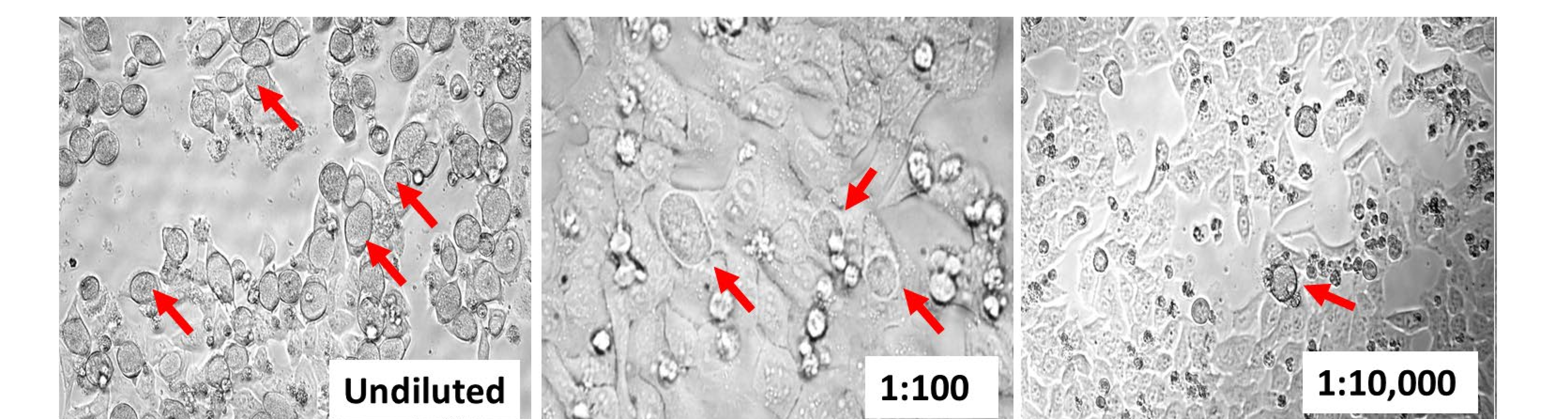
HeLa-229 cells were infected with the dilution series and incubated until inclusion bodies formed. Clones were selected for passaging and expansion.

4- MycoStrip, real-time qPCR, and whole genome sequencing for purity and identity confirmation

InvivoGen MycoStrip™
Realtime PCR
qPCR
GENOME SEQUENCING

5- RESULTS

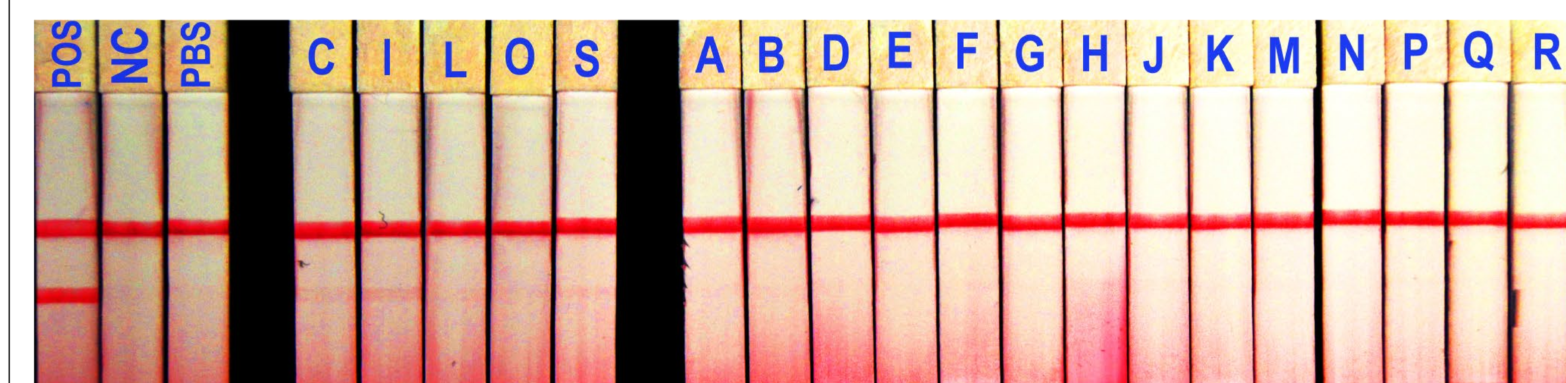
PROPAGATION OF TREATED STOCK



Inclusion bodies in HeLa229 cells after infection with limiting dilutions of TX-100 – treated chlamydia.

Some elementary bodies retained infectivity after TX-100 treatment. Single inclusion bodies were observed at dilutions from 1,000 to 10,000 folds. These wells were separately collected and propagated for several passages to generate stocks. Samples from a total of 19 wells were collected and expanded, and coded with litter from A to S.

MYCOSTRIP ASSAY (ISOTHERMAL PCR BASED)



Post-treatment mycoplasma negative clones were isolated

All controls performed as expected. Some clones (C, I, L, O, S) showed faintly positive bands and they were discarded. Clones A, B, D, E, F, G, H, J, K, M, N, P, Q, and R did not show any observable positive bands. The negative clones were selected for qPCR analysis to confirm the mycoplasma-free status.

