Pathogenesis of *Streptococcus infantarius* subspecies *coli* Isolated from Sea Otters with Infective Endocarditis

Katrina L. Counihan a,*,1, Verena A. Gill b,2, Melissa A. Miller c, Kathleen A. Burek-Huntington d, Rance B. LeFebvre a, Barbara A. Byrne a

a University of California Davis, School of Veterinary Medicine, Department of Pathology, Microbiology and Immunology, 4206 VM3A, One Shields Avenue, Davis, CA 95616, USA
b US Fish and Wildlife Service Marine Mammals Management, 1011 East Tudor Road, MS 341, Anchorage, AK 99503, USA
c Marine Wildlife Veterinary Care and Research Center, California Department of Fish and Wildlife, 1451 Shaffer Road, Santa Cruz, CA 95066, USA
d Alaska Veterinary Pathology Services, 23834 The Clearing Drive, Eagle River, AK 99577, USA

**A R T I C L E   I N F O**

Article history:
Received 4 November 2014
Received in revised form 24 February 2015
Accepted 11 March 2015

Keywords:
*Streptococcus infantarius* subspecies *coli*
Infective endocarditis
Sea otter
Pathogenesis
Macrophage
Extracellular matrix
Epithelial
Endothelial

**A B S T R A C T**

The Gram positive bacterial coccus *Streptococcus infantarius* subspecies *coli* is increasingly linked with development of fatal vegetative infective endocarditis and septicemia in humans, sea otters (*Enhydra lutris*) and other animals. However, the pathogenesis of these infections is poorly understood. Using *S. infantarius* subsp. *coli* strains isolated from sea otters with infective endocarditis, this study evaluated adherence and invasion of epithelial and endothelial cells, adherence to extracellular matrix components, and macrophage survival. Significant adherence to endothelial-derived cells was observed for 62% of isolates, 24% adhered to epithelial cell lines, and 95% invaded one or both cell types in vitro. The importance of the hyaluronic acid capsule in host cell adherence and invasion was also evaluated. Capsule removal significantly reduced epithelial adherence and invasion for most *S. infantarius* subsp. *coli* isolates, suggesting that the capsule facilitates attachment to and invasion of epithelium. Enzyme-linked immunosorbent assay testing revealed that all isolates adhered significantly to the extracellular matrix components collagen IV, fibronectin, laminin and hyaluronic acid. Finally, significant bacterial survival following phagocytosis by macrophages was apparent for 81% of isolates at one or more time points. Taken collectively these findings indicate that *S. infantarius* subsp. *coli* has multiple pathogenic properties that may be important to host colonization, invasion and disease.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

*Streptococcus infantarius* subspecies *coli* is a member of the *Streptococcus bovis*-equinus complex (SBEC) and was previously classified as *Streptococcus bovis* biotype II/1 [1]. Members of SBEC are commensals in the gastrointestinal tract of many mammals, including humans, but they are sporadically invasive and are associated with endocarditis, septicemia, and bacteremia in humans, pigeons, ruminants, and mink [2–5]. Infective endocarditis (IE) and septicemia due to *S. infantarius* subsp. *coli* have been major causes of
mortality in northern sea otters (*Enhydra lutris kenyoni*) where 30% of 613 carcasses collected and necropsied in Alaska during 2004–2010 had died due to the bacterium [6]. Additionally, four cases have been recognized in southern sea otters (*Enhydra lutris nereis*) from coastal California where 281 carcasses were examined between 2004 and 2008 [6]. Reports of endocarditis in humans due to SBEC have also significantly increased in prevalence in recent years, especially in southern Europe and South America [7,8]. There has been very little research conducted on the pathogenesis of SBEC infections in humans or other animals, particularly on *S. infantarius* subsp. *coli*.

Host colonization is necessary for pathogens such as *S. infantarius* subsp. *coli* to establish an infection. This bacterium is often isolated from the intestinal tract, cardiac valve lesions, heart blood, brain, and other organs of sea otters that died due to IE, suggesting that *S. infantarius* subsp. *coli* can colonize multiple sites in the host. *S. infantarius* subsp. *coli* may initially adhere to and invade intestinal epithelium as reported for other intestinal pathogens [9,10]. Bacteria that enter the intestinal wall may then disseminate hemogenously and infect the endocardium and heart valves by adhering directly to endothelium or exposed extracellular matrix (ECM), as described for other cardiovascular pathogens [11].

Following cell attachment and tissue invasion, bacterial survival is dependent on mechanisms to evade the host immune system. The ability to evade phagocytosis-mediated macrophage killing can be vital to bacterial persistence and systemic spread. The related Group A (GAS) and Group B (GBS) streptococci can survive for at least 12 h in macrophages [12,13]. *S. bovis*, associated with septicemia in pigeons, has also been shown to survive in macrophages for 7 h [2].

