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(54) Title: *LAWSONIA INTRACELLULARIS* PROPAGATION

(57) Abstract: The present invention relates to the use of cell lines for growing *Lawsonia intracellulars* bacteria, to methods for the attenuation of Lawsonia intracellulars bacteria and methods for growing *Lawsonia intracellulars* bacteria.

Lawsonia intracellularis propagation.

The present invention relates to the use of cell lines for growing *Lawsonia intracellularis* bacteria, to methods for the attenuation of *Lawsonia intracellularis* bacteria and methods for growing *Lawsonia intracellularis* bacteria.

Porcine proliferative enteropathy (PPE or PE) has become an important disease of the modern pig industry world-wide. The disease affects 15% to 50% of the growing herds and up to 30% of the individual animals in established problem herds. Today annual economical losses have been estimated US$ 5-10 in extra feed and facility time costs per affected pig. PPE is a group of chronic and acute conditions of widely differing clinical signs (death, pale and anemic animals, watery, dark or bright red diarrhea, depression, reduced appetite and reluctance to move, retarded growth and increased food conversion rate). However there are two consistent features. The first, a pathological change only visible at necropsy, is mainly a thickening of the small intestine and ileum mucosa. The second is the occurrence of intra-cytoplasmic small-curved bacteria in the enterocytes of the affected intestine. These bacteria have now been established as the etiological agent of PPE and have been name *Lawsonia intracellularis*.

Over the years *Lawsonia intracellularis* has been found to affect, next to pigs, a large group of animals including monkeys, rabbits, ferrets, hamsters, fox, horses, and other animals as diverse as ostrich and emoe. *Lawsonia intracellularis* is a gram-negative, flagellated bacterium that multiplies in eukaryotic enterocytes only and no cell-free culture has been described. In order to persist and multiply in the cell *Lawsonia intracellularis* must penetrate dividing crypt cells. The bacterium associates with the cell membrane and quickly enters the enterocyte via an entry vacuole. This then rapidly breaks down (within 3 hours) and the bacteria flourish and multiply freely in the cytoplasm. The mechanisms by which the bacteria cause infected cells to fail to mature, continue to undergo mitosis and form hypoplastic crypt cells is not yet understood.

The current understanding of *Lawsonia intracellularis* infection, treatment and control of the disease has been hampered by the fact that *Lawsonia intracellularis* can not be cultivated in cell-free media.

As a consequence, for the propagation of *Lawsonia intracellularis*, one has to rely on cell culture methods. Since *Lawsonia intracellularis in vivo* grows in epithelial cells and is very selective

with regard to its host cells, the bacterium is usually grown in pig epithelial cells, swine intestinal epithelial cells, rat IEC-18 epithelial cells, Intestine 407 human embryonic intestinal epithelial cells or in GPC-16 guinea pig colonic adenocarcinoma epithelial cells. All these cells are anchorage-dependent: they need a substrate to attach to so they can only be grown if a substrate is available. HEp-2 cells, again epithelial cells and originating from a human larynx carcinoma can be infected as monolayer cells and can thereafter be grown in suspension.

All cells mentioned above have their own difficulties and their own unusual and specific growth requirements.

But in addition to their demanding growth requirements, they all share the characteristic of being intestinal epithelial cells. This may be an advantage in the sense that epithelial cells are the target cells of *Lawsonia intracellularis* and therefore bacteria will easily grow on these cells.
This advantage however turns out to be a disadvantage if one wants to select for live attenuated *Lawsonia intracellularis* cells: serial passaging of bacteria on their target cells hardly gives any attenuation over time. Passaging over non-target cells leads to relatively quick adaptation of the bacteria to the new cells. Such bacteria are consequently less adapted to the original target cell. Such bacteria will need to re-adapt to the intestinal epithelium when administered to their in vivo host animal, e.g. the pig, and thus they will by definition behave in an attenuated manner, at least during the first few infection cycles. This leaves the immune system time to build up and remove the bacteria before they become fully pathogenic again.

A serious problem however is that there are hardly any non-epithelial cells known to sustain the growth of Lawsonia. It is easy to understand why: *Lawsonia intracellularis* attaches and invades only its target cell: the (intestinal) epithelial cell. If it does not recognize a cell as (intestinal) epithelial cell, it will not attach in the first place. The only exception to that rule found so far are McCoy cells, cells that were shown to sustain *Lawsonia intracellularis* growth although they are of mouse fibroblast origin.

