Assessment of *Bdellovibrio bacteriovorus* 109J killing of *Moraxella bovis* in an in vitro model of infectious bovine keratoconjunctivitis

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Abstract

The objective of this study was to determine the potential of *Bdellovibrio bacteriovorus* 109J as an alternative non-chemotherapeutic treatment of infectious bovine keratoconjunctivitis (IBK). To accomplish this, various parameters of *B. bacteriovorus* predation of *Moraxella bovis* were determined in vitro. Initial passage of *B. bacteriovorus* using *M. bovis* as prey required 10 d for active cultures to develop compared with 2 d for culture on normal *Escherichia coli* prey; however by the 5th passage, time to active predatory morphology was reduced to 2 d. This high passage *B. bacteriovorus* culture [1×10^{10} plaque forming units (PFU)/mL] killed 76% of *M. bovis* [1×10^{7} colony forming units (CFU)/mL] present in suspension broth in a 4 h assay. The minimal level of *M. bovis* supporting *B. bacteriovorus* predation was 1×10^{4} CFU/mL. To assess the ability of *B. bacteriovorus* to kill *M. bovis* on an epithelial surface mimicking IBK, an in vitro assay with Madin-Darby bovine kidney (MDBK) cells inoculated with 4×10^{7} CFU/mL *M. bovis* was used. Treatment with a *B. bacteriovorus* suspension (1.6×10^{11} PFU/mL) decreased adherence of *M. bovis* to MDBK cells by 6-fold at 12 h of treatment, as well as decreased the number of unattached *M. bovis* cells by 1.4-fold. This study demonstrates that *B. bacteriovorus* has potential as an effective biological control of *M. bovis* at levels likely present in IBK-infected corneal epithelia and ocular secretions.

Résumé

Cette étude visait à déterminer le potentiel de Bdellovibrio bacteriovorus *109J comme traitement alternatif non-thérapeutique de la kérato-conjonctivite infectieuse bovine (IBK). À cet effet, divers paramètres de prédation de* B. bacteriovorus *envers* Moraxella bovis *ont été déterminés in vitro. Le premier passage de* B. bacteriovorus *utilisant* M. bovis *comme proie nécessitait 10 j pour qu'une culture active se développe comparativement à 2 j pour une culture utilisant* Escherichia coli *comme proie; toutefois, rendu au 5e passage, le temps requis pour obtenir la morphologie de prédateur actif était réduit à 2 j. Cette culture de passage élevé de* B. bacteriovorus *(1×10^{10} unités formatrices de plaques (PFU)/mL) a tué 76 % des* M. bovis *(1×10^{7} unités formatrices de colonies (CFU)/mL) présents dans un bouillon lors d'un essai d'une durée de 4 h. Le nombre minimal de* M. bovis *permettant la prédation par* B. bacteriovorus *était de 1×10^{4} CFU/mL. Afin d'évaluer la capacité de* B. bacteriovorus *à tuer* M. bovis *sur une surface épithéliale imitant IBK, une épreuve in vitro avec des cellules rénales bovines Madin-Darby (MDBK) inoculées avec 4×10^{7} CFU/mL* M. bovis *fut utilisée. Le traitement avec une suspension de* B. bacteriovorus *($1{,}6 \times 10^{11}$ PFU/mL) a réduit l'adhérence de* M. bovis *aux cellules MDBK par un facteur de 6 après 12 h de traitement, et a également diminué le nombre de cellules de* M. bovis *non-attachées par un facteur de 1,4. Cette étude démontre que* B. bacteriovorus *a le potentiel d'être un moyen de réduction biologique efficace de* M. bovis *à des niveaux susceptibles d'être présents sur l'épithélium cornéen et dans les sécrétions oculaires d'animaux infectés par l'IBK.*

(Traduit par Docteur Serge Messier)

Introduction

Infectious bovine keratoconjunctivitis (IBK) is a widespread, severe, contagious eye disease of bovine species that causes significant economic loss worldwide (1–3). Ocular infection of cattle with *Moraxella bovis (M. bovis)* is associated with the development of IBK, a significant treatment problem which may be amenable to a novel non-chemotherapeutic treatment therapy by a predatory bacterium *Bdellovibrio bacteriovorus* 109J *(B. bacteriovorus)*. The clinical manifestations of IBK range from mild unilateral or bilateral conjunctivitis to central corneal ulceration and perforation (4). Bacterial culture collected from ocular secretions of calves with corneal ulcer due to *M. bovis* has been reported to harbor 1×10^{9} to 1×10^{10} *M. bovis* per sample (5). Prerequisites for induction of ocular lesions by *M. bovis* include microbial adhesion to the corneal surface and cytotoxicity, both mediated by several virulence factors. *Moraxella bovis* fimbriae (Q pili) allow for bacterial adherence to the bovine conjunctival mucosa (6). Cytopathic effect of *M. bovis* on bovine corneal epithelial cells (BCEC), neutrophils, and erythrocytes is mediated by a cytotoxic and leukotoxic hemolysin, hydrolytic and lipolytic enzymes, proteases, and collagenases (4,7–9). However, this process can be reversible as the corneal epithelium may regenerate once

