**BRIEF COMMUNICATION**

**Brucella pinnipedialis** in hooded seal (**Cystophora cristata**) primary epithelial cells

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**Abstract**

**Background:** Marine *Brucella* spp. have been isolated from numerous pinniped and cetacean species, but pathological findings in association with infection with *Brucella pinnipedialis* in pinnipeds have been sparse. The capacity of brucellae to survive and replicate within host macrophages underlies their important ability to produce chronic infections, but previous work has shown that *B. pinnipedialis* spp. are rapidly eliminated from hooded seal (**Cystophora cristata**) alveolar macrophages.

**Results:** To investigate if multiplication could take place in other hooded seal cell types, primary epithelial cells were isolated, verified to express the epithelial marker cytokeratin and challenged with three different strains of *B. pinnipedialis*; *B. pinnipedialis* sp. nov., *B. pinnipedialis* hooded seal strain B17, and *B. pinnipedialis* hooded seal strain 22F1. All strains were steadily eliminated and the amounts of intracellular bacteria were reduced to less than one-third by 48 h post infection. Intracellular presence was verified using immunocytochemistry.

**Conclusions:** So far, intracellular multiplication in seal cells has not been documented for *B. pinnipedialis*. The lack of intracellular survival in macrophages, as well as in epithelial cells, together with the fact that pathological changes due to *B. pinnipedialis* infection is not yet identified in seals, suggests that the bacteria may only cause a mild, acute and transient infection. These findings also contribute to substantiate the hypothesis that seals may not be the primary host of *B. pinnipedialis* and that the transmission to seals are caused by other species in the marine environment.

**Keywords:** *Brucella pinnipedialis*, Epithelial cells, Hooded seal, Infection, In vitro, Marine ecosystem, Pinnipeds, Spillover

**Findings**

*Brucella* spp. were first isolated from marine mammals in 1994 [1] and were validly published as the species *Brucella pinnipedialis* sp. nov. and *Brucella ceti* sp. nov. in 2007 [2]. The bacteria have been isolated from numerous organs in pinniped and cetacean species, but pathological findings in association to infection with *B. pinnipedialis* in pinnipeds has only once been reported in eared seals (otariids) [3] and never, to date, in true seals. In dolphins, however, *B. ceti* have been shown to cause pathology in the central nervous system and the reproductive system [4, 5].

The capacity of brucellae to survive and replicate within host macrophages underlies their important ability to produce chronic infections [6], yet in vitro work has revealed that *B. pinnipedialis* hooded seal (HS) strain and *B. pinnipedialis* sp. nov. do not multiply in murine or human macrophage cell lines [7]. Brucellae are shown to exhibit a host preference [8], however, in vitro work with *B. pinnipedialis* HS strain, *B. pinnipedialis* sp. nov., *B. ceti* sp. nov., and *B. ceti* Atlantic white-sided dolphin (**Lagenorhynchus acutus**) strain in HS primary macrophages revealed no multiplication [9]. *Brucella* spp. may invade many cell types [10], but *B. pinnipedialis* HS strain and *B. pinnipedialis* sp. nov. were likewise rapidly eliminated from a human epithelial cell line [7]. The aim of the current study is to investigate whether *B. pinnipedialis* multiply in primary epithelial cells from HS.
The Brucella strains used were B. pinnipedialis sp. nov. (NCTC 12890\textsuperscript{3}, BCCN 94–73\textsuperscript{3}) [2] and two B. pinnipedia-
lis HS isolates (spleen B17, and lung 22F1) [11]. The strains were kept and handled, and the final infective
solutions were prepared, as previously described [7].

Epithelial tissue was collected from esophagus post mortem on two HSs (the same animals as previously
described [9]). Approval of capture and import of ani-
mals was given by the appropriate authorities, and all
animal use was in accordance with the Norwegian Ani-
mal Welfare Act and the regulations for use of animals in
experimentation (permit no. 2402). Tissue cultures were
prepared according to published protocols [12], and cul-
tured in RPMI 1640, 10 % fetal bovine serum, 100 IU/ml
penicillin, and 100 μg/ml streptomycin at 37 °C, 5 % CO₂.

