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A fast and simple assay to quantify bacterial leukotoxin activity

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ABSTRACT

Background: Mannheimia haemolytica is the primary bacterial pathogen in causing bovine respiratory disease with tremendous annual losses in the cattle industry. The leukotoxin from *M. haemolytica* is the predominant virulence factor. Several leukotoxin activity assays are available but not standardized regarding sample preparation and cell line. Furthermore, these assays suffer from a high standard error, a prolonged time consumption and often complex sample pretreatments, which is important from the bioprocess engineering point of view.
Results: Within this study, an activity assay based on the continuous cell line BL3.1 combined with a commercial available adenosine triphosphate viability assay kit was established. The leukotoxin activity was found to be strongly dependent on the sample preparation. Furthermore, the interfering effect of lipopolysaccharides in the sample could be successfully suppressed by adding polymyxin B. We reached a maximum relative P95 value of 14%, which is more than seven times lower compared to current available assays as well as a time reduction up to 88%.
Conclusion: Ultimately, the established leukotoxin activity assay is simple, fast and has a high reproducibility. Critical parameters regarding the sample preparation were characterized and optimized making complex sample purification superfluous.

1. Introduction

Mannheimia haemolytica is the primary bacterial pathogen causing bovine respiratory disease (BRD) and its primary virulence factors are leukotoxin (LKT) and lipopolysaccharides (LPS) [1,2,3].

The LKT of *M. haemolytica* belongs to the repeat-in-toxin (RTX) family [4,5,6]. All serotypes of *M. haemolytica* produce a 102–105 kDa heat labile LKT during the logarithmic phase of growth [2]. In contrast to other RTX toxins the LKT of *M. haemolytica* is specific for ruminant leukocytes and the cytotoxicity is limited to ruminant lymphocytes, macrophages, neutrophils and platelets due to the specific expression of β2 integrins as a binding partner for the LKT [2,3,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20]. Especially, the leukocyte function associated antigen 1 (LFA1) is involved in causing the leukotoxic effect. LFA1 is a heterodimer compound of a CD11a and a CD18 subunit. Binding of LKT to both subunits causes the highest cytotoxic effect [9,14,16,19,20]. The effect of LKT is strongly dose-dependent [4]. Low concentrations activate neutrophils and macrophages, induce the release of histamine by mast cells and inhibit the mitogen mediated lymphoid proliferation [2]. The consequences are respiratory burst, degranulation and release of pro inflammatory cytokines (tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-8 (IL-8)). High concentration causes apoptosis of bovine leukocytes by extrinsic and intrinsic mechanisms and lead to pore formation, cell swelling and ultimately to necrosis [2,10,12].

Besides LKT, LPS is a major actor of the cytotoxic effect. LKT and LPS are the most prominent components in the supernatant of *M. haemolytica* and are able to complex increasing the cytotoxicity compared to native LKT [5,15]. LPS can also bind to β2 integrins whereby the CD18 subunit does not seem essential [18,21]. The spectrum of efficacy of LPS and LKT overlaps. Both can stimulate alveolar macrophages to produce reactive oxygen and nitrogen mediates. Furthermore, LPS can also induce the production of IL-1,

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IL-8, leukotriene 4 and TNF-α, resulting in inflammation and apoptotic cell death [15,22,23,24].

The majority of LKT activity assays are based on the measurement of cytotoxicity. LKT sensitive cells are incubated with various concentrations of the LKT followed by a cytotoxicity assay. In general isolates of peripheral blood monocular cells (PBMCs) from cattle and the bovine B-lymphoblastoid cell line (BL3) are utilized [25,26,27,28,29,30,31]. Currently available cytotoxicity assays for determining the LKT activity are the Chromium-51 release assay [31], lactate dehydrogenase (LDH) release assay [27], neutral red uptake assay [25], 3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphenyl] tetrazolium bromide (MTT) [30] and the nitroblue tetrazolium (NBT) assay [28]. Non-cytotoxicity assays are based on the morphological change of BL3 cells after incubation with LKT and the inhibition of the luminol dependent chemiluminescence of LKT incubated bovine neutrophils [5,10].

All LKT activity assays outlined above suffer from a high standard error and a high time consumption caused by a complex sample pretreatment. The overlapping cytotoxic effects and a molar ratio of LPS/LKT of *ca.* 60:1 in concentrated culture supernatants from *M. haemolytica* place a high demand on the sample preparation to measure just the single effect of LKT [27]. However, assays enabling activity measurements of the pure LKT are beneficial for bioprocess applications. Furthermore, a time and temperature dependent decrease in LKT activity is mentioned in the literature [26,32,33] but remained uncharacterized with respect to activity assays. Therefore, especially the sample preparation could influence the LKT activity leading to a reduced reproducibility and comparability.