Therefore, there clearly is a need for other non-epithelial cells that sustain the growth of *Lawsonia intracellularis* bacteria, for reasons of easier production and selection of live attenuated bacteria, or even for the purpose of growing *Lawsonia intracellularis* bacteria under less demanding conditions in the first place.

It is an objective of the present invention to provide new cell lines that are not of epithelial origin, that can be used for growing *Lawsonia intracellularis* bacteria.

It was surprisingly found now, that *Lawsonia intracellularis* bacteria can very efficiently be grown on non-epithelial ferret tumor cells.

Cell lines on the basis of non-epithelial ferret cells can be obtained along various routes known in the art. A well-established method is the growth of ferret tissue in the presence of high doses of mutagenic agents. Cells that lose their controlled life cycle will immortalize, whereas non-mutated cells will die after several divisions. Serial passaging of cells that were subjected to mutagenesis therefore auto-selects for immortalized cell lines.
There is however an attractive, quicker alternative to this method: the use of non-epithelial ferret tumor tissue as a starting material for the isolation of immortalized cells provides a quicker way of obtaining cell lines.
This option is the preferred option for the development of new cell lines. Raw non-epithelial ferret tumor tissue however comprises several different cell types, including non-tumor cells. Therefore, cells obtained from this kind of tissue will have to be passaged several times in order to get rid of non-immortalized cells. After serial passage for at least ten, preferably more, such as twenty or more times, non-immortalized cells will practically be lost. From that moment on, individual cells can be picked and submitted to further serial passaging.

It goes without saying that if ferret tumor tissue is used as the starting material for a ferret cell line according to the invention, this tissue should preferably not be of intestinal epithelium tumor origin. A ferret cell line originating from a ferret skin tumor would however be a suitable cell line. A method for selecting and growing ferret cell lines, including non-epithelial ferret cell lines is described in PCT/EP/2008/064804.
Another Ferret cell line is i.a. described, by Trowbridge, R.S. et al. (In Vitro Vol. 18: 952-960, 1982). This cell line is available through the American Type Culture Collection under number ATCC CRL 1656. This cell line; Mpf, originates however from a brain cell, and thus is clearly of non-epithelial origin and therefore not expected to support *Lawsonia intracellularis* growth.

Surprisingly it was found now, that non-epithelial ferret cell lines in general, including the Mpf cell line, are very well capable of supporting *Lawsonia intracellularis* growth.

They also offer a significant advantage over growth on McCoy cells, if only because of their undemanding growth requirements.

A first embodiment of the present invention therefore relates to the use of a non-epithelial ferret cell line for growing a *Lawsonia intracellularis* bacterium.

Preferably, the ferret cell line Mpf is used for growing the *Lawsonia intracellularis* bacterium.

Therefore, a preferred form of this embodiment relates to the use of a ferret cell line for growing a *Lawsonia intracellularis* bacterium, wherein that ferret cell line is an Mpf cell line.

It was also found that ferret cell lines other than the Mpf cell line, such as the cell lines that were derived from ferret tumor tissue, and that have chromosome numbers of 2N = 40 (the standard chromosome number of ferret cells), 2N = 38 and 2N = 36 are also suitable for propagating the *Lawsonia intracellularis*.

Therefore, another preferred form of this embodiment relates to the use of a ferret cell line for growing a *Lawsonia intracellularis* bacterium, wherein said ferret cell line has a chromosome number of 2N = 40, 2N = 38 or 2N = 36.

Finally it was unexpectedly found that, since ferret cells evidently are not of porcine origin, they are ideally suited for the attenuation of *Lawsonia intracellularis*. *Lawsonia intracellularis* grown on ferret cells becomes ferret-cell adapted during passaging and therefore loses its specific adaptation to porcine cells. This loss of specific adaptation to its natural target cell provides a very suitable means of attenuating *Lawsonia intracellularis*.
Therefore, another embodiment of the present invention relates to methods for attenuating *Lawsonia intracellularis* bacteria, wherein that method comprises the serial passaging of said bacterium on a non-epithelial ferret cell line. Preferably, the ferret cell line is the Mpf cell line or a ferret cell line that has a chromosome number of 2N = 40, 2N = 38 or 2N = 36.

Still another embodiment relates to methods for growing *Lawsonia intracellularis*. Such methods comprise the steps of infecting a non-epithelial ferret cell line with said *Lawsonia intracellularis* followed by harvesting the progeny bacteria.

In a preferred form of this embodiment, the ferret cell line is an Mpf cell line or a ferret cell line that has a chromosome number of 2N = 40, 2N = 38 or 2N = 36.