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Received July 2, 2010. Accepted October 19, 2010.

ocular *M. bovis* infection is cleared (10). Parenteral administration of oxytetracycline (10,11), florfenicol (12,13), ceftiofur crystalline free acid (14), and tulathromycin (15) have been shown effective in the treatment of experimentally induced or naturally occurring IBK in cattle. However, the predatory bacterium *B. bacteriovorus* administered via ocular instillation may provide an attractive alternative to antimicrobials, especially in case of multidrug-resistant IBK infection. Its use would prevent tissue and milk residues, decrease the opportunity for development of bacterial resistance, and avoid local and systemic side effects associated with antibiotic administration.

The genus *Bdellovibrio* was first described by Stolp and Starr in 1963 as bacteriolytic organisms capable of attacking a living bacterium, attaching to its surface, penetrating the cell wall, multiplying inside the host, and causing lysis of the infested cell (16) within approximately 3.5 to 4 h (17). More recent reviews describe the predatory lifestyle of *B. bacteriovorus* characterized by 2 differentiated cell stages (attack and growth phase) (18–21), as well as methods for its laboratory maintenance (22). *Bdellovibrio bacteriovorus* is a small (0.35 × 1.2 μm), obligate aerobe, motile (polar flagellum) gram-negative bacterium with obligate host-dependency on a wide range of gram-negative prey bacteria. *Bdellovibrio bacteriovorus* are ubiquitous and have been isolated from terrestrial and aquatic environments including soils, rice paddies, rhizosphere of plants, rivers, sewage, fish ponds, and irrigation water (16,23–25). Despite the use of *Escherichia coli (E. coli)* as the model prey bacterium in the majority of in vitro experiments published (17), *B. bacteriovorus* is selectively active against most *Pseudomonas* spp. and enterobacteria (16–17). Although variable between prey cells, a minimum prey density is required to sustain *B. bacteriovorus* life cycle. In 2 different studies, a prey concentration of approximately 1.5×10^5 *E. coli* per mL (26) and 3.0×10^6 *Photobacterium leignathi* per mL (27), was required for 50% survival of *B. bacteriovorus*. Optimal growth of *B. bacteriovorus* is seen at 30°C (range: 20°C to 45°C) and pH of 6.8 to 7.2 (range: 5.6 to 8.6) (27); values observed for the temperature of the corneal surface in horses (28) and pH of tears (29) in cattle, respectively. *Bdellovibrio bacteriovorus* is unlikely to cause mammalian cell toxicity because of its outer membrane characteristics and failure to grow in eukaryotic cells in vitro (23).

Reports of the use of *B. bacteriovorus* as a biological control or therapeutic agent are limited. Fratamico (30) demonstrated the potential use of *B. bacteriovorus* as biological control for pathogenic and spoilage organisms in food. Kadouri (31) demonstrated that *B. bacteriovorus* could attack and reduce an existing *E. coli* biofilm in as little as 30 min of exposure. Nakaruma (32) successfully treated *Shigella flexneri*-induced keratoconjunctivitis (KC) in rabbits with *B. bacteriovorus*. In a similar in vivo model, *B. bacteriovorus* effectively treated experimentally induced *Pseudomonas aeruginosa* KC (John J. Iandolo, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA; personal communication, November 2007).

To our knowledge, in vitro or in vivo research on the use of *B. bacteriovorus* on pathogenic isolates of *M. bovis* have not been conducted. In order to assess the potential for *B. bacteriovorus* for treatment of IBK, we used an in vitro IBK tissue culture model previously developed by Annuar and Wilcox (33). In vitro experiments investigating the adherence of *M. bovis* to cultured BCEC (8,9,33–35) and Madin-Darby bovine kidney (MDBK) cells (33), as well as associated cytopathic effects induced, have been previously published.

