Mutagenicity test of *Streptomyces Species* culture filtrate using mouse lymphoma cells

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Abstract: Introduction: *Streptomyces* species culture was isolated and identified. Its filtrate was found to be able to damage *Odontoglossum ringspot virus* and *Cymbidium mosaic virus* coat protein in our previous study. It is absolutely necessary to establish its biohazard level and toxicological profile before this filtrate or its active compound can be used for immediate disinfection of the equipment and bench surface, or even technician's skin. Aim: In this study we intend to test the mutagenicity of this culture filtrate using the mouse lymphoma assay (MLA).

Material and methods: *Streptomyces* species was cultured for 14 days, and the medium was collected for XTT assay to determine the highest concentration of the culture filtrate that do not cause reduction in cell number. *In vitro* mammalian cell gene mutation test using Mouse lymphoma L5178Y TK⁺/- was used to evaluate the mutagenic potential of the culture filtrate in accordance with OECD (1997), test No. 476: *in vitro* Mammalian Cell Gene Mutation Test. Result: Our cytotoxicity assay revealed that 1.33% of the culture filtrate was the maximal concentration that would not inflict reduction in cell number. The result of mutation test showed that with 3-hour treatment and S9 metabolic activation, the culture filtrate did not show significant mutagenicity when compared to the negative controls (ANOVA and Student t-test, p<0.05). Summary: This data suggested that the culture filtrate is not mutagenic.

1. Introduction

Many viruses that infect economic crop are transmitted through mechanical wound as well as insect bites to the plant surface. Some virus species are very stable and can survive for a very long period of time on farm tools. We proposed to use a sterilizing material of microbial source to remove the contaminating virus in the environment, preferably the handling surface or on the orchid [1].

We previously discovered that a *Streptomyces species* culture filtrate was able to destroy *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CYmMV) virus coat protein. The initial test discovered that the concentrated filtrate could destroy the infectivity of both viruses to the orchid within one minute [1, 2]. The active ingredient was possibly a protein, as it was sensitive to temperature higher than 70℃ (2019, August, C.A. Chang, personal communications). The activity was stable for up to 6 months storage at 4℃. The virus eradication was not restricted to ORSV and CymMV, as the filtrate was able to reduce the infectivity of other 13 viruses [2].

In the past, heat inactivation, chemical treatment such as bleach or alkaline solution were used to treat virus contaminated equipment and benches. These chemical possess environmental risk or is impractical to use for all possible surfaces, which includes plants, animals and humans. They were also impossible to use on living plants, nor were they ideal for use on the skin of human. Our proprietary agent was active within 1 minute of contacting virus [1], and therefore is suitable to use for immediate
disinfection of solid surfaces or biological tissues. To commercialize this agent, the knowledge of its biohazard level is needed. In our preliminary test this agent did not yield immediate obvious cytotoxic effect. However, for future application for long term and in close contact with human, genotoxicity should be considered. In this paper we report the mutagenicity of our proprietary *Streptomyces* species culture filtrate by the mouse lymphoma assay (MLA) [3, 4].

2. Materials and Methods

2.1. Preparation of the test agent
*Streptomyces* spp. was cultured in a soybean based medium for 14 days, and the medium was collected, filtered through 0.22µm membrane (Amicon). Control treatment was prepared by using the bacterial culture medium incubated at 37°C for 14 days without inoculation with the bacteria.

2.2. MLA culture
Mouse lymphoma L5178Y (TK+-/-) cell line purchased from ATCC Taiwan was used for testing mutagenicity. The cells were cultured in RPMI 1640 supplemented with 10% horse serum (HS), Pluronic F-68 1 mg/mL (Sigma), 200 µg/mL sodium pyruvate, 2mM L-glutamine, and 100 units/mL of penicillin and streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO₂ in air atmosphere at 37 °C.