Using 21 isolates of *S. infantarius* subsp. *coli* isolated from sea otters from Alaska and California with IE, we examined potential pathogenic mechanisms for *S. infantarius* subsp. *coli* infections and assessed inter-strain variation in virulence properties. We hypothesized *S. infantarius* subsp. *coli* could adhere and invade host cells, bind ECM and survive in macrophages. The ability of these isolates to adhere to and invade epithelial and endothelial cells and survive in macrophages was evaluated through standardized in vitro eukaryotic cell:bacterial culture assays. Five isolates were further tested to determine the role of capsular hyaluronic acid in adherence and invasion. Ten isolates were also included in enzyme-linked immunosorbent assays (ELISAs) to examine their binding to collagen IV, fibronectin, laminin, and hyaluronic acid.

### 2. Materials and methods

#### 2.1. Bacterial strains and growth conditions

*S. infantarius* subsp. *coli* isolates were obtained from either northern (*E. l. kenyoni*) or southern (*E. l. nereis*) sea otters that died due to IE and/or septicemia during 2004–2008. Isolates were identified utilizing Gram staining, catalase test results, conventional biochemical testing, (Rapid ID32 Strep System, bioMérieux Inc., Durham, NC, USA) and sequencing of 16S rRNA genes [14–16]. All isolates were stored as frozen stabiles at −80°C until utilized (Microbank Bacterial and Fungal Preservation System, ProLab Diagnostics, Round Rock, TX, USA). Isolates were selected from various years, source-tissues, and three different genotypes, as determined by pulsed-field gel electrophoresis (PFGE) [6]. Two sea otters included in the study each had two separate *S. infantarius* subsp. *coli* isolates obtained from different anatomical locations, and both were included so these isolates could be compared. Twenty-one isolates were used in the macrophage survival, and adherence and invasion assays. Five isolates that exhibited high levels of adherence and/or invasion to human epithelial or endothelial cell lines were selected for adherence/invasion blocking assays. Ten isolates that exhibited high and low levels of adherence and/or invasion to epithelial or endothelial cell lines, including the 5 used in the blocking assays, were used in the ELISAs and their capsular hyaluronic acid content was measured. The anatomical source of the isolates and the assays they were used in are summarized in Table 1. All isolates were grown in brain-heart infusion (BHI) broth at 37°C overnight on a shaker table at 150 rpm and washed twice in phosphate buffered saline (PBS) before use.

#### 2.2. Cellular adherence and invasion assays

Two human cell lines, Caco-2 and HUV-EC-C, and two mouse cell lines, CMT-93 and EOMA, were used in the adherence assays and the human cell lines were also used in invasion assays. Prior to the assays, the optimal incubation time and multiplicity of infection (MOI; bacteria:cell ratio) for use with each cell line was determined. The cell line was grown to confluence overnight in the appropriate growth medium. The optimal MOI was determined by incubating 1 MOI, 10 MOI, 50 MOI, 100 MOI, 500 MOI, or 1000 MOI of a selected *S. infantarius* subsp. *coli* strain (10423C), *Lactococcus lactis* (ATCC 19435, Manassas, VA, USA, negative control for all adherence and invasion assays), and *Salmonella enterica* serotype Typhimurium (clinical isolate, positive control for epithelial assays) or *Staphylococcus aureus* (ATCC 25923, positive control for endothelial assays) with the cells. The optimal incubation time for adherence was determined by incubating the selected MOI of *S. infantarius* subsp. *coli*, *L. lactis*, and the positive control for 0 h, 30 min, 1 h, 2 h, 4 h, or 6 h with the cell line [17,18]. The MOI and incubation time at which *S. infantarius* subsp. *coli* exhibited the best adherence, and the positive control did not cause obvious cell death, was selected for use in the experiments. The optimal incubation time was determined to be 1 h, and the optimal MOI was 50 for the mouse epithelial and endothelial cell lines and the human endothelial cell line. For the human endothelial cell line, the optimal incubation time was 1 h, and the optimal MOI was 100. None of the strains replicated when incubated in media alone during the incubation times employed. The cell lines were lysed with sterile, Millipore water to count bacteria that had invaded, and it was determined that none of the bacterial strains were lysed by exposure to the water. It was also determined prior to the assays that the *S. infantarius* subsp. *coli* isolates, *L. lactis*, *S. enterica* serotype Typhimurium, and
Table 1
Summary of sea otter S. infantarius subsp. coli isolates used in this study.