On the basis of preliminary experiments which confirmed that *B. bacteriovorus* grows on *M. bovis* isolates in vitro, its successful use as treatment for gram-negative bacterial KC in vivo and its reported non-toxic effect on mammalian cells, we propose that *B. bacteriovorus* administered by ocular instillation may represent an effective non-chemotherapeutic alternative treatment for IBK. The purpose of the study reported here was to determine the potential of *B. bacteriovorus* in the treatment of IBK associated with *M. bovis* in vitro. The first objective was to determine whether *B. bacteriovorus* can be trained to kill *M. bovis* as effectively as its normal prey *E. coli*. The second objective was to estimate the efficiency of low and high passage *B. bacteriovorus* predation on *M. bovis* in suspension broth culture and to establish the minimum prey concentration needed to sustain the *B. bacteriovorus* life cycle. The third objective was to determine the predatory effect of *B. bacteriovorus* on adherence of *M. bovis* to MDBK cells.

Materials and methods

Bacterial strains

Non-hemolytic *M. bovis* strain M^- was kindly provided by J. Angelos (University of California at Davis, School of Veterinary Medicine, Davis, California, USA) and *B. bacteriovorus* strain 109J and *E. coli* strain 012207 by J. Iandolo (University of Oklahoma, Health Sciences Center, Oklahoma City, Oklahoma, USA).

Routine cultivation of *B. bacteriovorus*

Cultivation of *B. bacteriovorus* on *E. coli* was modified from that described by Ruby (21). *Escherichia coli* was grown overnight as lawns on 5% brain heart infusion agar with 5% sheep blood (BAP) at 37°C. Bacteria were swabbed from the surface of the BAP and used to inoculate 45 mL of dilute nutrient broth (0.16% nutrient broth, 0.02% yeast extract, and 0.1% casitone supplemented with 2 mM $CaCl_2$ and 3 mM $MgCl_2$) to an absorbance reading of 0.4 to 0.6 at 600 nm. The cells were immediately harvested by centrifugation (1800 × *g* for 5 min), the supernatant was discarded and the pellet was resuspended in 35 mL of peptone yeast extract (PYE) (1% Bacto peptone and 0.3% yeast extract supplemented with 2 mM $CaCl_2$ and 3 mM $MgCl_2$). The bacterial suspension was centrifuged as described, and resuspended in 5 mL of PYE to a final concentration of approximately 1×10^9 *E. coli*/mL. A 75 cm^2 flask (Cell star tissue culture flask; Greiner Bio-One, Frickenhansen, Germany) containing 20 mL of PYE was inoculated with 5 mL of *E. coli* and 250 μL of an active 7 to 10 d old stock culture of *B. bacteriovorus* [Multiplicity of Infection (MOI) = 1; ratio 1 *E. coli:* 1 *B. bacteriovorus*], and incubated with shaking (180 rpm) at 30°C. After approximately 48 h of incubation, the solution was examined microscopically (Olympus BX41 laboratory microscope; Hitschfel Instruments, St-Louis, Missouri, USA) and considered active and ready to use when it contained motile, active attack-phase *B. bacteriovorus* (1×10^9 plaque forming units (PFU)/mL) with no visible *E. coli* or bdelloplasts (infected

E. coli cells). Once active, *B. bacteriovorus* culture was stored at 4°C for a maximum period of 1 mo.

Cultivation of *B. bacteriovorus* using *M. bovis* as prey

Moraxella bovis prey cells were prepared exactly as the *E. coli* inoculum. Motile and active *B. bacteriovorus* previously grown on *E. coli* were harvested as described by Rogosky (17). The *E. coli* cell debris was removed by centrifugation (1500 × *g* for 5 min). The *B. bacteriovorus*-rich supernatant was saved and the cells were centrifuged (8500 × *g* for 20 min), washed in PYE, centrifuged again (8500 × *g* for 20 min), and resuspended in PYE. The washed cells were filtered 3 times sequentially through 0.8 μm, then 0.45 μm, and finally 0.45 μm membranes (Acrodisc syringe filters; Pall Corporation, Ann Arbor, Michigan, USA) pre-wetted with PYE to remove residual *E. coli* cell debris. A 75 cm^2 flask containing 15 mL of PYE was inoculated with 5 mL of *M. bovis* and 5 mL of triple filtered *B. bacteriovorus* (MOI = 0.2; ratio 1 *M. bovis:* 5 *B. bdellovibrio*), and incubated with shaking (180 rpm) at 30°C for a period of 5 to 7 d or until active. Once grown on *M. bovis*, subsequent subcultures were done using the same protocol, without filtration.

Preparation of *M. bovis* inoculum

Lawns of *M. bovis* were grown for 24 h at 37°C on BAP. Cells were harvested by centrifugation (13 000 × *g* for 15 min), washed then diluted in Hank's balanced salt solution containing phenol red and supplemented with 25 μg/mL of glucose (HBSS + G) to an absorbance reading of 0.14 at 600 nm, for a final bacterial concentration of 4×10^7 *M. bovis*/mL.