2.3. Cytotoxicity assay
The XTT assay was performed using the Cell proliferation kit (Biological Industries). The cells (2*10⁴ cells/well in 96-well plates) were transferred to serum free medium for 24 hours, before treated with test filtrate, control soybean medium for 24 h followed by treatment with XTT solution according to manufacturer’s suggestion. After gentle agitation the absorbance was measured at 500 nm (630 nm for background reading) with a microplate reader (SpectraMax 250, Molecular Device, Sunnyvale, CA, USA). The results are expressed as the percentage of XTT reduction relative to the absorbance of the control cells.

2.4. Mutagenicity assay using mouse lymphoma cells
The mutagenicity test consists of 3 steps; Cell cleansing, Treatment and Mutagenicity testing. The complete protocol was illustrated in Figure 1.

2.4.1. Cell cleansing: The cleansing procedure is carried out on two consecutive days. The cells are first treated with THMG (thymidine, hypoxanthine, methotrexate, glycine) solution to cleanse the culture of spontaneous TK -/- mutants, and treated by THG in the following day.

2.4.2. Treatment of MLA cells with test agent and expression of phenotype: The cells were exposed to the test agent (the *Streptomyces* spp. culture filtrate). A negative control was prepared using cells without treatment to the test agent. They were cultured in duplicate plate with 1% S9 (Rat liver S9, aroclor-1254-induced, Moltox) for metabolic activation and 5% HS for 3 hours, before they were plated without the test reagent and S9 activation in RPMI+10 % HS for 48 hours to observe expression of phenotypes. Cells (1.6 cells/well) were plated in 96 wells for 2 plates at the end of test agent treatment for the relative survival test. Cells were counted and re-inoculated at 6x10⁵ cells/mL (6-10⁵/dish) at 24 and 48 hours of expression time for determination of suspension growth.

2.4.3. Viability test and mutation frequency test: At the end of the 48 hours the cells were plated at 1.6 cell/well in 96 wells for 2 plates, for the viability test. In another test 2000 cells were plated per well (1*10⁴ cells/mL) in 96 wells in RPMI supplemented with 10% HS and 4 µg/mL 5-trifluorothymidine (TFT) (Sigma), to test the TFT resistance and mutation frequency. Both cells plated for viability and mutation frequency were cultured for 13 days, and the viable clones within each well were counted.
3. Results
The lowest observed adverse effect level for the culture filtrate treated Mouse lymphoma L5178Y TK+/- cells was between 1.33%-3.33%.

*Streptomyces* spp from single colony was cultured for 14 days and used as test agent. Mouse lymphoma L5178Y (TK+/-) cells were cultured in 0.2% serum containing medium, and test agent was added for 24 hours, and the cell survival was tested using XTT assay. The survival rate was calculated as the percentage to the control, 1.0=100%. The cells survived well with 1.33% fresh bacterial culture medium (98%), while with 3.33% cell survival was 87%. This is regarded as the starting concentration which may cause cell damage (Figure 2).

Figure 1: Overview of the mutagenicity assay.

Figure 2: The lowest observed adverse effect level.
3.1. MLA report

3.1.1. Empty wells (EW): After 3 hours of test agent treatment, and expression time of 2 days, at the end of 13-day culture in TFT, the wells containing viable clones were identified by naked eye and the number was recorded precisely. The 1.6 cell/well plated for survival, and 1.6 cell/well plated at the end of 2-day expression period for viability, were counted as well. The large and small colony were recorded separately. The empty wells were listed in table 1. Two plates were tested for each experiment.

Table 1. Numbers of empty wells (EW) and total wells (TW) in the 3 tests performed for the MLA.

| Parameters | +S9 | Empty wells (EW) | Total wells (TW) |
|------------|-----|-----------------|-----------------|
| 1.6 cell/well (plating for survival test) | Negative control | 31 | 96 |
| Treatment (S9+ culture filtrate) | 33 | 96 |
| 2000 cells/well (TFT resistance) | Negative control | 159 | 192 |
| Treatment (S9+ culture filtrate) | 157 | 192 |
| 1.6 cell/well after 2 day expression (viability) | Negative control | 1 | 192 |
| Treatment (S9+ culture filtrate) | 1 | 192 |

