Decontamination of mycoplasma-contaminated *Orientia tsutsugamushi* strains by repeating passages through cell cultures with antibiotics

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**Abstract**

**Background:** Mycoplasmas-contamination of *Orientia tsutsugamushi*, one of the obligate intracellular bacteria, is a very serious problem in *in vitro* studies using cell cultures because mycoplasmas have significant influence on the results of scientific studies. Only a recommended decontamination method is to passage the contaminated *O. tsutsugamushi* strains through mice to eliminate only mycoplasmas under influence of their immunity. However, this method sometimes does not work especially for low virulent strains of *O. tsutsugamushi* which are difficult to propagate in mice. In this study, we tried to eliminate mycoplasmas contaminants from both high virulent and low virulent strains of the contaminated *O. tsutsugamushi* by repeating passage through cell cultures with antibiotics *in vitro*.

**Results:** We cultured a contaminated, high virulent strain of *O. tsutsugamushi* using a mouse lung fibroblasts cell line, L-929 cell in the culture medium containing lincomycin at various concentrations and repeated passages about every seven days. At the passage 5 only with 10 μg/ml of lincomycin, we did not detect mycoplasmas by two PCR based methods whereas *O. tsutsugamushi* continued good growth. During following four passages without lincomycin, mycoplasmas did not recover. These results suggested that mycoplasmas were completely eliminated from the high virulent strain of *O. tsutsugamushi*. Furthermore, by the same procedures with 10 μg/ml of lincomycin, we also eliminated mycoplasmas from a contaminated, low virulent strain of *O. tsutsugamushi*. Our additional assay showed that 50 μg/ml of lyncomycin did not inhibit the growth of *O. tsutsugamushi*, although MICs of many mycoplasmas contaminantts were less than 6 μg/ml as shown previously.

**Conclusion:** Our results showed an alternative method to eliminate mycoplasmas from the contaminated *O. tsutsugamushi* strains in place of *in vivo* passage through mice. Especially this notable method works for the decontamination not only from the high virulent strain also from the low virulent strain of *O. tsutsugamushi*. For further elimination, lincomycin at the limit concentration, which does not inhibit the growth of *O. tsutsugamushi*, can possibly eliminate most mycoplasmas from contaminated *O. tsutsugamushi* strains.

**Keywords:** *Orientia tsutsugamushi*, Intracellular bacteria, Mycoplasma, Contamination, Elimination, Cell culture, Antibiotics

**Background**

The contamination of cell cultures by mycoplasmas is a serious problem because these bacteria have multiple effects on cell cultures and also have a significant influence on the results of scientific studies. The mycoplasmas are not harmless bystanders and thus cannot be ignored in the cell cultures.

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Various elimination methods were previously reported [1-3]. These methods are mainly based on four general procedures, physical, chemical, immunological and chemotherapeutic treatment. The physical procedures include heat treatment and filtration. The chemical procedures, treatments to detergents and other chemicals which are effective only against mycoplasmas, but not against host cells. The immunological procedures include *in vitro* co-culture with macrophages and specific anti-mycoplasmas antisera and *in vivo* passage thorough mice. The chemotherapeutic procedures are mainly antibiotics treatments that are kills mycoplasmas.
Orientia tsutsugamushi, which is the causative agents of scrub typhus is one of the obligated intracellular bacteria [4]. The mycoplasmas-contaminations of O. tsutsugamushi is also very serious in the *in vitro* studies using cell cultures. Furthermore the most effective methods for elimination of mycoplasmas can not be applied for decontamination of *O. tsutsugamushi* strains because these methods also inhibit the growth of *O. tsutsugamushi*. Decontamination methods should have strong effect on mycoplasmas, but have minimum or no effect on *O. tsutsugamushi*. Only the recommended decontamination method is to passage the contaminated *O. tsutsugamushi* strains through mice. Mouse immunity possibly affects on mycoplasmas, but have minimum or no effect on *O. tsutsugamushi* strains because they are generally difficult to propagate in mice.

Some of the antibiotics, which are used for elimination of mycoplasmas from tissue culture, are supposed to have less effect against *O. tsutsugamushi* according to the differences of minimum inhibitory concentrations (MICs) of antibiotics between mycoplasmas [5-7] and *O. tsutsugamushi* [8]. In this study, we tried to eliminate mycoplasmas from contaminated *O. tsutsugamushi* strains by repeating passages through cell cultures with antibiotics *in vitro*.