Preparation of *B. bacteriovorus* inoculum

Ten milliliters of active and motile *B. bacteriovorus* previously grown on *M. bovis* was centrifuged (1500 × *g* for 5 min) to remove cell debris. The *B. bacteriovorus*-rich supernatant was saved and the cells were centrifuged (8500 × *g* for 20 min) and resuspended in PYE. The washed *B. bacteriovorus* cells were filtered twice (0.8 μm, 0.45 μm) to remove residual *M. bovis* cell debris. This suspension was cultured on BAP and was shown to be free of *M. bovis*. However, this filtration process also decreased the number of *B. bacteriovorus* present by 30% or greater. Therefore, the number of active *B. bacteriovorus* in the filtered bacterial suspension was estimated under light microscopy (100×).

Enumeration of *B. bacteriovorus* using plaque assays

A modification of the double–layer plaque assay technique used for counting bacteriophage described by Varon and Shilo (36) was used for enumeration of *B. bacteriovorus*. A 200 μL sample of *B. bacteriovorus* dilutions 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} and 200 μL of the *E. coli* suspension (as prepared in routine cultivation of *B. bacteriovorus*) were mixed in 3 mL of liquefied overlay agar (PYE medium containing 0.6% agar) kept on a hot plate at 45°C. The mixtures were inverted 6 or 7 times to allow proper mixing and immediately spread over the surface of PYE medium containing 1.5% agar in 92 × 16 mm in Petri dishes. Plates were incubated upright at 30°C for 3 to 7 d until clear circular plaques appeared in the lawn of prey cells.

Efficiency of killing assay

Bdellovibrio bacteriovorus and *M. bovis* inocula were prepared as described except that prey cells were resuspended in HBSS to a final *M. bovis* concentration of approximately 1×10^8/mL. The number of live *B. bacteriovorus* present in the inoculum was determined via plaque assay technique. Five hundred microliters of the original *M. bovis* inoculum was serially diluted in 4.5 mL of HBSS (10^{-1} to 10^{-4}). In group A (control group), 50 μL of PYE was added to triplicate tubes of 250 μL of each *M. bovis* dilutions (1×10^4/mL to 1×10^8/mL). The same method was used for group B (treatment group), except that PYE was replaced by 50 μL of *B. bacteriovorus* inoculum (1×10^{10} PFU/mL), resulting in a final 1:1 predator to prey ratio. Serial dilution tubes were incubated at 35°C for 4 h with shaking (180 rpm). To determine the *M. bovis* colony forming units (CFU)/mL at time 0 and 4 h, each *M. bovis* dilution from the 3 replicates of group A and B were further serially diluted, and serial dilution 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were plated on BAP and incubated at 37°C for 24 h. The percent of *M. bovis* killed by *B. Bdellovibrio* predation was calculated using the formula described previously by Rogosky (17):

$$\% \text{ killed} = \frac{(1 - B_4/B_0)}{A_4/A_0} \times 100$$

where: A_0 and A_4 are the mean CFU/mL *M. bovis* in the absence of *B. bacteriovorus* (group A) at 0 and 4 h, respectively, and B_0 and B_4 are the mean CFU/mL *M. bovis* in the presence of *B. bacteriovorus* (group B) at 0 and 4 h.

Madin-Darby bovine kidney cell culture

Immortalized MDBK cells (50th to 60th passage levels) were kindly provided by R. Fulton (Oklahoma State University Center for Veterinary Health Sciences, Stillwater, Oklahoma). Tissue culture growth media consisted of Dulbecco's modification of Eagle's medium with glucose (4.5 g/L) and L-glutamine (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) of penicillin-streptomycin (10 000 IU/mL, 10 000 μg/mL). For the adherence assay, antibiotic-free media was used. Cells were incubated at 37°C, 5% CO_2 and 90% to 100% humidity and cell culture media was changed 3 times weekly until cells reached 90% confluency. Cells formed monolayers within 3 to 4 d of incubation, and additional passages of the cells were done using conventional procedures at a split ratio of 1:14.