3.1.2. Determination of mutant frequency: The mutant frequency (MF) for the culture filtrate treated group and the negative control (treated with cultured medium without bacterial inoculation same as the treated group) was calculated as follows. MF was expressed as 'mutant per 10^6 viable cells'. It was calculated as plating efficiency of mutants divided by plating efficiency of viable cells as described below,

\[ MF = \left( \frac{PE(\text{mutant})}{2000} \right) \times 10^6 \]

2000 cells were plated per well for 5-trifluorothymidine resistance while 1.6 cells were plated per well for viability. PE (mutant)=p(mutant)/2000, and PE(viable)=P(viable)/1.6. In each case, \( P=-\ln(EW/TW) \) (EW=empty wells, TW=total wells). Therefore,

\[ MF = \left[ \frac{P(\text{mutant})}{2000} \right] \times \left[ \frac{1.6}{P(\text{viable})} \right] \times 10^6 = \left[ -\ln[EW/TW(\text{mutant})] / +\ln[EW/TW(\text{viable})] \right] \times 800. \]

3.1.3. Statistics for mutant frequency: The mutant frequency for the treated group and the negative control were calculated using raw data listed in table 1, was listed in the following table. They were not significantly different (pair-wise Student t test, \( P=0.29\), two tails, method 1)

For calculating the mutant frequency (MF) for the treated group, the PE for mutant is calculated as \( p(\text{mutant})=\ln \left( \frac{EW}{TW} \right) \) mutant/2000, where \( \ln \left( \frac{EW}{TW} \right) \) is listed as in table 2
Table 2. Calculating the ln(EW/TW) for mutant.

| Empty wells (EW) | Total wells (TW) | ln(EW/TW) |
|------------------|------------------|-----------|
| 167              | 192              | -0.13950156 |
| 157              | 192              | -0.201249567 |
| 158              | 192              | -0.194900339 |

The PE for viable cells is calculated as p(viable)/1.6= -ln (EW/TW)/1.6, where ln(EW/TW) is listed in table 3.

Table 3. Calculating the ln (EW/TW) for viable.

| Empty wells (EW) | Total wells (TW) | ln(EW/TW) |
|------------------|------------------|-----------|
| 1                | 192              | -5.257495 |
| 1                | 192              | -5.257495 |
| 1                | 192              | -5.257495 |

The mutant frequency was calculated as ln(EW/TW)mutant/ln(EW/TW)/2000*1.6*10^6 (Table 4).

Table 4. Mutant frequency for the treated cells.

|            | Exp 1 | MF   |
|------------|-------|------|
|            | 1     | 21.22|
|            | 2     | 30.62|
|            | 3     | 29.66|

For calculating the mutant frequency (MF) for the negative control group, the PE for mutant is calculated as p(mutant)/2000= -ln (EW/TW) mutant/2000, where ln (EW/TW) is listed in table 5.

Table 5. Calculating the ln (EW/TW).

| EW     | TW     | ln(EW/TW)  |
|--------|--------|------------|
| 159    | 192    | -0.18859117|
| 153    | 192    | -0.227057451|
| 159    | 192    | -0.18859117|

The PE for viable cells is calculated as p(viable)/1.6= -ln (EW/TW)/1.6, where ln(EW/TW) is listed in table 6.

Table 6. Calculating the ln(EW/TW) viable.

| EW | TW | ln(EW/TW) |
|----|----|-----------|
| 1  | 192| -5.257495 |
| 1  | 192| -5.257495 |
| 1  | 192| -5.257495 |
The mutant frequency was calculated as $\ln(\text{EW/TW})_{\text{mutant}}/\ln(\text{EW/TW})_{2000\times1.6\times10^6}$ (Table 7).