<table>
<thead>
<tr>
<th>Sea otter ID</th>
<th>Year isolated</th>
<th>Sea otter type</th>
<th>Tissue of isolation</th>
<th>PFGE type*</th>
<th>Macrophage assays</th>
<th>Adherence/ invasion assays</th>
<th>Blocking assays</th>
<th>ELISAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>3353</td>
<td>2004</td>
<td>Northern</td>
<td>Heart</td>
<td>3</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3506</td>
<td>2004</td>
<td>Northern</td>
<td>Blood</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5253</td>
<td>2004</td>
<td>Southern</td>
<td>Heart</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7074</td>
<td>2005</td>
<td>Northern</td>
<td>Heart</td>
<td>1</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10050</td>
<td>2004</td>
<td>Northern</td>
<td>Heart</td>
<td>3</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1816</td>
<td>2005</td>
<td>Northern</td>
<td>Heart</td>
<td>1</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2481A</td>
<td>2005</td>
<td>Northern</td>
<td>Heart</td>
<td>1</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2481E</td>
<td>2005</td>
<td>Northern</td>
<td>Feces</td>
<td>1</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7930</td>
<td>2005</td>
<td>Northern</td>
<td>Heart</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1801</td>
<td>2006</td>
<td>Northern</td>
<td>Lymph node</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1802</td>
<td>2006</td>
<td>Northern</td>
<td>Heart</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47166</td>
<td>2006</td>
<td>Northern</td>
<td>Blood</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5413</td>
<td>2006</td>
<td>Northern</td>
<td>Brain</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8029</td>
<td>2006</td>
<td>Northern</td>
<td>Blood</td>
<td>3</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9144</td>
<td>2006</td>
<td>Northern</td>
<td>Heart</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10172</td>
<td>2006</td>
<td>Southern</td>
<td>Heart</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10423A</td>
<td>2006</td>
<td>Northern</td>
<td>Heart</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10423C</td>
<td>2006</td>
<td>Northern</td>
<td>Uterus</td>
<td>2</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>2007</td>
<td>Northern</td>
<td>Blood</td>
<td>1</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1805</td>
<td>2007</td>
<td>Northern</td>
<td>Heart</td>
<td>1</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2212</td>
<td>2007</td>
<td>Southern</td>
<td>Heart</td>
<td>1</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* PFGE types determined in previous study [6].

S. aureus were completely killed by 10 µg ml⁻¹ gentamicin, which was used in the invasion protection assays.

The mouse epithelial cell line, CMT-93 (ATCC CCL-223), derived from mouse rectal tissue was maintained in 75 cm² flasks with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 2.5 µg ml⁻¹ amphotericin B. The cells were grown at 37 °C in a 5% carbon dioxide atmosphere. For the adherence assays, the CMT-93 cells were inoculated into 24-well plates, and grown overnight to confluence. An extra set of wells was seeded so a count of the CMT-93 cells could be performed to calculate the 50 MOI amount prior to the experiment. Bacteria were inoculated into antibiotic-free DMEM with 10% FBS to make a 50 MOI inoculum. One ml of bacterial inoculum was added to wells containing confluent CMT-93 cells. Each bacterial strain was tested in triplicate. The CMT-93 cells were incubated with the bacteria for 1 h, the supernatant was removed, and the cells were washed 5 times with PBS. The CMT-93 cells were lysed with 1 ml of cold, sterile, Millipore water. The lysed cell solution was serially diluted and plated in duplicate on 5% sheep blood agar to determine cfu ml⁻¹ of bacteria that adhered to CMT-93 cells.

The mouse endothelial cell line, EOMA (ATCC CRL-2586), derived from mouse heart tissue was maintained in 75 cm² flasks with DMEM containing 10% FBS, 100 IU ml⁻¹ penicillin, 10 µg ml⁻¹ streptomycin, and 2.5 µg ml⁻¹ amphotericin B. The mouse endothelial cell adherence assay was conducted as described for the mouse epithelial assay.

The human epithelial cell adherence assay was performed as described above for the mouse epithelial adherence assay with the following modifications. The human epithelial cell line, Caco-2 (ATCC HTB-37), derived from human colon tissue was maintained in 75 cm² flasks with Eagle’s minimum essential medium (EMEM) adjusted to contain 10% FBS, 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 2.5 µg ml⁻¹ amphotericin B. For the invasion assay, bacteria were allowed to adhere/invade as described above, then the Caco-2 cells were rinsed 1 time with PBS after the removal of the supernatant, and then 1 ml of EMEM with 10 µg ml⁻¹ gentamicin and 10% FBS was added. The Caco-2 cells were further incubated for 1 h to kill extracellular bacteria, the supernatant was removed, and the cells were washed 5 times with PBS. The Caco-2 cells were lysed with 1 ml of cold, sterile, Millipore water. The lysed cell solution was serially diluted and plated in duplicate on 5% sheep blood agar to determine cfu ml⁻¹ of bacteria that invaded the Caco-2 cells.

The adherence assay with the human endothelial-derived cells was carried out as described for the mouse epithelial adherence assay, with the following exceptions. The human endothelial cell line, HUV-EC-C (ATCC CRL-1730), derived from human umbilical vein, was maintained in 75 cm² flasks with Kaighn’s modification of Ham’s F-12 medium (F-12K) adjusted to contain 10% FBS, 0.1 mg ml⁻¹ heparin, 0.04 mg ml⁻¹ endothelial cell growth supplement, 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 2.5 µg ml⁻¹ amphotericin B. Bacteria were inoculated into antibiotic-free F-12K with 10% FBS to make a 100 MOI inoculum. The invasion assay was conducted as described for the human epithelial cell line with the exception of media.