Cell monolayers on coverslips

Madin-Darby bovine kidney cells were grown as monolayers on coverslips for adherence experiments as described by Moore and Rutter (35). Prior to cell seeding, 13 mm round coverslips (Thermanox cell culture coverslips; Nalge Nunc International, Rochester, New York, USA) were placed at the bottom of each well of four 24-well tissue culture plates coated with type 1 collagen (Greiner Bio-One, Frichenhansen, Germany). The cell culture was maintained coated side up. Ninety percent confluent MDBK monolayers were released from their original tissue culture flask with trypsin-EDTA (0.05%, 0.53 mM EDTA). The detached cells were centrifuged (200 × *g* for 5 min) and then resuspended in fresh tissue

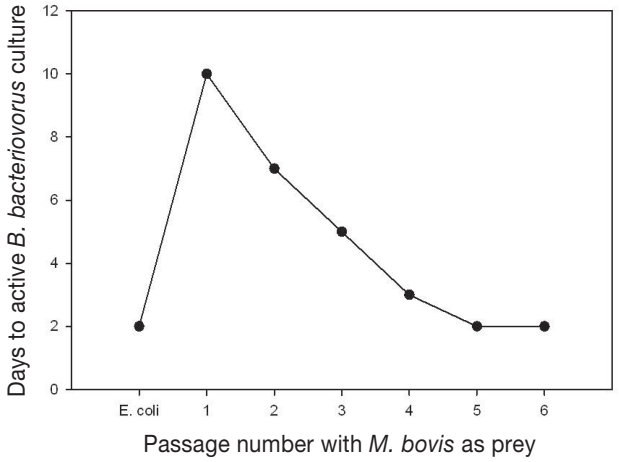

Figure 1. Time to active *B. bacteriovorus* predatory morphology for passages 1 through 6 on *M. bovis* with comparison with normal prey *E. coli*.

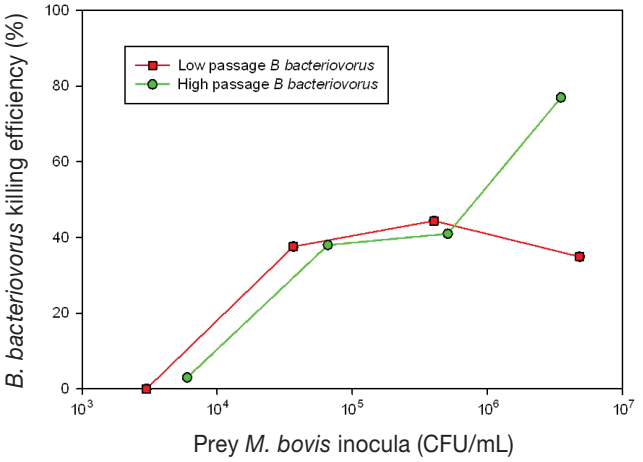

Figure 2. Efficacy of *B. bacteriovorus* predation of *M. bovis* in broth cultures. Initial inocula levels of *M. bovis* (CFU/mL) are plotted on the x-axis versus the *B. bacteriovorus* killing efficacy (%) at 4 h of exposure on the y-axis. Low and high passages of *B. bacteriovorus* on *M. bovis* correspond with 2 to 3 and 6 passages, respectively.

culture growth media. The suspension was adjusted to 2×10^5 viable cells per mL. Four 24-well plates were seeded with 1×10^5 cells per coverslip. Each coverslip was incubated in their respective well with 0.5 mL of cell suspension at 37°C, 5% CO_2, and 90% to 100% humidity. The cells were used after 36 to 48 h when the monolayers were 75% to 80% confluent as estimated by light microscopy (Olympus CKX-41 inverted microscope; Hitschfel Instruments, St. Louis, Missouri, USA).

Attachment assay

Attachment of *M. bovis* to MDBK cells was done as described by Annuar and Wilcox (33), with slight modifications. *Bdellovibrio bacteriovorus* and *M. bovis* inocula, as well as MDBK monolayers grown on coverslips, were prepared as described. The media type and amount per well, incubation temperature and time, and the concentration of the bacterial suspension used were determined by preliminary experiments (data not shown) to give optimum MDBK cells viability, preserve *M. bovis* viability and prevent overgrowth, provide optimum *M. bovis* attachment to MDBK cells, and mimic what would occur at the level of the ocular surface in case of naturally occurring IBK.