**Results and discussion**

According to the MICs of antibiotics in the previous reports [5,7-9], we used two antibiotics, lincomycin and ciprofloxacin for elimination of mycoplasmas from the contaminated *O.tsutsugamushi* strains (Table 1). Both lincomycin and ciprofloxacin are effective against mycoplasmas. Unfortunately there is no available information about the MICs of lincomycin against *O. tsutsugamushi*. However, according to the MICs of lincomycin against gram-negative bacteria [10], lincomycin is supposed to be much less effective against *O. tsutsugamushi* because *O. tsutsugamushi* is one of the gram-negative bacteria. For the example, the MICs of lincomycin against *Escherichia coli*, one of the typical gram gram-negative bacteria are more than 50 times higher than those against mycoplasmas. Ciprofloxacin was also less effective against *O. tsutsugamushi*. The MICs of ciprofloxacin against *O. tsutsugamushi* are about 3 to 200 times higher than those against mycoplasmas (Table 1).

Our result of the direct sequencing showed that Ikeda and Kuroki strains of *O. tsutsugamushi* were contaminated with *Mycoplasma hominis* and *M. orale* respectively. *M. hominis* and *M. orale* are 10 to 30% of contaminants of cell cultures (Table 2) [11]. Previous reports showed that *M. fermentas*, *M. hyorhinis*, *M. arginini* and *Acholeplasma laidlawii* are the most common contaminants as well as *M. hominis* and *M. orale*. More than 90% of the contaminants were caused by these six mycoplasmas [11,12]. The TaqMan PCR and the nested PCR can detect not only all the 6 most common contaminants also some other mycoplasmas. These facts suggested that the detection methods were very reliable to monitor mycoplasmas-contaminations in this study.

For elimination of mycoplasmas, we first cultured a contaminated, high virulent Ikeda strain of *O. tsutsugamushi* using L-929 cell in the culture medium containing lincomycin and ciprofloxacin and repeated the passages (Figure 1). Lincomycin and ciprofloxacin were used at 100, 10 and 1 μg/ml. However, ciprofloxacin at 100 μg/ml were cytotoxic against L-929 cell in the first assay and was omitted from the further analyses. We checked mycoplasma-contaminations and *O. tsutsugamushi-growth* at each passage by the two PCR based methods and/or an immunofluorescent (IF) staining (see Additional file 1). From the passage 1 to 2 with 10 μg/ml of lincomycin, the real-time PCR showed that mycoplasmas decreased, whereas *O. tsutsugamushi* did not decrease. At the passage 4 with the same concentration of lincomycin, the real-time PCR did not detect mycoplasmas, however the nested PCR still detected them. At the passage 5, both the real-time PCR and the nested PCR did not detect mycoplasmas, whereas the flourish growth of *O. tsutsugamushi* was observed by IF staining. We continued to culture with lincomycin until the passage 6. During following passages from 7 to 10 without lincomycin, mycoplasmas did not recover. These results clearly showed that mycoplasmas were completely eliminated from *O. tsutsugamushi*-infected cells. However, the cultivation with 100 μg/ml of lincomycin as well as 10 and 1 μg/ml of ciprofloxacin decreased both mycoplasmas and *O. tsutsugamushi-growth*, whereas the cultivation with 1 μg/ml of lincomycin did not influence the neither growths.

By the same procedure of Ikeda strain, we cultured a contaminated, low virulent Kuroki strain of *O. tsutsugamushi* with lincomycin at 10 μg/ml (Figure 1). Mycoplasmas and

**Table 1 Minimum inhibitory concentrations (MICs) of antibiotics used in this study**

| Antibiotics   | Drug class | MICs against Orientia<sup>a</sup> | MICs against mycoplasmas<sup>b</sup> |
|---------------|------------|----------------------------------|-------------------------------------|
| Lincomycin    | Lincosamide| No available data                 | 0.25–2 μg/mL                        |
| Ciprofloxacin | New Quinolone| 6.25–25 μg/mL                    | 0.125–2 μg/mL                        |
| Gentamicin    | Aminoglycoside| No available data<sup>c</sup> | 2.5–500 μg/mL                       |
| Kanamicin     | Aminoglycoside| No available data                | 2.5–500 μg/mL                       |
| Minocycline   | Tetracycline| 0.024–0.195 μg/mL               | 0.016–32 μg/mL                      |