2.3. Capsule hyaluronic acid measurement

The S. infantarius subsp. coli isolates were grown to exponential phase in BHI broth and washed twice with sterile Millipore water. The bacteria were suspended in 0.5 ml of sterile Millipore water, 1 ml of chloroform was added and the mixture shaken vigorously and allowed to stand for 1 h. The mixture was centrifuged and the aqueous phase collected. Absorbance at 640 nm was measured on a spectrophotometer after adding 2 ml of staining solution, which
consisted of 20 mg Stains All (Sigma, St. Louis, MO, USA) and 60 μl glacial acetic acid in 100 ml of 50% formamide. Cappular hyaluronic acid concentration was determined using a standard curve generated with purified hyaluronic acid (Sigma) [19].

2.4. Hyaluronic acid blocking assays

Two methods were used to evaluate the role of hyaluronic acid in adherence and invasion. The S. infantarius subsp. coli isolates were treated with 200 U ml⁻¹ hyaluronidase (MP Biomedicals, Solon, OH, USA) in PBS at 37 °C for 3 h. Each isolate was stained using India ink prior to and after the hyaluronidase treatment to confirm removal of the capsule. The adherence and invasion assays using the Caco-2 and HUV-EC-C cell lines were conducted as described above using the hyaluronidase-treated bacteria [20].

The second method treated Caco-2 cells with 500 μg ml⁻¹ hyaluronic acid or HUV-EC-C cells with 100 μg ml⁻¹ hyaluronic acid (Sigma) for 1 h at 37 °C. The cells were washed with PBS to remove unbound hyaluronic acid, and then the adherence and invasion assays were conducted as described above [21].

2.5. Anti-S. infantarius subsp. coli antibody preparation

Antibody to S. infantarius subsp. coli was generated in a 12–15 week old New Zealand white rabbit at the University of California Davis Comparative Pathology Laboratory. The S. infantarius subsp. coli isolates were heat-inactivated at 60 °C for 1 h and an equal mixture of the 10 isolates was prepared at a concentration of 1 × 10⁸ cfu ml⁻¹. The rabbit was injected subcutaneously with an equal mixture of Freund’s complete adjuvant and inactivated S. infantarius subsp. coli for the first injection. Subsequent immunizations used an equal mixture of Freund’s incomplete adjuvant with the bacteria. The rabbit was immunized every two weeks for a total of six immunizations and was bled prior to each injection. The fourth and fifth bleeds were pooled and used in the ELISA assays. A titration, ranging in concentration from 1:10–1:10,000, was conducted to determine the optimal concentration of rabbit serum for use in the ELISA. Animal use was carried out with approval from the University of California Davis Institutional Animal Care and Use Committee.

2.6. Enzyme-linked immunosorbent adherence assays

Ninety-six well flat-bottom plates were purchased from BD Biosciences (Bedford, MA, USA) pre-coated with 5 μg cm⁻² of collagen type IV (mouse tumor), fibronectin (human plasma), or laminin (mouse tumor). Ninety-six well flat-bottom plates (BD Biosciences) were coated with 5 μg cm⁻² of hyaluronic acid (Sigma) in carbonate buffer overnight at 4 °C. The plates were washed 3 times with 0.05% Tween-20 (Sigma) washing solution after coating. All plates were blocked for 1 h with 5% bovine serum albumin (BSA, Sigma). A suspension of each isolate was inoculated into triplicate wells at a concentration of 1 × 10⁷ cfu ml⁻¹ and incubated for 1 h at room temperature. After unbound bacteria were washed from the plate, the rabbit anti-S. infantarius subsp. coli serum was added at a dilution of 1:100 for 1 h at room temperature, and then 500 ng ml⁻¹ of phosphatase-labeled goat anti-rabbit F(ab')₂ fragment (KPL, Gaithersburg, MD, USA) was incubated for 1 h at room temperature. After each incubation the ELISA plate was washed 3 times with 0.05% Tween-20 washing solution, and the second wash was allowed to soak for 5 min. BluePhos Microwell Phosphatase Substrate (KPL) was added to the wells and developed for 1 h, and then the plates were read on a VersaMax tunable microplate reader ( Molecular Devices) at a wavelength of 650 nm. All incubation times and bacteria and reagent concentrations were selected after validating what provided the optimal signal. A standard curve had been determined for each S. infantarius subsp. coli isolate in order to determine the cfu ml⁻¹ represented by the optical density measured on the microplate reader. Three independent experiments were conducted with each strain tested in triplicate in all experiments. Uncoated ELISA plates that were blocked with 5% BSA were included in each experiment as a control for nonspecific binding [22,23].