To begin the assay, tissue culture growth media was removed from each well of 24-well plate group 1 (control plate) and group 3 (*B. bacteriovorus* plate), and replaced by 400 μL of HBSS + G. The same process was repeated for group 2 (*M. bovis* plate) and group 4 (*M. bovis* + *B. bacteriovorus* plate) except that each well received 400 μL of *M. bovis* inoculum. All 4 plates were incubated at 37°C for 45 min. Following incubation, coverslips were transferred to 4 new corresponding 24-well tissue culture plates using 25 gauge needles and sterile tissue forceps with non-serrated tips leaving the majority of the non-adherent *M. bovis* inocula in the original wells. The coverslips were not washed to remove residual bacteria inocula because this process also resulted in detachment of variable numbers of MDBK cells. At time zero, 250 μL of HBSS + G and 50 μL of PYE was added to each well of plate 1 and plate 2, and 250 μL of HBSS + G and 50 μL of *B. bacteriovorus* inoculum was added to each well of plate 3 and plate 4. To determine the CFUs per well at 0, 6, and 12 h, media from 6 randomly selected wells from each treatment group was removed, serially diluted (up to 10^{-3}), and plated on BAP, which were incubated at 37°C for 24 h. The MDBK coverslips from corresponding wells were fixed in methanol and acetone (1:1 ratio) for 2 min and stained with Wright-Giemsa stain with phosphate buffered saline added (3:1 ratio) for 10 min. To quantify the mean number of adherent bacteria per MDBK cell, attached *M. bovis* were counted on 40 MDBK cells per coverslip. The number of live *B. bacteriovorus* present in the inoculum in the combined media from 2 randomly selected wells in plate 3 (*B. bacteriovorus* group) and plate 4 (*M. bovis* + *B. bacteriovorus* group) at time 0 and at 12 h, was determined via plaque assay technique.

Results

Efficiency of *B. bacteriovorus* predation following serial passages on *M. bovis*

As shown in Figure 1, initial passage of *B. bacteriovorus* using *M. bovis* as prey required 10 d for active cultures to develop compared with 2 d for culture on normal *E. coli* prey; however, by the 5th passage of *B. bacteriovorus* on *M. bovis*, time to active predatory morphology was reduced to 2 d.

Efficacy of *B. bacteriovorus* predation of *M. bovis* in broth cultures

The level of *M. bovis* prey required for *B. bacteriovorus* to demonstrate predation was assessed in suspension broth cultures for *M. bovis* prey levels of 1×10^3 to 1×10^7 CFU/mL with a *B. bacteriovorus* inoculum of 1×10^{10} PFU/mL. As shown in Figure 2, *B. bacteriovorus* passaged on *M. bovis* 3 times showed killing of ~40% for *M. bovis* prey levels greater than 4×10^4 CFU/mL. However after 6 passages, killing efficiency increased to ~76% only for *M. bovis* prey levels greater than 9×10^6 CFU/mL.

Efficacy of *B. bacteriovorus* predation of *M. bovis* in an in vitro model of IBK

Moraxella bovis attaches specifically to epithelial cells of bovine origin (8,33–35), and MDBK cells have been used by others as an in vitro model of *M. bovis* attachment for IBK (33). Using this model *M. bovis* attached to MDBK cells appeared as darkly stained rods or coccobacillary shapes, characteristically in pairs, adhered to the surface of the larger polygonal MDBK cells (Figure 3). At the time of inoculation in the presence of 1.6×10^{11} PFU/mL of *B. bacteriovorus*, the mean number of bacteria attached per MDBK cell was ~4 *M. bovis* per MDBK cell (Table I). The number of *M. bovis* attached per MDBK cell remained relatively constant during the 12 h incubation period with no statistical difference between the 0, 6, and 12 h time points for the controls lacking *B. bacteriovorus*. When compared with the presence of *B. bacteriovorus*, there was 1.9-fold decrease of *M. bovis* attached to MDBK cells after 6 h and 6-fold decrease after 12 h, which were statistically significant at $P < 0.05$ and $P < 0.001$, respectively.

In addition to *M. bovis* attached to MDBK cells, 0.8×10^5 to 1×10^6 CFU/mL *M. bovis* were present in the culture media at the beginning of the incubation period (Table I). In the absence of *B. bacteriovorus*, the *M. bovis* level increased to 1.5×10^6 CFU/mL at 12 h, whereas in the presence of *B. bacteriovorus* the supernatant *M. bovis* declined at 6 h and were near the pre-inoculation value of 0.7×10^6 CFU/mL at 12 h. These values were significantly different ($P < 0.005$) from that for the supernatant *M. bovis* CFU/mL in the absence of *B. bacteriovorus*.

The supernatant *B. bacteriovorus* level increased from 8×10^9 PFU/mL at 0 h to 3×10^{10} PFU/mL and 5×10^{10} PFU/mL at 12 h in the absence and presence of *M. bovis*, respectively. The number of active *B. bacteriovorus* in the original filtered bacterial suspension ranged from 15 to 20 per 100 × field under light microscopy.