PCR RESULTS

Samples	Chlamydia Ct value	Mycoplasma Ct value
A	13.740	Undetermined
B	14.649	Undetermined
D	17.118	Undetermined
E	14.067	44.846
F	14.206	Undetermined
G	13.982	Undetermined
H	14.784	Undetermined
J	13.614	Undetermined
K	14.425	Undetermined
M	14.401	Undetermined
N	14.545	Undetermined
Q	15.519	Undetermined
R	13.887	Undetermined
Cell only control	29.557	Undetermined
NTC	Undetermined	Undetermined

The qPCR results confirmed the MycoStrip assay results

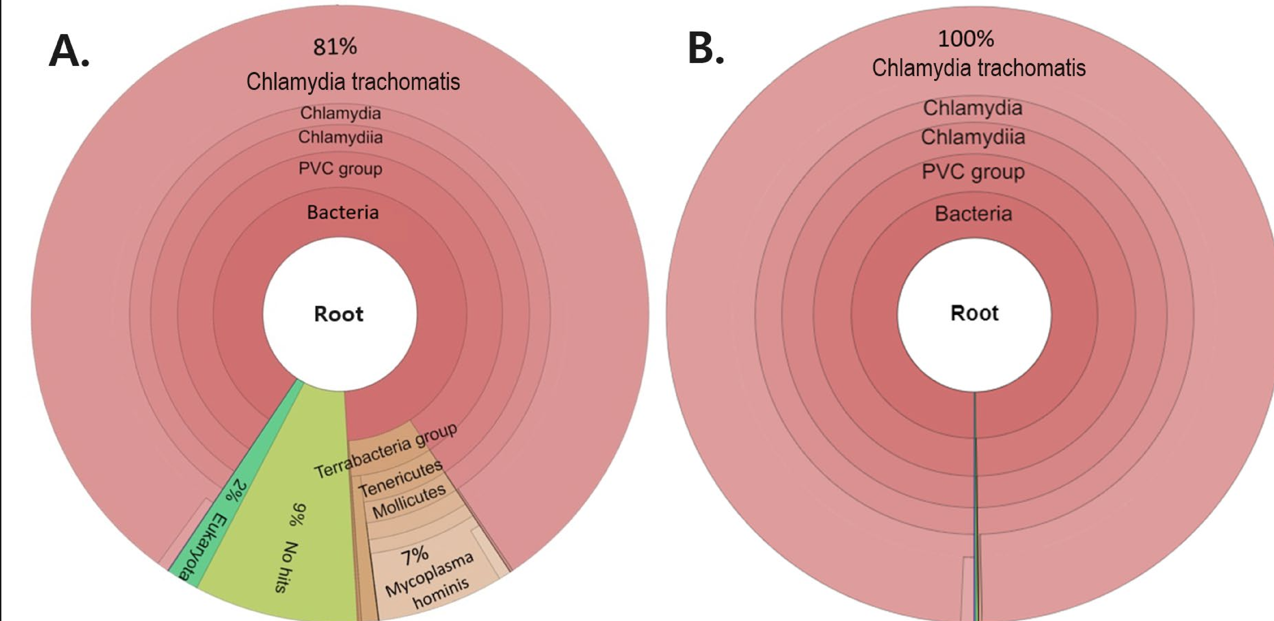
As a confirmation to the MycoStrip assay results, qPCR was performed. The results showed that all clones that tested negative using MycoStrip were also tested negative by real-time qPCR. Although sample E showed a PCR signal curve, its Ct value was above the positive Ct value threshold (i.e. 40)

GENOME IDENTITY

Genome identity preserved post-treatment

Next-generation sequencing (NGS) libraries for whole genome sequencing were prepared for from DNA extracts of both pre-treatment and post-treatment samples. Sequencing of the libraries was performed using an Illumina MiSeq sequencer. Reference-based genome assembly and alignment (DNASTAR) of the *Chlamydia trachomatis* genomes revealed **99.81%** similarity using Mauve alignment (Multiple Alignment of Conserved Genomic Sequence With Rearrangements).

METAGENOMICS



Metagenomics supports mycoplasma elimination

Metagenome analysis (Illumina DRAGEN Metagenomics) of the NGS data was performed to examine genomic content. Pre-treatment **(A)** sequencing resulted in 81% corresponding to *C. trachomatis* and 7% aligned to *Mycoplasma hominis.* Post-treatment **(B)**, nearly 100% of reads match *C. trachomatis* and <0.001% aligned to *Mycoplasma hominis*.

6- CONCLUSIONS

1. Using a method combining treatment with Triton X-100 and limiting dilution approach, we were able to eradicate mycoplasma from our stock of *Chlamydia trachomatis*. This was demonstrated by the results of both isothermal PCR (MycoStrip) and real-time PCR to persist over 5 passages.
2. Future lots of this material will continue to be tested for mycoplasma to ensure purity.
3. The genome sequence identity of the mycoplasma-free Chlamydia culture was characterized by the next generation sequencing of the pre- and post- treatment cultures.
4. The purity of the mycoplasma-free Chlamydia culture was further supported by the results of metagenome analysis.
5. This method could potentially be applied to other mycoplasma-contaminated chlamydia strains that we have in-house, namely other serovars of *Chlamydia trachomatis* and *Chlamydia pneumoniae*.