2.7. Macrophage survival experiments

Mouse macrophage-like cell line, J774 (ATCC TIB-67), was maintained in 75 cm² flasks with DMEM containing 10% FBS, 100 IU ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and 2.5 μg ml⁻¹ amphotericin B. The cells were grown at 37 °C in a 5% carbon dioxide atmosphere. For the survival assays, macrophages were inoculated into 24-well plates and grown overnight to confluence (1 × 10⁶ cells ml⁻¹). Bacteria were suspended in antibiotic-free DMEM with 10% FBS, and 2 × 10⁵ cfu were inoculated into each well. L. lactis (ATCC 19435) was used as a negative control. Listeria monocytogenes (ATCC 19115), a facultative intracellular bacterium, was used as a positive control to verify phagocytic activity by the macrophages and intracellular survival. Each bacterial strain was tested in triplicate. The bacteria were inoculated with the macrophages for 1 h, the supernatant removed and the macrophages washed twice with PBS. It was determined none of the strains replicated in DMEM during the 1 h incubation period. A set of wells were selected for the 0 h time point, and the remainder of the wells had 1 ml of DMEM with 2 μg ml⁻¹ ampicillin and 10% FBS added to them to kill extracellular bacteria. All strains were confirmed to be completely killed by 2 μg ml⁻¹ ampicillin. Wells were then sampled at 6 h, 12 h, 24 h and 48 h. Macrophages were washed twice with PBS, then lysed by adding 1 ml of cold, sterile, Millipore water. The lysed macrophage solution was then serially diluted and plated in duplicate on 5% sheep blood agar to determine cfu ml⁻¹ of bacteria that survived within the macrophages [2,24].

2.8. Statistical analysis

A two-tailed t-test was conducted to determine which isolates adhered or invaded epithelial or endothelial cell lines significantly versus the control, L. lactis. In the blocking assays, a paired t-test was used to determine significant differences in adherence and invasion for each
S. infantarius subsp. coli isolate, versus its untreated control. A two-tailed t-test was conducted to determine if the S. infantarius subsp. coli isolates used in the ELISAs adhered significantly as compared to the BSA control, and a one-way analysis of variance (ANOVA) with a Bonferroni’s multiple comparison post-test was conducted to determine significant differences in adherence between the isolates. In the macrophage assays, an ANOVA with a Dunnett’s multiple comparison post-test was performed to determine significant differences in bacterial survival between the S. infantarius subsp. coli isolates and the L. lactis control. Gaussian distribution of data was determined with the Kolmogorov–Smirnov test, except for the ELISA data where the D’Agostino Pearson omnibus test was used. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Adherence and invasion of S. infantarius subsp. coli to epithelial and endothelial cell lines

Four of the 21 S. infantarius subsp. coli isolates tested for adherence to the human epithelial cell line adhered significantly, as compared to the negative control, L. lactis, and at levels similar to the positive control, S. enterica serotype Typhimurium (Fig. 1). One isolate adhered significantly to the mouse epithelial cell line, as compared to the negative control, L. lactis, but it did not adhere at the same level as S. enterica serotype Typhimurium (Fig. 2). Eighteen of the 21 isolates demonstrated significant invasion of human epithelial-derived cells, as compared to L. lactis, with ranges similar to S. enterica serotype Typhimurium (Fig. 1).

More isolates adhered to the endothelial cell lines with 7 of the 21 S. infantarius subsp. coli isolates adhering significantly to human endothelial-derived cells, and 10 adhering significantly to the mouse endothelial cell line, as compared to the negative control, L. lactis (Figs. 1 and 2). The isolates that adhered significantly adhered at lower levels than the positive control, S. aureus. As seen with the epithelial cell line, most of the isolates invaded, with 16 isolates significantly invading the human endothelial cell line, as compared to L. lactis, but at levels below S. aureus (Fig. 1).

An association was observed between the tissue source for each isolate and strain-specific performance in adherence and invasion assays. We predicted that S. infantarius subsp. coli isolates obtained from more than one location in the same animal would perform similarly. Strains 2481A (cardiac isolate) and 2481E (fecal isolate) were isolated from different tissues in the same animal and adhered and invaded at similar levels. However, isolates 10423A and 10423C, two isolates from another otter, demonstrated significant differences in attachment and invasion.

Fig. 1. Percent of S. infantarius subsp. coli inoculum (mean and SD) adhering to and invading CACO-2 (human epithelial cell line) and HUV-EC-C (human endothelial cell line) cells. * Indicates significant adherence or invasion (P<0.05), as compared to the negative control, L. lactis.
properties. The cardiac isolate, 10423A, adhered significantly to the human endothelial cell line, but not to the epithelial line, while the opposite results were obtained for the gastrointestinal ulcer isolate, 10423C. In general, isolates exhibiting greater adherence to endothelial cell lines were isolated from the heart or blood. However, only one of the five isolates that demonstrated adherence to epithelial-derived cells was isolated from gastrointestinal tract, and no isolates were able to adhere and invade all of the cell types.