Discussion

Efficacy of low and high passages *B. bacteriovorus* predation of *M. bovis* in suspension broth cultures

It is difficult to replicate the in vivo conditions of IBK in an in vitro model for assessing the efficiency of predation of *M. bovis* by *B. bacteriovorus*. The 4 h time period was selected to provide sufficient time for *B. bacteriovorus* to complete its life cycle and the incubation temperature of 35°C was selected (instead of the 30°C optimum for *B. bacteriovorus*) for optimal *M. bovis* growth and viability. *Bdellovibrio bacteriovorus* requires adequate levels of prey bacteria, typically $> 1 \times 10^6$ CFU/mL (26), in order to maintain its predatory lifestyle. The efficiency of predation followed a predicable trend in that at low prey levels ($< 10^4$ CFU/mL) there were insufficient prey to either trigger or sustain *B. bacteriovorus* predation, but at higher prey levels ($> 10^5$ CFU/mL and $> 10^7$ CFU/mL) efficiency of predation increased from ~40% to ~76%, respectively. The range of this amount is less efficient compared with the reported *B. bacteriovorus* predation efficiency of other gram-negative bacteria such as *E. coli*, but comparable to various strains of *Enterobacter* spp, *Erwinia* spp, and *Salmonella* spp. Rogosky et al (17) reported that *B. bacteriovorus* does have prey preference, killing some prey more efficiently than others. *Pantoea agglomerans*, *E. coli*, and *S. marcescens* were reported to be preferred prey for *B. bacteriovorus* with efficiency of predation of ~90% compared with *E. aerogenens*, *E. carotova* subsp. *carotova*, and *S. enterica* with efficiency of predation of ~60%. It has been observed here and by others that *B. bacteriovorus* can be trained to kill less preferred prey more efficiently by continuous passage on that prey. For this study, *B. bacteriovorus* was initially passaged on *M. bovis* 3 times with modest shortening of the passage time (from 10 d to 5 d after 3rd passage) to fully active *B. bacteriovorus* cultures. Improved efficiency of predation at high prey concentration ($> 10^7$ CFU/mL) was attained when *B. bacteriovorus* number of passages on *M. bovis* increased to 5 times, with corresponding passage time of 2 d to fully active *B. bacteriovorus* culture (Figure 1).

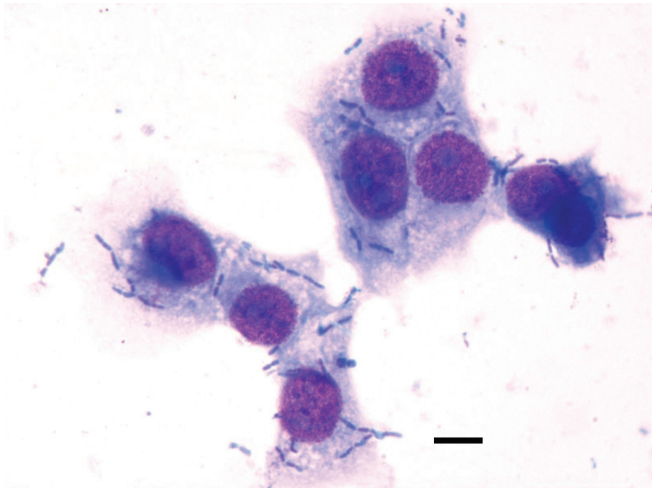

Figure 3. Light microscopic appearance of *M. bovis* adhering to Madin-Darby bovine kidney (MDBK) cells. *Moraxella bovis* attached to MDBK cells appeared as darkly stained rods or coccobacillary shapes, characteristically in pairs, adhered to the surface of the larger polygonal MDBK cells. Wright-Giemsa stain. Magnification: 1000×. Bar = 4.5 μm.

Efficacy of *B. bacteriovorus* predation of *M. bovis* in an in vitro model of IBK

Our initial experiments with the in vitro IBK model used a β-hemolytic strain of *M. bovis*, but this strain produced significant cytotoxicity resulting in MDBK cell detachment from coverslips such that the mean number of *M. bovis* attached per MDBK cell could not be consistently determined. Therefore, a non β-hemolytic strain of *M. bovis* was used in this study. No difference in efficiency of predation by *B. bacteriovorus* of hemolytic versus non-hemolytic strains of *M. bovis* was observed.

A paramount question for the use of *B. bacteriovorus* as a non-chemotherapeutic alternative therapy for IBK is whether *B. bacteriovorus* can efficiently decrease the number of *M. bovis* adhered to corneal epithelial cells in infected bovine eyes. To study this in vitro, a co-culture model of *M. bovis* with MDBK cells was used, with the media selected to support both MDBK and *M. bovis* viability while attempting to mimic the ocular environment. To reproduce the physical ocular environment, round coverslips with the approximate dimension of the bovine cornea were used and to mimic the ocular tear film, the media column above the coverslip was kept to the

Table I. *Bdellovibrio bacteriovorus* clearance of *Moraxella bovis* attached to Madin-Darby bovine kidney (MDBK) cells