The epithelial origin of the cell culture was verified
by immunocytochemistry using the epithelial marker
cytokeratin. Adherent cells were fixed for 5 min at
room temperature using 4 % paraformaldehyde (0.02 M
sucrose, pH 7.2) and washed once in phosphate-buffered
saline (PBS). Immune labeling was performed using
4 % paraformaldehyde (0.02 M
sucrose, pH 7.2) and washed once in phosphate-buffered
saline (PBS). Immune labeling was performed using
mouse anti-pan cytokeratin antibody (PKC-26, Sigma–
Aldrich, St. Louis, USA, 1:100). Secondary antibody
was Alexa Fluor 546 goat anti-mouse IgG (Molecular
Probes, Life Technologies, Paisley, UK, 1:500). For veri-
fication of intracellular bacterial localization, epithelial
cells were challenged with B. pinnipedialis HS strain B17
as described in the gentamicin protection assay. Immune
labeling was done using rabbit polyclonal anti-Brucella
antibody and Alexa Fluor 488 goat anti-rabbit IgG (Molecular
Probes, 1:500). The fluorescent DNA dye DRAQ5 (Cell
Signaling, Danvers, USA, 1:1000) was used for visualiza-
tion of nuclei.

Primary HS epithelial cells were seeded (10⁵ cells/well) in
24 well plates and cultured for 8 days prior to infec-
tion. The cells were challenged as previously described
for HeLa cells [7] and incubated for 1.5, 7, 24, and 48 h.
Harvesting of cells and plating for evaluation of the num-
ber of intracellular bacteria were as previously described
[7].

After 8 days in culture the majority of the cells
expressed the epithelial marker cytokeratin as illustrated
by the anti-pan cytokeratin staining (Fig. 1). At day 12
the cultures contained a large amount of cells with a
fibroblast-like morphology staining negative for anti-pan
cytokeratin. Bacterial challenge was thus performed after
8 days in culture to ensure that the correct cell type was
evaluated.

The results from the gentamicin protection assay
revealed that all B. pinnipedialis strains were able to
enter HS epithelial cells in vitro. When challenging the
cells with a MOI of 500, B. pinnipedialis HS strains B17
and 22F1, and B. pinnipedialis sp. nov. showed moderate
ability to enter, yielding log CFUs of 3.16, 2.87 and 2.82
at 1.5 h post infection (pi), respectively (Fig. 2). All three
B. pinnipedialis strains were steadily eliminated and by
24 h pi the retrieved CFUs were reduced with 1.05–1.39
log CFUs. By 48 h pi, the amount of intracellular bacte-
ria were reduced to less than one-third of the numbers of
CFU at 1.5 h pi, yielding log CFUs of 0.66, 0.89 and 0.72.
No significant differences (Student’s t test, P < 0.05 was
considered significant) could be detected and the pattern
of entry and elimination was similar for the three strains
investigated (Fig. 2).

The intracellular localization of B. pinnipedialis HS
strain B17 was confirmed by confocal microscopy (Fig. 3).
Double immune labeling with anti-Brucella antibody and
anti-pan cytokeratin antibody revealed intracellular pres-
bence of bacteria in cells staining positive for the epithelial
marker.

For the first time, we present the results of infecting HS
epithelial cells with B. pinnipedialis HS strain in vitro.
Compiled with existing information from field research
and in vitro macrophage infection assays, our results
contribute to further understanding of marine Brucella
infections in seals, a condition where epidemiology,
pathogenesis and clinical importance are still unclear.

Brucella pinnipedialis HS strain seems to have a
restricted, if any, ability to establish chronicity as the bac-
teria fail to multiply intracellularly in human and murine
macrophages [7, 13]. The low pathogenicity of HS B. pin-
nipedialis has also been confirmed in a mouse model of
infection [14]. Little information is available regarding the
pathogenicity of these bacterial strains in seals, which
are assumed to be the natural hosts of B. pinnipedialis,
and the ability of the marine mammal brucellae to enter
and multiply in host cells has been largely unexplored.

In addition to trophoblasts, which are target cells in the
female reproductive organ, interaction with different cell
types are shown for the pathogenic terrestrial brucellae.
Macrophages are believed to be preferred as long time
survival in the mononuclear phagocyte system of spleen,
liver and bone marrow will sustain a chronic infection
[6]. Brucella pinnipedialis HS isolate B17 and sp. nov.
are previously shown to enter HS alveolar macrophages,
but are rapidly eliminated [9]. As HSs are believed to
be the primary host of B. pinnipedialis HS strain, it is
intriguing that the HS isolate were not able to multiply in
macrophages. In this work we aimed to explore if multi-
plication could take place in primary epithelial cells from
a tentative host species, as shown for terrestrial patho-
genic brucellae [15]. Epithelial cells would be the first cell
type encountered given an exposure route through the
food web, and both fish [16] or invertebrates, and possi-
ibly lungworms [17, 18], may be involved in transmission
of marine brucellae. Although intracellular bacteria were not eliminated as quickly as reported for HS alveolar macrophages, the amount of viable intracellular bacteria steadily decreased during 48 h pi and no multiplication was detected. Entry of HS epithelial cells by *B. pinnipedialis* HS strain was verified by confocal microscopy, where intracellular bacteria were detected within cells staining positive for the epithelial marker (Fig. 3).