3.2. Role of hyaluronic acid in S. infantarius subsp. coli adherence and invasion

Capsular hyaluronic acid was measured in 10 S. infantarius subsp. coli isolates. The amount of hyaluronic acid each isolate possessed ranged from 0.46 fg cfu⁻¹ to 4.90 fg cfu⁻¹ (Fig. 3). The majority of the isolates contained around 1 fg cfu⁻¹, but 3 isolates expressed much higher levels of capsular hyaluronic acid with concentrations of 2.71, 3.17 and 4.90 fg cfu⁻¹.

Five S. infantarius subsp. coli isolates that exhibited high levels of adherence and/or invasion to human epithelial or endothelial cell lines were tested in two assays to determine the role of hyaluronic acid in adherence and invasion. Pre-treatment with hyaluronidase prior to incubation with the human epithelial cell line resulted in significantly decreased adherence for all 5 isolates, and significantly decreased invasion for 1 isolate, as compared to adherence or invasion of isolates not treated with hyaluronidase. When hyaluronidase-treated bacteria were incubated with the human endothelial cell line, adherence was significantly increased for 1 isolate and decreased for another, while invasion was not significantly impacted, as compared to adherence or invasion of isolates not treated with hyaluronidase. (Fig. 4).

Efforts to block bacterial adherence through addition of excess hyaluronic acid to monolayers of human epithelial- derived cells significantly increased both adherence and invasion for all isolates, when compared to untreated controls. In contrast, the addition of excess hyaluronic acid to monolayers of human endothelial-derived cells resulted in no significant change in adherence, and a significant increase in invasion for 1 isolate (Fig. 5).

3.3. Adherence of S. infantarius subsp. coli to extracellular matrix

Ten isolates, including the 5 used in the assays designed to block adherence via hyaluronic acid, were evaluated in ELISAs to determine their ability to adhere to collagen IV, fibronectin, laminin, and hyaluronic acid. All 10 isolates demonstrated significant levels of adherence to collagen, fibronectin, laminin, and hyaluronic acid, as compared to the BSA control (Fig. 6). For all isolates, the greatest degree of binding was to collagen and fibronectin.

A range of adherence to each ECM component was observed among the S. infantarius subsp. coli isolates indicating strain differences exist. Adherence to collagen ranged from 1.4% to 23.2% of the inoculum, laminin binding spanned 0.4 to 8.1% of inoculum, fibronectin adherence varied from 1.5% to 25.0% of inoculum, and hyaluronic acid binding ranged from 1.2% to 21.7% of inoculum.
Four isolates, 7074, 2481A, 2481E, and 5413 demonstrated significantly higher adherence to all ECM substrates, when compared to the other six isolates, when analyzed with a one-way ANOVA with Bonferroni’s post-test, with the exception that adherence of 7074 to laminin was not significant when compared to isolate 2212. There was no relationship between the sea otter source (northern or southern subspecies) or the tissue origin and ECM adherence. The four isolates exhibiting the strongest ECM adherence were obtained from brain (1), feces (1), and heart (2).

3.4. Survival of S. infantarius subsp. coli in macrophages

All 21 S. infantarius subsp. coli isolates were tested in the macrophage survival assay, and 17 demonstrated significantly higher survival at one or more time points, as compared to the negative control, L. lactis. The 17 isolates consisted of 3 of 4 southern sea otter isolates and 14 of 17 northern sea otter isolates used in the assays. Significant S. infantarius subsp. coli survival was observed at the following time points: 6 h (10 isolates), 12 h (8 isolates), 24 h (11 isolates), and 48 h (6 isolates) as compared to the survival of the negative control, L. lactis, at the same time points (Fig. 7).

4. Discussion

This study provides important new insights into the pathogenesis of S. infantarius subsp. coli infection of humans and animals. Several isolates were capable of adhering to epithelial and endothelial-derived cells, but many more demonstrated cellular invasion. All tested isolates adhered to a range of ECM components, including collagen IV, fibronectin, laminin and hyaluronic acid. The majority of S. infantarius subsp. coli isolates exhibited statistically significant intracellular macrophage survival, compared to controls, and several isolates were able to survive in macrophages for up to 48 h post-exposure. Collectively, these virulence properties may facilitate host invasion, persistence and systemic spread by S. infantarius subsp. coli.

