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Salmonella infection in grey seals (*Halichoerus grypus*), a marine mammal sentinel species: Pathogenicity and molecular typing of *Salmonella* strains compared with human and livestock isolates

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Originality-Significance Statement. This field based study in an unusual and poorly explored ecosystem investigates the prevalence and types of Salmonella in grey seal pups in Scottish waters. The findings raise serious concerns regarding the spread of human and livestock pathogens to wildlife marine sentinel species in coastal areas.

Abstract:

Microbial pollution of the marine environment through land-sea transfer of human and livestock pathogens is of concern. *Salmonella* was isolated from rectal swabs of free-ranging and stranded grey seal pups (21.1%; 37/175) and compared to strains from the same serovars isolated from human clinical cases, livestock, wild mammals and birds in Scotland, UK to characterise possible transmission routes using pulsed-field gel electrophoresis (PFGE) and multi-locus variable number of tandem repeat (MLVA) analyses. A higher prevalence of *Salmonella* was found in pups exposed to sea-water, suggesting that this may represent a source of this pathogen. *Salmonella* Bovismorbificans was the most common isolate (18.3% pups; 32/175) and was indistinguishable from isolates found in Scottish cattle. *Salmonella* Typhimurium was infrequent (2.3% pups; 4/175), mostly similar to isolates found in garden birds and, in one case, identical to a highly multidrug resistant strain isolated from a human child. *Salmonella* Haifa was rare (1.1% pups; 2/175) but isolates were indistinguishable from that of a human clinical isolate. These results suggest that *S.* Bovismorbificans may circulate between grey seal and cattle populations and that both *S.* Typhimurium and *S.* Haifa isolates are shared with humans, raising concerns of microbial marine pollution.

Introduction

Infection with *Salmonella* spp. is a major, global, human and animal health concern causing more than 90 million human cases of clinical disease, annually, worldwide (Majowicz et al., 2010). It is the second most commonly reported cause of bacterial infectious intestinal disease in Scotland after *Campylobacter* spp. and most identified cases can be attributed to contaminated food products (Browning et al., 2012). While generally leading to transient gastro-intestinal symptoms such as diarrhoea or vomiting,

fever, anorexia and malaise, it can produce potentially fatal invasive infections (Coburn et al., 2007).

Salmonella spp. have been isolated from several pinniped species including grey seals (*Halichoerus grypus*), harbour seals (*Phoca vitulina*), Stellar sea lions (*Eumetopias jubatus*), New Zealand sea lions (*Phocarctos hookeri*), Antarctic fur seals (*Arctocephalus gazella*), northern elephant seals (*Mirounga angustirostris*) and California sea lions (*Zalophus californianus*) in locations remote from human habitation and with increased frequency in recent years (Baker et al., 1995; Palmgren et al., 2000; Fenwick et al., 2004; Stoddard, Atwill, et al., 2008; Carrasco et al., 2011). Increased sampling effort may well explain this apparent increase in infection but it may also be the result of human activity or exposure between marine mammals and other animals, marine or terrestrial. Furthermore, the detection of multidrug resistant strains of *Salmonella* spp. from several marine mammal species (Foster et al., 1998; Johnson et al., 1998; Stoddard et al., 2005) raises serious concerns about microbial environmental pollution and the potential impact upon the increasingly important global topic of antimicrobial resistance.

Within the UK pinniped populations *Salmonella* spp. has been isolated from faeces and faecal swabs of both healthy and clinically ill grey and harbour seals with *Salmonella enterica* ssp. *enterica* serovar Bovismorbificans, *S.* Newport, *S.* Tennessee, *S.* Typhimurium definitive type 49 and *S.* Typhimurium definitive type 104 detected to date (Anderson et al., 1979; Baker et al., 1980, 1995; Foster et al., 1998, 1999). *Salmonella* Enteritidis PT8 was isolated also from infected bite wounds in a grey seal

(Davison et al., 2010). The pathogenicity of these bacteria for seals and their relationship with known terrestrial and human isolates remains largely unknown.