This study provides a novel assay for a fast and reproducible determination of the LKT activity. The assay is based on a commercial available adenosine triphosphate (ATP) viability assay kit. Important parameters affecting the LKT activity such as time and temperature throughout the sample preparation were characterized and optimized. In order to repress the interfering effect of LPS the addition of polymyxin B (PB) as a LPS inhibitor was evaluated making further complex and time consuming sample purification superfluous.

2. Material and methods

2.1. Preparation of the LKT activity standard

The LKT was obtained by growth of *Mannheimia haemolytica* (ATCC® 43,270, American Type Culture Collection, USA) in RPMI-1640 medium (R6504, Sigma-Aldrich, Germany) in a 0.5 L stirred tank reactor (MiniBio 500, Applikon, Netherlands) under similar conditions as described previously [34,35,36]. Harvest occurred at the end of the exponential growth phase. The supernatant was centrifuged (5000×*g*, 10 min, 4°C), afterwards filtered through a 0.22 μm bottle top filter (SCGVT05RE, Merck Millipore, Germany) and aliquoted and frozen at -85°C in cryo-vials (72.379, Sarstedt, Germany) [25].

2.2. Preparation of the cell culture

The BL3.1 cells were grown in RPMI-1640 (RPMI 1640 FG 1385, Biochrom, Germany) supplemented with 10% (v/v) FBS (FBS Superior, Biochrom, Germany) at 37°C and 5% CO_2 in T75-flasks (REF 83.3911.502, Sarstedt, Germany) with a working volume of 25 mL. The cells were passaged three days before the activity assay was performed. Cell counts and viability were determined in a hemocytometer using the trypan blue exclusion assay. Criteria for passaging was a cell viability of ≥80%. The cell suspension was diluted to a viable cell density of 0.15–0.30 $*$ 10^6 cells/mL to ensure a high growth rate as well as a high viability.

2.3. General procedure of the ATP assay

Within this study a viable cell density of ≥0.60 $*$ 10^6 cells/mL and a viability of ≥95% was set to be optimal for the assay. The cell suspension was diluted in fresh medium to a viable cell density of 0.60 $*$ 10^6 cells/mL. The LKT activity standard (LKTAS) vial was thawed in a water bath (23°C) until the ice was nearly gone. Afterwards the LKTAS was supplemented with 1% (v/v) of a 5 mg/mL concentrated solution of polymyxin B (Cat# 420,413, Calbiochem, USA) in PBS (Biochrom, Germany) and incubated for 15 min on ice. A twofold serial dilution of the cell suspension (0.60 $*$ 10^6 cells/mL) served as a calibration and RPMI-1640 supplemented with 10% (v/v) FBS was used as a blank. As the positive control 50 μL of a 4% Triton X-100 (Sigma-Aldrich, Germany) solution dissolved in PBS was supplemented with 50 μL of the cell suspension. A twofold serial dilution with PBS was carried out for all samples in a white 96 well plate (Nunc 136102, Thermo Fisher Scientific, Germany) to a final volume of 50 μL/well. Each sample well was then supplemented with 50 μL of the cell suspension and the plate was incubated for 2 h at 37°C and 5% CO_2. Afterwards, 100 μL of the working solution of the viability assay (CellTiter-Glo®, Promega, Germany) was added to each well. The plate was shaken for 3 min and remained for further 10 min in the 30°C prewarmed plate reader (Synergy HT, BioTek Instruments, USA), followed by the luminescence measurement.

The calibration line for cell count was forced through zero and a cell number of 3 $*$ 10^4 cells/well was the upper value of the calibration. The data were linearized by plotting the logarithm of the dilution factor of the sample to the base of 2 on the abscissa according to [Equation 1].

$$Y = \log\left(\frac{{}^{\text{Deathrate}}/_{100\%}}{1-{}^{\text{Deathrate}}/_{100\%}}\right) \qquad \text{[Equation 1]}$$

One unit/mL of LKT activity is defined as the concentration of biological active leukotoxin which causes death of 50% of the target cells. As an alternative calculation for the LKT activity the EC50 of the dose response function in OriginPro 8.5 was tested (data not shown) and rejected because the relative P95 value was higher compared to the linearization method. The P95 value represents the interval of the sample data which covers the true value 95% of the time.