**Table 7.** Mutant frequency for the negative control.

| Exp | MF  |
|-----|-----|
| 1   | 28.70 |
| 2   | 34.55 |
| 3   | 28.70 |

3.1.4. **Calculation of plating efficiency:** The plating efficiency (PE) for the treated group (culture filtrate) and the negative control (treated with cultured medium without bacterial inoculation same as the culture filtrate group) are calculated using raw data in table 1 as follows. The PE in any given test was

$$\text{PE} = \frac{P}{\text{number of cells plated per well}}.$$  

For Day 2, an average of 1.6 viable cells per well were plated, therefore

$$\text{PE} = \frac{P}{1.6} = \frac{-\ln(\text{EW/TW})}{1.6}$$

3.1.5. **Statistics for plating efficiency:** The average plating efficiency for the treated is 66.16%, and for the negative control is 72.23%. Both are larger than 65%, which is an acceptance criterion for the MLA. The plating efficiency was not significantly between the two sample (Student t test, $p=0.24$) (Table 8).

**Table 8.** The plating efficiency.

| Plating efficiency | Treated | Negative control | t-test |
|--------------------|---------|------------------|-------|
| 1                  | 66.74   | 70.64            |       |
| 2                  | 68.66   | 66.74            |       |
| 3                  | 63.06   | 79.28            |       |
| Average            | 66.15   | 72.22            | 0.2389 |

4. **Discussion**

As the MLA tests indicated, the culture filtrate collected from the *Streptomyces* species used at 1.33% concentration, which falls within the lowest observed adverse effect level, did not yield a mutant frequency different to that of the non-treated one (Student t test, $p=0.39$). This suggested that at this given concentration the filtrate will not give more mutant than the spontaneous mutation that was observed for this cell line. Spontaneous mutation is common for any given cell lines, therefore a mutation rate similar to the spontaneous mutation will not be explained as a tendency for cytotoxic mutation. This suggested that the bacterial culture filtrate we obtained is not cytotoxic, and has the potential to develop into a product that could be applied on human skin.

OECD requires the MLA report to fulfil the following criteria for the mutation test to be considered valid. Firstly, the mean mutant frequency in the negative control culture fall within the normal range (approximate 20-200 mutants per $10^6$ viable cells but not more than threefold the historical mean value). Secondly, the positive control chemical induces a clear increase in mutant frequency. Third, the absolute cloning efficiencies (% absolute CE, i.e. Day 2 plating efficiency of viability) of the negative control obtained from the mutant selection must be greater than 65%.

For the first criteria, our data showed that the mean mutant frequency of the negative control fell within the normal range. As demonstrated in table 3 and the calculation for it, for the 3 tests we have
done, the mutant frequencies are 28.70, 34.55, and 28.70. These satisfy the normal range of approximate 20-200 mutant per 10^6 viable cells as the first criteria. As we are not routinely doing the MLA test, there was not historical data for comparison. However, our numbers were in the lower side of the normal range, and it is highly unlikely that they would be more than three folds of the established historical mean value had we have accumulated them.

For the third criteria, the absolute cloning efficiencies (% absolute CE, i.e. Day 2 plating efficiency of viability) of the negative control obtained from the mutant selection must be greater than 65%. This is calculated by the day 2 plating efficiency of the viability. Our data and calculation is presented in table 4. The average plating efficiency of viability for the 3 experiments was 72.22%, which was greater than 65%, and therefore fulfilled this criterion.

Unfortunately, we didn’t do a positive control to induce a clear increase in mutant frequency. For this culture filtrate to be developed as a product, we need to submit mutagenicity tests that followed OECD regulations. Our lab is not certified to do so, therefore it was not our intention to do the proper test in this project. Rather, it was a pre-test for the future buyer of the patent or the right for this culture filtrate to evaluate whether to spend money on the test, which is not cheap. This aside, the different treatments were done in the same experiments and were using the same condition, therefore the comparison of culture filtrate tested and the negative control results were still valid.

Our tests provide evidence to support a notion that, under the similar condition, the mutagenicity of the filtrate treated cells were not different from the negative control cells, which suggests that the culture filtrate is not mutagenic. For this culture filtrate to be developed as product it still needs a certified lab to carry out a procedure with proper positive control to meet the OECD criteria, and our experiments suggest that this filtrate has the potential to be invested and developed into product.

5. Conclusion
In conclusion, our MLA test was performed to meet two criteria out of the three OECD requirement. Under the same condition the bacterial culture filtrate did not give significantly different result to that of the negative control for the mutagenicity test.

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