The aim of this study was to determine the prevalence, serotypes and antimicrobial resistance of *Salmonella* spp. in live and dead, free-ranging grey seal pups and yearlings within a breeding colony, in stranded live pups presented to a rehabilitation centre and those that subsequently died. To elucidate the origin of seal isolates and their relationship with known terrestrial and human isolates, typing was performed with four different but complementary typing methods recommended by the European Centre for Disease Prevention and Control for molecular surveillance of these pathogens: serotyping, phage typing, pulsed-field gel electrophoresis (PFGE) and multilocus variable number of tandem repeat analysis (MLVA).

Results

Prevalence and serotypes

Three serotypes of *Salmonella* were isolated from the 196 selenite enriched rectal swabs and 6 sediment samples: *S.* Bovismorbificans (n=32), *S.* Typhimurium (n=4) and *S.* Haifa (n=2) (Table 1 and Table S 1). The overall prevalence of *Salmonella enterica* ssp. *enterica* in grey seal pups was 21.1% (37/175) and 0% (0/19) in grey seal yearlings (Table 1). The prevalence of *S.* Bovismorbificans was higher in stranded live seal pups arriving at the rehabilitation centre (26.9%, 7/26) than in other groups (live free-ranging pups: 16.6% and dead pups on the colony: 18%). No significant differences were recorded between groups except between stranded live pups (26.9%) and live free-ranging yearlings (0%) (Fisher's exact test, p=0.016). Although the positive cases of

Salmonella spp. (n=7) in the 26 pups submitted to the rehabilitation centre were predominantly found in pups rescued from the South-East region of Scotland (see Figure 1), the small number of samples from stranded seals precluded meaningful spatial statistical analysis.

The prevalence of *S.* Bovismorbificans in live free-ranging grey seal pups was significantly higher at the tidal boulder beach site when compared to the grassy slope site (p=0.021; Fisher's exact test) and was subjectively higher than that seen on the stagnant rocky pool site with a difference approaching statistical significance (p=0.057; Fisher's exact test) (Figure 2). For the live animals, pups sampled at the tidal boulder beach and stranded pups admitted to the rehabilitation centre were considered to have been exposed to seawater. The prevalence of *Salmonella* spp. was significantly higher in live pups exposed to sea water when compared to those not exposed to sea water (p=0.004, Fisher's exact test) with a 3.92 times higher odds ratio of carrying *Salmonella* spp. than those not exposed to sea water (OR=3.92, 95% CI: 1.42, 10.85, glm, p=0.01). There was a statistically significant increase in the prevalence of free-ranging live grey seal pups carrying *S.* Bovismorbificans on the Isle of May between the early and late sampling periods (p=0.005; Fisher's exact test).

In the final multivariate logistic regression model, an increased risk for *Salmonella* Bovismorbificans infection in live, free-ranging grey seal pups was associated with sampling time and sampling site. The odds of shedding *S.* Bovismorbificans were 14.5 times higher in seals sampled in the late pupping season than in seals sampled in the early pupping season (OR=14.5, 95% CI: 1.72, 122.4, p=0.008). Seals sampled on the