MICs were obtained from previous reports. a) from [8] and b) from [5-7]. c) Gentamicin was not effective against Orientia tsutsugamushi in a mouse model [25].
Otsutsugamushi were monitored by the nested PCR and the IF assay respectively (see Additional file 2). At the passage 8, the nested PCR did not detect mycoplasmas. We then continued to cultivate it with lincomycin until the passage 11. During following passages from 12 to 14 without lincomycin, mycoplasmas did not recover. These results showed that we successfully eliminated mycoplasmas also from the low virulent Kuroki strain. The elimination length of Kuroki strain was longer than that of Ikeda strain probably because numbers and/or antibiotics-susceptibility of the contaminated mycoplasmas were different. For further elimination of mycoplasmas from other strains of Otsutsugamushi, we should first evaluate a maximum concentration of lincomycin that does not influence Otsutsugamushi-growth, and then apply it for decontamination because maximum effects against mycoplasmas are necessary to eliminate them for a short time and to avoid producing lincomycin-resistant mycoplasmas [13-15] during repeating passages. Our additional assay showed that lincomycin at 25 μg/ml did not affect the growth (the virulent strain), whereas 50 μg/ml slightly decreased (did not inhibit) the growth in the IF assay (Table 3). Many previous reports about antibiotics-susceptibilities of isolated mycoplasmas showed that MICs of lincomycin against M. hominis, M. fermentans and A. laidlawii, which are the major contaminants, were less

| Species                              | Frequency of contamination (a) | tuf gene (TaqMan PCR)(b) | 16S-23S ribosomal RNA intergenic region (nested PCR)(c) | Matching of new PCR primers | Strains | Sequence ID |
|--------------------------------------|--------------------------------|--------------------------|-------------------------------------------------------|----------------------------|---------|-------------|
| Mycoplasma fermentans                | 10%-20%                        | +                        | +                                                     | Match                     | human B cell lymphoma contaminants, 16054780 | AY838558 |
| Mycoplasma hyorhinis                 | 10%-40%                        | +                        | +                                                     | Match                     | HUB-1   | NC_014448.1 |
| Mycoplasma arginine                  | 20%-30%                        | +                        | +                                                     | Partial Match             | ATCC 23714D | gi|315440428 |
| Mycoplasma orale                     | 20%-30%                        | No Data                  | +                                                     | Partial Match             | G230    | gi|290575476 |
| Acholeplasma laidlawii               | 5%-20%                         | +                        | +                                                     | Match                     | PG-8A   | CP000896    |
| Mycoplasma hominis                   | 10%-20%                        | +                        | +                                                     | Match                     | ATCC 23114 | M57675    |
| Other species                        |                                |                          |                                                       |                           |         |             |
| Mycoplasma arthritidis               | No Data                        | +                        | No Data                                               | Match                     | 158L3-1 | NC_011025.1 |
| Mycoplasma bovis                     | No Data                        | +                        | No Data                                               | Match                     | PG45    | NC_014760.1 |
| Mycoplasma buccale                   | No Data                        | +                        | No Data                                               | -                         | -       |             |
| Mycoplasma fauclium                  | No Data                        | +                        | No Data                                               | -                         | -       |             |
| Mycoplasma gallisepticum             | No Data                        | +                        | No Data                                               | Match                     | PG31    | X16462     |
| Mycoplasma genitalium                | No Data                        | +                        | +                                                     | Match                     | ATCC33530 | X16463    |
| Mycoplasma hyopneumoniae             | No Data                        | +                        | No Data                                               | Match                     | 7448    | NC_007332.1 |
| Mycoplasma penetans                  | No Data                        | +                        | No Data                                               | Match                     | FH      | X55768     |
| Mycoplasma pneumoniae                | No Data                        | +                        | +                                                     | Match                     | 3259    |             |
| Mycoplasma primatum                  | No Data                        | +                        | No Data                                               | Match                     | 3259    |             |
| Mycoplasma salviarium                | No Data                        | +                        | +                                                     | Partial Match             | ATCC 23064D | gi|313575713 |
| Ureaplasma parvum                    | No Data                        | +                        | No Data                                               | Match                     | ATCC 33697 | AF270770  |
| Mycoplasma zalophi                   | No Data                        | No Data                  | Match                                                 | C8L 4296                  | gi|148536300 |
| Mycoplasma mycoides                  | No Data                        | No Data                  | Match                                                 | PG1                       | gi|126252003 |
| Mycoplasma capricolum                | No Data                        | No Data                  | Match                                                 | ATCC 27343                | gi|83319253  |
| Mycoplasma agalactiae                | No Data                        | No Data                  | No Data                                               | Match                     | PG2     | gi|148291314 |
| Mycoplasma pyrurn                    | No Data                        | No Data                  | +                                                     | No data                   | -       |             |