EXAMPLES.

Example 1

Preparation of cells from a ferret tumor

Skin tumor material was obtained from ferrets.

Fat and dead tissue were removed from the tumors and the remaining tissue was cut into small pieces and placed into 100 ml pet-flasks.

Tissue samples were washed 3 times with PBS phenol red as follows: 50 ml PBS was added to the tissue samples and the pet-flask was softly stirred. Thereafter, tissue samples were allowed to settle under 1*gravity and PBS phenol red was carefully decanted.

After washing, 50 ml PBS phenol red with 0.1% trypsin and 0.02% EDTA was added to the tissue samples. The tissue samples were stirred for 5 minutes at 37^0C. Tissue lumps were allowed to settle under 1*gravity and supernatant was decanted into a falcon tube with 2 ml FCS (Biochrom).

This procedure was repeated 3 times, The supernatants were pooled and spun down for 10 minutes at 200xg at room temperature. The supernatant was decanted and the cell pellet was resuspended in 5 ml medium (RPMI 1640 (Gibco), 10% fetal calf serum (Biochrom), penicillin/streptomycin. The number of cells was counted with a Burker-Turk counting chamber. A total number of 10^4 cells per cm^2 were plated in a culture flask.

Cell growth was screened daily and when a confluence of 80-90% was achieved, the cells were trypsinized and propagated as described below.

Cultivation and propagation of cells

Cells were seeded onto a culture flask in a concentration of 10000 cells per cm^2 in RPMI medium with 10% FCS and penicillin/streptomycin and incubated at 5% CO_2 and 37°C until a confluence of 80-90% was achieved, then the cells were passed by trypsinization as follows: cells were

washed twice with PBS phenol red. Thereafter, 5 ml PBS with 0.1% trypsin and 0.01% EDTA was added in such a way that the bottom was fully covered. After decanting this solution, the culture flasks were incubated for 5-10 minutes at 37°C . Detached cells were resuspended in 10 ml medium en spun down for 5 min at 200*g. 10 ml of medium was added and cells were brought in suspension and seeded onto a new culture flask in a concentration of 10000 cells/cm^2. 75% fresh medium and 25% conditioned medium (1000xg 10 min) from the harvested cells was added.

After ±20 passages no further conditioned medium was added to the fresh medium.

After 40 passages, stable cell lines were obtained that did not change in growth characteristics or karyotype.

conclusion

Stable cell lines originating from tumor cells were established.

Example 2

This example describes how a second ferret tumor cell line was made. For the preparation of cells, the procedure as describe in Example 1 was repeated. Growth conditions and media were slightly changed as described below.

Cultivation and propagation of cells

Cells were seeded onto a culture flask in a concentration of 20000 cells per cm^2 in medium free of animal components, in the presence of penicillin/streptomycin and incubated at 5% CO_2 and 37°C until a confluence of 80-90% was achieved, then the cells were passed by trypsinization. Cells were washed twice with PBS phenol red. Thereafter, 5 ml PBS with 0.1% trypsin and 0.01% EDTA was added in such a way that the bottom was fully covered. After decanting this solution, the culture flask was incubated for 5-10 minutes at 37°C . The detached cells were resuspended in 4 ml soybean trypsin inhibitor solution and spun down for 5 min at 200xg. 10 ml of animal component free medium was added and cells were brought in suspension and seeded onto a new culture flask in a concentration of 20000 cells/cm^2.

Example 3

Cultivation of cells

Mpf cells (ATCC CRL 1656) and McCoy ATCC CRL 1696 were obtained from the American Tissue Culture Collection. The cells were sub-cultured using 75 cm^2 flasks. They were incubated in cell growth medium MEM with 5% Fetal Calf Serum, without any antibiotics in atmospheric conditions in 5% CO_2 at 37°C. At day 0, one day before infection, cells were seeded in 25 cm^2 (T25) flasks at 1.0 x 10^4 cells/ml.

Infection with Lawsonia

At day 1, cells were infected with $10^{3.8}$ TCID50 of *Lawsonia intracellularis*. The medium was poured of the cells, 1 ml of *Lawsonia intracellularis* suspension containing $10^{3.8}$ TCID50 was put onto the cells and incubated for 1 hour at 37°C in atmospheric environment. Subsequently 4 ml medium was added to the inoculated cells. T25 flasks were incubated for 7 days at 37°C in a special micro-aerophilic system made by an airtight jar containing Campypak Plus Micro-aerophilic System Envelopes.