One can only speculate whether other cell types could be the target of *B. pinnipedialis* infection, supporting intracellular survival and multiplication. *Brucella abortus* is shown to survive within murine and human B-cells [19, 20] creating an intracellular niche that contributes to a chronic infection. Specific subpopulations of peripheral blood mononuclear cells (PBMCs) are not yet identified in the HS, however preliminary results indicate that *B. pinnipedialis* HS strain is quickly eliminated from infected HS PBMCs, reaching lysosomal compartments already at 1 h pi (Larsen, unpublished results). In light of the unusual high hematocrit found in hooded seals [21], erythrocytes could be a target for infection, as shown for *B. melitensis* in mice [22].

The lack of intracellular survival, together with the fact that pathological changes due to *B. pinnipedialis* infection is not yet identified in true seals, suggests that the
bacteria cause a mild acute and transient infection. Given that \emph{B. pinnipedialis} is unable to multiply intracellularly in macrophages and epithelial cells derived from other seal species, we argue that seals may not be the primary host for \emph{B. pinnipedialis}, but rather a “dead-end” or spill-over host being susceptible to infection transmitted from other hosts in the marine environment. Age-dependent serological and bacteriological patterns for \emph{B. pinnipedialis} have been identified in HSs with a low probability of being positive for pups, a high probability for yearlings, followed by a decreasing probability with age, suggesting an environmental exposure post weaning during the first year of life followed by clearance of infection before the age of reproduction [23]. A similar age-dependent pattern of anti-\emph{Brucella} antibodies was also identified in harbor seals [24, 25]. This raises the question of a reservoir of \emph{B. pinnipedialis} in the food web, a hypothesis that is strengthened by the results presented herein and warrants further investigations.

Authors’ contributions
IHN and AKL drafted the manuscript. AKL isolated hooded seal primary epithelial cells and performed the in vitro cell work. JG, IHN and AKL participated in the study design and provided critical evaluation of the manuscript. All authors have read and approved the final manuscript.