(a) Upper 6 species of mycoplasmas are the most common contaminants of cell cultures [11,12].
(b) This broad-range TaqMan PCR can detect many species of mycoplasmas [22].
(c) This nested PCR is highly sensitive, and it is used to check for mycoplasma contamination in the Cell Bank of BioResource Centre, Riken Tsukuba Institute, Tsukuba, Ibaraki, Japan [21].
(d) PCR assay for sequencing of mycoplasmas designed in this study. Partial Match means that 2 or 3 of the total of 4 nested-PCR primers match to available regions of the tuf gene on the public database.
than 6 μg/ml (0.025 to 6 μg/ml) [5, 16-18]. In actual, a previous report showed that lincomycin at 50 μg/ml successfully eliminated the other major contaminants of mycoplasmas, M. hyorhinis and M. hominis from cell cultures [19]. However, a previous report showed that some isolates of M. hyorhinis were highly resistant to lyncomycin (MICs > 100 μg/ml) [14] and a few reports showed that other species of mycoplasmas but not major species of contaminants were highly resistant to lyncomycin [13, 15]. Considering these facts, lincomycin at 50 μg/ml can possibly eliminate the contaminants from many of other contaminated strains of O. tsutsugamushi, although it might not be effective for all the cases.

Conclusions

Our results showed an alternative method to eliminate mycoplasmas from the mycoplasma-contaminated strains of O. tsutsugamushi in place of in vivo passage through mice. Especially this new method works for the decontamination not only from the high virulent strain also from the low virulent strain of O. tsutsugamushi, which is difficult to propagate in mice. For further elimination, lincomycin at the limit concentration, which does not inhibit the growth of O. tsutsugamushi, can possibly eliminate most mycoplasmas from contaminated O. tsutsugamushi strains.

Methods

Cell lines

A mycoplasmas-free L-929 cell (a mouse fibroblast cell line, JCRB9003) [20] was grown in Eagle’s minimum essential medium (MEM, Wako Co. Ltd., Tokyo, Japan) supplemented with 5 to 10% of mycoplasma-free, heat-inactivated FCS (Sigma-Aldrich Japan Co. LCC., Tokyo, Japan) at 37°C in 5% CO₂.

Mycoplasmas-contaminated O. tsutsugamushi strains for elimination

A mycoplasmas-contaminated high virulent Ikeda strain and a low virulent Kuroki strain of O. tsutsugamushi were used for elimination. These strains were accidentally contaminated during a long passage history probably because mycoplasmas-contaminated cell culture was used for propagation of these strains. The mycoplasma-free L-929 cell was used for propagation as mentioned in the previous section.
Detection and quantification of mycoplasmas

Major mycoplasmas are listed in Table 2. Upper 6 species are the most common contaminants in cell cultures [11,12]. In order to monitor mycoplasmas, we extracted DNA from O. tsutsugamushi-infected L-929 cell with a commercial DNA extract kit (Tissue genomic DNA extraction mini kit, Favorgen biotech corporation, Ping-Tung, Taiwan) and detected mycoplasmas by two high sensitive and broad range PCR based methods for detection, the nested PCR [21] and the real-time PCR (TaqMan PCR) [22]. The nested PCR is used to check mycoplasma-contaminations in the Cell Bank of Bioresource Centre, Riken Tsukuba institute, Tsukuba, Ibaraki, Japan. For determination of mycoplasma species, we designed new sequencing primers against tuf gene (Table 2). These designed primers matched tuf gene of 19 mycoplasmas on the public database. All the primers and the probe are listed in Table 4.