	Attachment (*M. bovis* per MDBK)[e]		Unattached *M. bovis* (10^3 CFU/mL)	
Exposure time (h)	No *B. bacteriovorus*	With *B. bacteriovorus*	No *B. bacteriovorus*	With *B. bacteriovorus*
0	4.13 ± 2.62	4.62 ± 1.31	80 ± 152[c]	998 ± 571[c]
6	3.41 ± 1.27[a]	1.77 ± 0.45[a]	277 ± 65	213 ± 87
12	5.35 ± 1.96[b]	0.88 ± 0.11[b]	1.520 ± 860[d]	691 ± 72[d]

[a] Statistically significant at $P < 0.05$.
[b] Statistically significant at $P < 0.001$.
[c] Statistically significant at $P < 0.005$.
[d] Statistically significant at $P < 0.005$.
[e] Results represent mean ± 1 standard deviation from 6 coverslips.

minimum that supported MDBK cell viability for a 12 h exposure period. Selected media was also tailored to simulate tears. It contained minimum levels of nutrients in an aqueous salts composition to reduce *M. bovis* growth rate to one simulating the slower growth rate in vivo. This was accomplished in that the doubling time for *M. bovis* in the in vitro IBK model was 4.3 h (calculated from data for Table I). During the 12 h experimental period, *M. bovis* in the unexposed control IBK model underwent approximately 3 doublings, but *M. bovis* attachment to MDBK cells only increased 1.3-fold. However, exposure to *B. bacteriovorus* decreased the number of adherent *M. bovis* on MDBK cells in vitro by 6-fold (Table I).

Bdellovibrio bacteriovorus appears to be an effective predator of bacterial prey fixed on surfaces as demonstrated by its efficacy against bacteria in surface biofilms (31). Exposure to *B. bdellovibrio* increased the levels of planktonic *M. bovis* by 12-fold at the beginning of the experiment, suggesting that the predatory bacteria is efficient in preventing initial attachment of *M. bovis* on MDBK cells in vitro. Although *B. bacteriovorus* was not as effective in clearing *M. bovis* from the aqueous media above the coverslip, it did reduce the CFU/mL by 1.4-fold as compared to a 19-fold increase in the controls without *B. bacteriovorus* (Table I). The apparent bactericidal effect of *M. bovis* attached to bovine epithelial cells may be crucial for reducing *M. bovis* ulceration of the cornea of infected cattle because pathogenesis of these lesions appears to be the result of direct contact of *M. bovis* with corneal epithelial cells (37). In contrast, although *M. bovis* attached to corneal epithelia extend into the corneal stroma where they may be protected from innate and acquired immunity, *M. bovis* in the tear film will not only be exposed to *B. bacteriovorus* predation but also to innate immune response of infiltrating neutrophils; tear antimicrobial proteins and enzymes, such as lysozyme (29), the latter reported to increase up to 33-fold in cattle with inflamed corneas (38); and to acquired immunity through secreted IgA and leaked serum IgG (29,39), and therefore, bacteriostatic activity of *B. bacteriovorus* against *M. bovis* in the tear film may be adequate to resolve infection. Due to its poor specificity against gram-negative bacteria, the true protective effect of tear lysozyme against the invasion of the ocular surfaces by *M. bovis* is unknown (29).

In summary, the present study confirms that *B. bacteriovorus* can be trained to kill *M. bovis* as effectively as its normal prey *E. coli*. The efficiency of low passage *B. bacteriovorus* predation on *M. bovis* in suspension broth culture was ~40% at prey levels $> 4 \times 10^4$ CFU/mL though, at high passage on *M. bovis* and prey levels $> 9 \times 10^6$ CFU/mL, the efficiency was increased to ~76%. The minimum *M. bovis* concentration to sustain *B. bacteriovorus* life cycle was established to be $< 10^4$ CFU/mL. In the in vitro model of IBK, exposure to *B. bacteriovorus* not only significantly decreased the number of adherent *M. bovis* on MDBK cells, but also had a bacteriostatic effect on planktonic *M. bovis*. Therefore, we conclude that *B. bacteriovorus* can act as an effective *M. bovis* predator at levels present in IBK infected corneal epithelia and ocular secretions. Future studies are needed to evaluate the cytoprotective effects of *B. bacteriovorus* on *M. bovis* infected MDBK cells and to characterize the effect of high lysozyme concentration on *B. bacteriovorus* in vitro.

Acknowledgments

This research was supported by grant 5T20RR015564, Center of Biomedical Research Excellence (COBRE) from the National Center for Research Resources to the University of Oklahoma Health Sciences Center, and the Oklahoma State University Research Advisory Committee. The authors thank Huda Mussa and Asitha Vasudevan Pillai for technical assistance.

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