References
1. Ross HM, Foster G, Reid RJ, Jahans KL, MacMillan AP. \emph{Brucella} species infection in sea-mammals. Vet Rec. 1994;134:359.
2. Foster G, Osterman BS, Godfroid J, Jacques I, Cloeckaert A. \emph{Brucella ceti} sp. nov. and \emph{Brucella pinnipedialis} sp. nov. for \emph{Brucella} strains with cetaceans and seals as their preferred hosts. Int J Syst Evol Microbiol. 2007;57:2688–93.
3. Duncan CG, Tiller R, Mathis D, Stoddard R, Kersh GJ, Dickerson B, et al. \emph{Brucella} placentalis and serorelevance in northern fur seals (\emph{Callorhinus ursinus}) of the Pribilof Islands, Alaska. J Vet Diagn Invest. 2014;26:507–12.
4. Guzman-Verri C, Gonzalez-Barrientos R, Hernández-Mosa G, Morales JA, Baquerizo-Calvo E, Chaves-Quintero E, et al. \emph{Brucella ceti} and \emph{brucellosis} in cetaceans. Front Cell Infect Microbiol. 2012;2:3.
5. Nymo IH, Tryland M, Godfroid J. A review of \emph{Brucella} infection in marine mammals, with special emphasis on \emph{Brucella pinnipedialis} in hooded seal (\emph{Cystophora cristata}). Vet Res. 2011;42:93.
6. Roop RM, Gaines JM, Anderson ES, Caswell CC, Martin DW. Survival of the fittest: how \emph{Brucella} strains adapt to their intracellular niche in the host. Med Microbiol Immunol. 2009;198:211–38.
7. Larsen AK, Nymo IH, Briquemont B, Sorensen KK, Godfroid J. Entrance and survival of \emph{Brucella pinnipedialis} hooded seal strain in human macrophages and epithelial cells. PLoS One. 2013;8:e84861. doi:10.1371/journal.pone.0084861.
8. Larsen AK, Nymo IH, Briquemont B, Sorensen KK, Godfroid J. Entrance and survival of \emph{Brucella pinnipedialis} hooded seal strain in human macrophages and epithelial cells. PLoS One. 2013;8:e84861. doi:10.1371/journal.pone.0084861.
9. Roop RM, Gaines JM, Anderson ES, Caswell CC, Martin DW. Survival of the fittest: how \emph{Brucella} strains adapt to their intracellular niche in the host. Med Microbiol Immunol. 2009;198:211–38.
10. Corbel M, Banai M. \emph{Genus Brucella} Meyer and Shaw 1920. In: Garrity GM, Krieg NR, Staley JT, editors. Bergey’s Manual of Systematic Bacteriology. 2nd ed. New York: Springer; 2005. p. 370–85.
11. Larsen AK, Nymo IH, Boysen P, Tryland M, Godfroid J. Entrance and elimination of marine mammal \emph{Brucella} spp. by hooded seal (\emph{Cystophora cristata}) alveolar macrophages in vitro. PLoS One. 2013;8:e70186. doi:10.1371/journal.pone.0070186.
12. Nymo IH, Boysen P, Tryland M, Godfroid J. Entrance and survival of \emph{Brucella pinnipedialis} hooded seal strain in human macrophages and epithelial cells. PLoS One. 2013;8:e84861. doi:10.1371/journal.pone.0084861.
13. Roop RM, Gaines JM, Anderson ES, Caswell CC, Martin DW. Survival of the fittest: how \emph{Brucella} strains adapt to their intracellular niche in the host. Med Microbiol Immunol. 2009;198:211–38.
14. Corbel M, Banai M. \emph{Genus Brucella} Meyer and Shaw 1920. In: Garrity GM, Krieg NR, Staley JT, editors. Bergey’s Manual of Systematic Bacteriology. 2nd ed. New York: Springer; 2005. p. 370–85.
15. Roop RM, Gaines JM, Anderson ES, Caswell CC, Martin DW. Survival of the fittest: how \emph{Brucella} strains adapt to their intracellular niche in the host. Med Microbiol Immunol. 2009;198:211–38.
15. Pizarro-Cerda J, Meresse S, Parton RG, van der Goot G, Sola-Landa A, Lopez-Goni I, et al. Brucella abortus transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infect Immun. 1998;66:5711–24.
16. El-Tras WF, Tayel AA, Eltholth MM, Guisan J. Brucella infection in fresh water fish: evidence for natural infection of Nile catfish, Clarias gariepinus, with Brucella melitensis. Vet Microbiol. 2010;141:321–5.
17. Dawson CE, Perrett LL, Stubberfield EJ, Stack JA, Fareelly, SSJ, Cooley WA, et al. Isolation and characterization of Brucella from the lungs of a harbor porpoise (Phocoena phocoena). J Wildl Dis. 2008;44:237–46.
18. Garner MM, Lambourn DM, Jeffries SJ, Hall PB, Rhyian JC, Ewalt DR, et al. Evidence of Brucella infection in Paraspherae lung worms in a Pacific harbor seal (Phoca vitulina richardsi). J Vet Diagn Invest. 1997;9:298–303.
19. Goenka R, Guirnalda PD, Black SJ, Baldwin CL. B lymphocytes provide an infection niche for intracellular bacterium Brucella abortus. J Infect Dis. 2012;206:91–8.
20. Saldana MR, Santelises MA, Lafont MM, Argumedo VP, Cervantes VP, Santiago RL. Human B cells are targets for Brucella abortus infection. J Immunol. 2013;190 Meeting abstract suppl 186.

21. Burns JM, Leistikow LP, Hammill MO, Blix AS. Size and distribution of oxygen stores in harp and hooded seals from birth to maturity. J Comp Physiol B. 2007;177:687–700.
22. Vitry MA, Hanot MD, Dieghelt M, Hack K, Machelart A, Lhomme F, et al. Brucella melitensis invades murine erythrocytes during infection. Infect Immun. 2014;82:3927–38.
23. Nymo IH, Tryland M, Frie AK, Haug T, Foster G, Rodven R, et al. Age-dependent prevalence of anti-Brucella antibodies in hooded seals (Cystophora cristata). Dis Aquat Organ. 2013;106:187–96.
24. Lambourn D, Garner M, Ewalt D, Raverty S, Sidor I, Jeffries SJ, et al. Brucella pinnipedialis infections in Pacific Harbor Seals (Phoca Vitulina Richardsi) from Washington State, USA. J Wildl Dis. 2013;49:802–15.
25. Zarnke RL, Salki JT, MacMillan AP, Brew SD, Dawson CE, Hoef JWV, et al. Serologic survey for Brucella spp., phocid herpesvirus-1, phocid herpesvirus-2, and phocine distemper virus in harbor seals from Alaska, 1976–1999. J Wildl Dis. 2006;42:290–300.