Detection of O. tsutsugamushi

To monitor the growth of O. tsutsugamushi, we used a real-time PCR against the gene encoding 47kDa common antigen (Table 4). We extracted DNA from O. tsutsugamushi-infected L-929 cell as mentioned in the previous section and performed the real-time PCR according to the general procedure [23]. We also used an IF staining to monitor the growth of O. tsutsugamushi. In this staining, human convalescent sera of a scrub typhus patient, which were permitted by the ethics committee (number 255), and anti-human antibody conjugated with AlexaFluor®488 (Life technologies Japan Ltd, Tokyo, Japan) were used. A part of the infected cells were harvested and fixed on a glass slide with ice cold acetone and then the slide was applied for the IF staining according to the previous reports [24].

Antibiotics

Lincomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and ciprofloxacin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used for elimination of mycoplasmas in this study. Kanamycin and gentamycin are routinely used for propagation of O. tsutsugamushi to avoid accidental bacterial contamination in our laboratory because they do not influence O. tsutsugamushi-growth [25].

Elimination of mycoplasmas from O. tsutsugamushi-infected cells with antibiotics

We cultured the contaminated strains of O. tsutsugamushi using L-929 cell in the culture medium containing lincomycin and ciprofloxacin at 100, 10 and 1 μg/ml in 25cm² tissue culture flask, and repeated passages about every seven days. At each passage, the infected cells were harvested. One-third of the harvested cells was used for the next inoculation, another one-third was used for DNA extraction, and the remaining one-third was frozen and stocked. Elimination of mycoplasmas was checked by the nested PCR and/or real-time PCR. The growth of O. tsutsugamushi was monitored by the real-time PCR and/or the IF staining.

Table 4 Primers and probes for detection and sequencing in this study

| Targets | Assay | Name | Primers and probes |
|---------|-------|------|---------------------|
| **Mycoplasmas** | | | |
| tuf gene | real-time PCR | Mollicutes 414F | 5'-TCCAGGWCAYGCTGACTA-3' |
| | | Mollicutes 541R | 5'-GTTTWWGAACKCCWACTTG-3' |
| | | Probe 451Fa) | 5'-GCTGTCGCAACAGATTGG-3' |
| tuf gene | Sequencing 1st | Myco-tuf-F1 | 5'-HATHGGCCCAYRTTGAYCAYGGKAAAA-3' |
| | | Myco-tuf-F2 | 5'-ATGATYACHGDDGCWGCHCAAATGGA-3' |
| | Sequencing 2nd | Myco-tuf-R1 | 5'-CCRCCTCRCGRATDGAGAAYT-3' |
| | | Myco-tuf-R2 | 5'-TKRTGACGDCACCCTTCYCTC-3' |
| 16s-23s rRNA intergenic spacer region | nested PCR 1st | MCGpF11 | 5'-ACACCATGGGGAGYTGTTAAT-3' |
| | | R23-1R | 5'-CTTCTTAGCGAAAGSACATY-3' |
| | nested PCR 2nd | R16-2 | 5'-GTGSGMTGGATGACCTCTCCT-3' |
| | | MCGpR21 | 5'-GCATCCACCAWAWACYTCT-3' |
| **Orientia tsutsugamushi** | | | |
| 47kDa common antigen coding gene | real-time PCR | Ots-47k-F | 5'-AATTCTGTGTTGTGTATTGTTAATG-3' |
| | | Ots-47k-R | 5'-AGCAATCCACATTGCTG-3' |
| | | Ots-47k-P b) | 5'-GTCTTAATGATCTTACCTCCAAATT-3' |

a) Locked nucleic acid (LNA) bases (underlined) and was synthesized with the fluorescent reporter 6-carboxyfluorescein (FAM) covalently coupled to the 5' end and a dark quencher to the 3' end.

b) TaqMan probe was synthesized with the fluorescent reporter 6-carboxyfluorescein (FAM) covalently coupled to the 5' end and a dark quencher to the 3' end.
Additional files

Additional file 1: Decontamination of a mycoplasma-contaminated, high-virulent strain of Orientia tsutsugamushi (Ikeda strain) by repeated passages with antibiotics.

Additional file 2: Decontamination of a mycoplasma-contaminated, low-virulent strain of Orientia tsutsugamushi (Kuroki strain).

Competing interests
All authors declare that they have no competing interest.

Authors' contribution
MO carried out the entire part of this study. TU carried out DNA sequences of O.tsutsugamushi mycoplasmas and O.tsutsugamushi PCR assay. All authors read and approved the final manuscript.

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