2.4. Optimization of the sample preparation

The assay was carried out as previously described (2.3). To determine the optimal sample preparation various incubations conditions after thawing (10 min at 37°C + 50 min on ice/1 h at 23°C/ 1 h on ice) were evaluated.

2.5. Optimization of the LKT incubation time

The assay was carried out as previously described (2.3). The LKT incubation time was varied in an independent test serial between 1, 2 and 3 h.

2.6. Neutralization of LPS

Two experiments with minor changes from the general procedure of the ATP assay (2.3) were carried out to proof a sufficient neutralization of LPS with PB. For the first experiment the effect of various PB concentrations were evaluated. The incubation time was set to was incubated for 70 min at 37°C for a complete LKT inactivation. Afterwards the standard was split and one half was supplemented with 1% (v/v) PB in PBS at a concentration of 5 mg/mL.

Table 1
Determined LKT units of the mean of triplicates and measurement deviation.

Sample	1	2	3	4	5	6	7	8	9	Averaged rel. P95 value
LKT units/mL	29.5	31.4	32.8	28.8	32.4	34.7	31.2	34.5	35.0	14%

3. Results

3.1. Statistical evaluation of the assay

For the determination of the statistical values, nine samples with the same LKT concentration were analyzed in three independent test series. An averaged relative P95 value of 14% was calculated (Table 1).

3.2. Optimization of the sample preparation

The toxicity of the LKT standard shows a strong sensitivity to the sample incubation condition and time after thawing (Fig. 1). The storage for 1 h on ice did not show a significant reduction of biological LKT activity compared to an immediate use. Nevertheless, a temperature dependent reduction of the LKT activity is especially favored at T ≥ 37°C. The higher the temperature the faster the LKT inactivation.

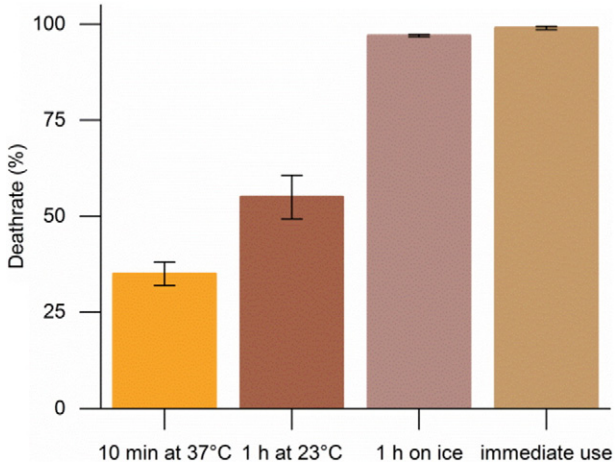

Fig. 1. Temperature and time depended effect of the sample preparation on the LKT activity at a relative LKTAS concentration of 0.25. The LKTAS was either incubated after thawing for 10 min at 37°C, 1 h at 23°C, 1 h on ice prior the general ATP assay procedure or immediately used.

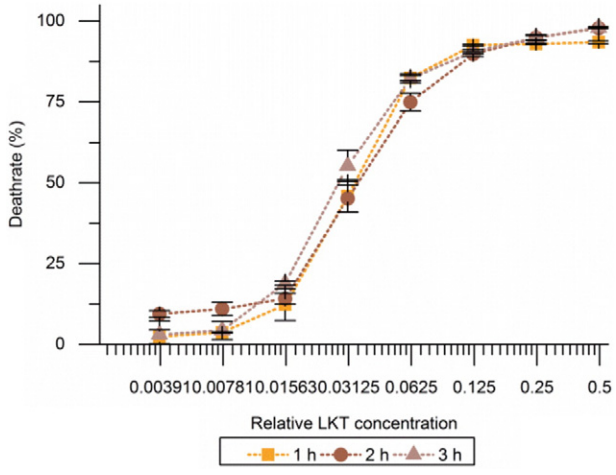

Fig. 2. Incubation time dependent effect on the LKT activity. Throughout the general ATP assay procedure the LKT incubation time was set to 1 h, 2 h and 3 h.

3.3. Optimization of the incubation time

Regarding the incubation time, no differences of the cytotoxicity could be observed between 1 and 3 h (Fig. 2). However, an artificial effect on the maximum death rate of the positive control (Triton X-100) depending on the incubation time could be seen (data not shown). At an incubation time of 1 h the death rate of the positive control was 96% instead of the anticipated 100%. An insufficient degradation of intracellular ATP after cell death could be assumed causing an interference in the viability assay and an inaccurate death rate of 96% is the result. Therefore, an incubation time of 2 h was set to be optimal.