A previous study of 122 S. infantarius subsp. coli isolates from sea otters, including the isolates used in this study, identified six major PFGE genotypes [6]. Three different PFGE groups were included in the macrophage assays,
which revealed that isolate genotype was not predictive of macrophage survival. For example, the six isolates that survived significantly at all time points represented all three genotypes. Thus, despite genetic similarities revealed by PFGE analysis, additional strain-specific differences appear to account for varying susceptibility to phagocytosis-mediated bacterial inactivation. Survival in the J774 macrophage cell line by *S. infantarius* subsp. *coli* was similar to that of GBS where intracellular survival was high during the first 8 h and then decreased rapidly until less than 5% of the inoculum survived after 48 h [13]. As with the *S. infantarius* subspecies *coli* isolates, GBS were phagocytosed without complement or antibody [13]. Some *S. infantarius* subsp. *coli* isolates also survived intracellularly longer than *L. monocytogenes*, the positive control (Fig. 7). The ability of *S. infantarius* subsp. *coli* to survive intracellularly in macrophages has implications for the clinical care of live sea otters in rehabilitation centers with IE. In order to optimize care, antibiotics with the ability to penetrate macrophages, such as enrofloxacin or doxycycline, should be selected for treatment.

Several *S. infantarius* subsp. *coli* isolates adhered to human or mouse epithelial and endothelial-derived cells, and invaded human cell lines. As with macrophage survival, no relationship was identified between genetic (PFGE) profile and adherence or invasion properties. Standardized mouse cell lines were used for adherence assays because no sea otter cell lines were available to the authors’ knowledge. Additionally, human cell lines were used because humans develop *S. infantarius* subsp. *coli* infections. While only 19% of isolates adhered to the human epithelial cell line, 86% invaded. Similarly, 29% of isolates exhibited adherence to human endothelial-derived cells, but 76% invaded. This higher level of invasion compared to adherence was notable, and has been observed in other bacteria, such as uropathogenic *Escherichia coli* [25].

Members of SBEC are often found as commensals in the intestinal tract of many mammals, including humans and ruminants [26]. However, *S. infantarius* subsp. *coli* is rarely isolated from healthy sea otters (V. Gill, personal communication). Sea otters that die due to IE often have *S. infantarius* subsp. *coli*-positive intestinal contents or feces, suggesting that the intestinal tract may be an important route of entry. In a study with *Streptococcus suis*, which can also cause IE, Lalonde et al. determined that approximately 1% of the inoculum adhered to epithelial cells; however, the bacteria were unable to invade [27]. *S. infantarius* subsp. *coli* were not only able to invade Caco-2 cells, but three isolates adhered at levels near the positive control, *S. enterica* Typhimurium. Adherence to epithelium could facilitate bacterial retention and proliferation in the intestinal tract, and the demonstrated ability of sea otter *S. infantarius*
Fig. 6. Percent of *S. infantarius* subsp. *coli* inoculum (mean and SD) adhering to ECM components collagen type IV, fibronectin, laminin, and hyaluronic acid. All isolates adhered significantly to each ECM component, as compared to the BSA coated plate (*P*<0.05).

*subsp. coli* strains to invade epithelium suggests they could pass through the mucosa and spread systemically. Other streptococci, enterococci, and *E. coli* appear to use similar methods to cross the mucosal barrier [10]. After invading the epithelium, the bacteria can spread to the submucosa, lamina propria and endothelium, then disseminate through the lymphatic and cardiovascular systems [10].

High levels of adherence to epithelial-derived cells were predicted because members of SBEC inhabit the intestinal tract, but *S. infantarius* subsp. *coli* isolates adhered more frequently to endothelial-derived cells. Nearly half of the isolates adhered to the mouse endothelial cell line, and approximately a third adhered to the human endothelial cell line. The two most common etiological agents of endocarditis, *staphylococci* and streptococci, utilize different strategies to colonize the heart. Viridans streptococci colonize the heart by targeting ECM components that have been exposed following physical or chemical damage, whereas *S. aureus* can adhere to exposed ECM or intact endothelium [11]. However, other streptococci that have been found to adhere or invade endothelial cell lines did so at similar levels to *S. infantarius* subsp. *coli*. Abranches et al. determined 0.05–0.22% of *Streptococcus mutans* inoculum invaded an endothelial cell line [28]. Another study demonstrated that 1–10% of *Streptococcus galolyticus* subsp. *galolyticus* inoculum adhered and 0.01–1% of its inoculum invaded endothelial cell lines [29]. Both studies used different cell lines and longer incubation times than were used in this project, but strain differences were noted in all the studies. The demonstrated ability of *S. infantarius* subsp.
coli isolates to adhere to endothelial-derived cells might allow them to colonize humans and sea otters with intact endothelium. The ability of these bacteria to adhere to intact endothelium is supported by descriptions of human SBEC endocarditis cases, which typically involve native valves in patients with no prior history of heart disease [7,30,31].