Subculture of Lawsonia in Mpf and McCoy cells

At day 6 Mpf and McCoy cells were seeded again in T25 flasks at 1.0 x 10^4 cells/ml. Lawsonia was harvest at day 7. The medium comprising *Lawsonia intracellularis* cells was collected, the cells were freeze-thawed twice, cells were scratched from the bottom of the T25 flasks, and the cell suspension was added to the medium. 1 ml of the harvest was used for infection of the freshly seeded cells and incubated at 37°C for one hour. Subsequently 4 ml of fresh medium was added to the infected cells. T25 flasks were incubated for 7 days at 37°C in a special micro-aerophilic system made by an airtight jar containing Campypak Plus Micro-aerophilic System Envelopes.

Titration of Lawsonia and IFA

Cells were seeded in microtiterplates in a concentration of 20000 cells/ml in MEM medium with 5% FCS without antibiotics, 100 µl/well. After one day the cells were inoculated with 10 fold dilutions of a *Lawsonia intracellularis* suspension.

The dilutions were made in MEM-medium with 5% FCS, per dilution 100 µl was added to 10 wells each. The plates were incubated at 37°C and micro aerophilic environment. After 7 days the cells were fixed with ice cold 96% alcohol.

An immune fluorescence assay (see below) was performed using a polyclonal anti *Lawsonia intracellularis* and an anti mouse antibody conjugated with fluorescin isothiocynate (FITC) (Nordic). Fluorescence was monitored with a UV microscope. The $TCID_{50}$/ml was calculated using Reed and Münch method.

Immune Fluorescence Test:

After 7 days of incubation the supernatant was removed, and the cells were fixed with 96% ethanol at 70°C. To visualise the virus for the titration, the plates were stained with a specific polyclonal antiserum against *Lawsonia intracellularis*. Therefore, the plates were adapted to RT, the alcohol was poured off and the plates were washed with PBS. A working dilution of a polyclonal antibody against *Lawsonia intracellularis* was prepared at a strength which was known to give good fluorescence, but little background. This first antibody was then brought onto the plates and the plates were incubated for 1 hours at 37°C in a moist atmosphere. The plates were then washed 3x with PBS. Then the second antibody-conjugate was prepared, a goat anti-mouse IgG-FITC conjugate (Nordic™). This was brought onto the plates in the required dilution, and was incubated again for 1 hour at 37 °C. After this incubation the plates were washed 3x with PBS, after which a 1:1 mixture of PBS:glycerol was added. Plates were then stored in the dark at 4°C until reading. Fluorescence was monitored with a UV microscope. Per dilution the amount of positive wells were counted. The $TCID_{50}$/ml was calculated using Reed and Münch method.

Results:

Table 1 shows the titers (TCID50) in 10 log/ml of the inoculum, the first, the second and the third passage of Lawsonia. For the inoculum and first passage the titers are comparable.

Titrations of harvest of Lawsonia grown on Mpf were done with both McCoy cells and Mpf cells. No differences in titers were measured.

	Lawsonia intracellularis grown on	
	McCoy cells	Mpf cells
	$_{10}$log $TCID_{50}$/ml	
inoculum	3.8	3.8
first passage	6.6	6.2

Table 1

Claims

1) use of a non-epithelial ferret cell line for growing a *Lawsonia intracellularis* bacterium.
2) use according to claim 1, characterized in that said ferret cell line is an Mpf cell line.
3) Use according to claim 1 or 2, characterized in that said ferret cell line has a chromosome number of 2N = 40
4) Use according to claim 1 or 2, characterized in that said ferret cell line has a chromosome number of 2N = 38
5) Use according to claim 1 or 2, characterized in that said ferret cell line has a chromosome number of 2N = 36
6) Method for attenuating a *Lawsonia intracellularis* bacterium, said method comprising the serial passaging of said bacterium on a non-epithelial ferret cell line.
7) Method according to claim 6, characterized in that said ferret cell line is an Mpf cell line or a ferret cell line that has a chromosome number of 2N = 40, 2N = 38 or 2N = 36.
8) Method for growing a *Lawsonia intracellularis* bacterium, characterized in that said method comprises the steps of infecting a non-epithelial ferret cell line with said *Lawsonia intracellularis* bacterium and harvesting the progeny bacteria.
9) Method according to claim 8, characterized in that said ferret cell line is an Mpf cell line or a ferret cell line that has a chromosome number of 2N = 40, 2N = 38 or 2N = 36.

INTERNATIONAL SEARCH REPORT

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PCT/EP2010/060019

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20 C12N1/36
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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