3.4. Neutralization of LPS

All samples supplemented with PB showed no difference to each other (Fig. 3). Consequently, a saturation with PB can be assumed

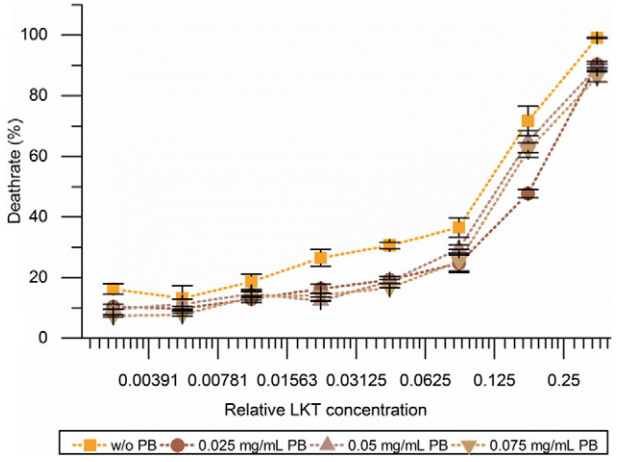

Fig. 3. Effect of different PB concentrations on the LKT activity. Throughout the general ATP assay procedure the LKTAS was supplemented with either 0.025 mg/ml PB, 0.05 mg/mL PB, 0.75 mg/mL PB or without PB.

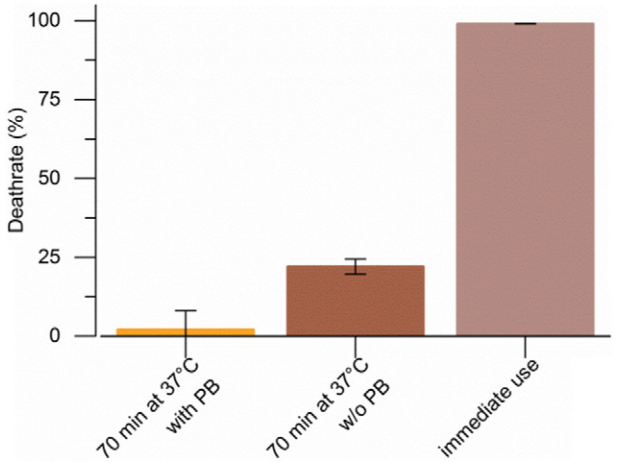

Fig. 4. Effect of different temperature and PB sample pretreatments on the LKT activity at a relative LKTAS concentration of 0.5. Throughout the general ATP assay procedure the LKTAS was incubated for 70 min at 37°C and either supplemented without or with 0.05 mg/mL PB and compared to an immediately used LKTAS.

at concentrations ranging from 0.025 mg/mL to 0.075 mg/mL. A PB concentration of 0.05 mg/mL was selected based on a safety factor to ensure neutralization under conditions with varying LPS concentrations.

A further proof of a complete LPS neutralization through PB can be seen in Fig. 4. The incubation of the LKTAS at 37°C for 70 min led to an inactivation of the LKT [32,37,38] and the remaining cytotoxicity of 22% can be attributed to the LPS. A further supplementation with 0.05 mg/mL PB led to a complete loss of cytotoxic activity. Therefore, a neutralization of LPS could be assumed.

4. Discussion and conclusion

Current available LKT activity assays are based on continuous cell lines and isolated leukocytes. BL3.1 cells have the lowest variability compared to other continuous leukocyte cell lines. The usage of BL3.1 cells makes the extraction of fresh leukocytes superfluous, reducing the effort enormously and ensure a reproducible and high quality of the target cells.

Most important is a standardized sample preparation and assay procedure as demonstrated in this study. Especially, the time and temperature throughout the sample preparation had a strong influence on the LKT activity. A gentle thawing procedure in combination with a sample preparation on ice is mandatory. This leads to a higher accuracy and reproducibility compared to currently available activity assays. We reached a maximum relative P95 value of 14%, which is more than seven times lower compared to previous data [30]. The time consumption for the ATP assay is ~30 min. Compared to the neutral red assay [39] and MTT [30] this corresponds to a reduction of 75% and respectively 88%. A further huge advantage of our established assay is the direct inactivation of LPS with PB, making further complex and time consuming sample purification superfluous.

In summary, the established LKT activity assay is simple, fast and sensitive overcoming all drawbacks of currently available activity assays. A complete automation of the ATP assay is possible [40] making the assay well suited for process monitoring (*e.g.* downstream) for industrial LKT production. A transfer into a 384 well plate format is conceivable for a high-throughput screening system and could further reduce the time consumption and material costs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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