The hyaluronic acid capsule can be an important virulence factor and some of the S. infantis subsp. coli isolates had a mucoid appearance; therefore, the levels of capsular hyaluronic acid and its role in adherence and invasion for S. infantis subsp. coli were investigated. Results of adherence and invasion blocking assays indicate that the hyaluronic acid capsule of S. infantis subsp. coli plays a role in epithelial adherence and invasion. Capsule removal reduced adherence to the human epithelial cell line by half for all isolates. Invasion was unchanged for two isolates, and decreased up to five times for the other three. The hyaluronic acid capsule of Streptococcus equi subsp. zoonepidemicus and Streptococcus pyogenes has been shown to be important for their adherence to host cells [21,32]. Highly mucoid, presumably with an abundant capsule, GAS are often more invasive than less mucoid strains and are associated with greater degrees of tissue necrosis and bacteremia [32].

Capsule removal prior to incubation with the human endothelial cell line produced more variable results with adherence and invasion increasing, decreasing, or remaining unchanged depending on the isolate. An apparent increase in adherence and invasion was observed for some isolates, possibly due to exposure of receptors during capsule removal. Interestingly, all isolates exhibiting increased adherence following capsule removal had capsular hyaluronic acid levels greater than 1 fg cfu⁻¹. Possessing a thicker capsule may sterically hinder access of adhesins to their target, while capsule removal would facilitate binding. Prior studies revealed that highly encapsulated GAS and S. suis have reduced levels of adherence to epithelial and endothelial cells [17,32,33]. However, a thicker capsule provides bacteria with enhanced protection against phagocytosis, and encapsulated strains are generally more invasive. A similar phenotypic tradeoff between epithelial infectivity and evasion of host immune responses may be occurring for various strains of S. infantarius subsp. coli.

Addition of excess hyaluronic acid to epithelial and endothelial cell monolayers to block adherence sites for capsular hyaluronic acid resulted in a paradoxical increase in bacterial adherence and invasion. This suggests that S. infantarius subsp. coli can use host ECM components, including exogenous hyaluronic acid, to mediate tissue adherence and invasion. Enzyme-linked immunosorbent assay testing of S. infantarius subsp. coli strains exposed to exogenous collagen IV, fibronectin, laminin, and hyaluronic acid demonstrated statistically significant adherence to all four ECM components, suggesting that ECM adherence could be important for tissue colonization, systemic spread, and pathogenesis of IE. As with all other assays, no association was found between the PFGE group and levels of adherence, reinforcing the concept that despite genetic similarities, important phenotypic differences exist between S. infantarius subsp. coli strains. All of the isolates adhered at higher levels to collagen IV, fibronectin, and hyaluronic acid, and at lower levels to laminin. It has been noted that other IE-associated streptococci also vary in their capacity to bind ECM components. Egleas et al. [34] determined that S. suis binds fibronectin, but not collagen IV, while Vollmer et al. [29] found that S. gallolyticus subsp. gallolyticus binds collagen IV at higher levels and laminin at lower levels. Components of the ECM can be exposed following physical or chemical damage to the endocardium, thus facilitating attachment and spread of S. infantarius subsp. coli [35]. Initial infection by another pathogen could result in endothelial damage and exposed ECM. Phocine distemper virus has been detected in northern sea otters in Alaska, and morbilliviruses can predispose hosts to infection by other opportunistic microorganisms [36,37]. The ability of S. infantarius subsp. coli to adhere to multiple ECM constituents would allow the bacteria to bind multiple targets on damaged endothelium and more efficiently colonize the heart.

4.1. Conclusions

The pathogenesis of IE/septicemia due to SBEC, and especially S. infantarius subsp. coli, has not been extensively studied. The knowledge gained from the current study is a major step in elucidating mechanisms for S. infantarius subsp. coli infection of humans and animals. The results of this study suggest S. infantarius subsp. coli can adhere to cardiac valves with pre-existing endothelial damage, but they may target intact endothelium, as well. Their ability to resist macrophage killing not only has implications about their pathogenesis, but for therapeutic selection, also. The results of these experiments have provided a basis for further study into the pathogenicity of S. infantarius subsp. coli. Taken collectively, these bacterial properties provide important new insight into the pathogenesis of S. infantarius subsp. coli infections in sea otters. Infective endocarditis is an important cause of illness and death for northern sea otters, other animals and humans, and understanding S. infantarius subsp. coli pathogenesis could facilitate prevention and treatment.
Acknowledgements

This project was supported by the California Department of Fish and Game's Oil Spill Response Trust Fund through the Oiled Wildlife Care Network at the Wildlife Health Center, School of Veterinary Medicine, University of California Davis. The Alaska portion of this work was conducted under Marine Mammal Protection Act permit number MA041309-4 issued to Verena Gill, USFWS.

We would like to gratefully acknowledge the work of others that made this project possible. Necropsies of the sea otters were performed by staff at the California Department of Fish and Wildlife, United States Fish and Wildlife Service, Alaska SeaLife Center, and Alaska Veterinary Pathology Services. Isolate identification was carried out at the Microbiology Laboratory at the University of California Davis. William R. Pritchard Veterinary Medical Teaching Hospital and Centers for Disease Control and Prevention. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

References