141 tidal boulder beach were more likely to be shedding *S.* Bovismorbificans than those
142 sampled on the rocky pools and muddy/grassy slope (Table S 2).
143
144 In addition, *S.* Bovismorbificans was isolated from visceral tissues of three pups and *S.*
145 Typhimurium was isolated from the tissues of one pup at post mortem examination
146 (Table 1).
147
148 **Plasmid profiling of *Salmonella* isolates and antimicrobial sensitivity**
149 Plasmid profiling of the *S.* Bovismorbificans strains revealed three distinct plasmid
150 banding patterns (Table S 1). Plasmid profiling and phage typing of the five *Salmonella*
151 Typhimurium isolates revealed three distinct patterns (Table S 1). The five *S.*
152 Typhimurium isolates corresponded to phage definitive type (DT) 104 (n=3), DT1
153 (n=1) and DT41 (n=1). The three DT104 isolates were resistant to 8/14 antimicrobial
154 compounds (Table S 1) but all remaining isolates of *S.* Typhimurium, *S.*
155 Bovismorbificans and *S.* Haifa were susceptible to all antimicrobials tested.
156
157 **Pulsed-field gel electrophoresis (**PFGE**)**
158 PFGE of the 36 *S.* Bovismorbificans isolates (n=32 rectal swabs, n=3 viscera, n=1
159 sediment) grouped them into two pulsotypes, both of which were identical to pulsotypes
160 previously recorded by the Scottish *Salmonella*, *Shigella* and *Clostridium difficile*
161 Reference Laboratory (SSSCDRL). Thirty-five isolates were classed as pulsotype
162 BmoX9 and a single isolate (or singleton) from a live seal pup, stranded at St Cyrus,
163 Aberdeenshire, was classified as pulsotype BmoX4 (Figure 3). These two pulsotypes

were closely related (~90 % similarity) with a single band difference at either 763 kbp or 683 kpb for BmoX9 and BmoX4, respectively.

Within the SSSCDRL database, other BmoX9 pulsotypes have been recorded from faecal samples from cattle in Orkney and Caithness (10 isolates), a sheep from Caithness and visceral tissue samples from four grey seal pups sampled in 2010 (Table 2). BmoX4 pulsotype had previously been recorded in visceral organs of a single grey seal in 2010 and in faecal samples of cattle from Dumfries and Galloway (5 isolates) in 2008 (Table 2). All strains of *S.* Bovismorbificans in the SSSCDRL database were sensitive to all antimicrobials tested, with the exception of a single, multi-drug resistant strain of pulsotype BmoX12 isolated from a dog.

PFGE of the five *S.* Typhimurium isolates grouped them into 3 distinct pulsotypes which correlated with the phage type described above (Figure 1). All three PFGE patterns were indistinguishable from isolates previously recorded in the SSSCDRL database. All three DT104s had identical PFGE profiles and were attributed to pulsotypes STYMXB.001; the DT41 was attributed to STYMXB.0029 and the DT1 to STYMXB.0146, a PFGE pattern typical of DT41 isolates. Phage typing of the latter strain was repeated in light of this finding and the isolate was confirmed as DT1.

PFGE of the two isolates of *Salmonella* Haifa showed they were of the same pulsotype: HaiX9 (Figure 3). This PFGE pattern was indistinguishable from that of a previously reported *S.* Haifa isolate isolated from an adult human, submitted from the East of Scotland. Furthermore, this HaiX9 pulsotype was very similar to a pulsotype named

"HaiX9+" isolated from two male human patients with a history of recent travel to Pakistan.

Multilocus variable number of tandem repeat analysis (MLVA)

A minimum spanning tree was generated using the MLVA profiles of 921 *S.* Typhimurium isolates recorded in the SSSCDRL database sampled between 1990 and 2013. The three DT104 isolates had identical MLVA profiles and were indistinguishable from an isolate from a human child from South-East Scotland submitted to the SSSCDRL in 2011 (Figure 4). The closest non-human related strain in this database was a *S.* Typhimurium DT104 from a sheep in Caithness and the three DT104 isolates clustered with the majority of Scottish bovine, ovine and human DT104 isolates in the database.

The DT1 isolate shared a MLVA pattern with one other *S.* Typhimurium isolate in the SSSCDRL database: an environmental isolate of phage type DT195 (Figure 4). However, the PFGE profiles of these two isolates were distinct. The MLVA pattern of this DT1 isolate was closely related to two DT40s differing only at one MLVA locus.

The DT41 isolate did not share a MLVA pattern with any other *S.* Typhimurium isolates in the database but was closely related to a DT2 (one MLVA locus difference) and two DT40 isolates (two MLVA loci difference).

209

210 **Systemic infection**

211 *Salmonella* spp. was cultured from rectal swabs of 13 of 59 (22.0%) pups presented for
212 post-mortem examination. Three of the nine (33.3%) dead pups positive for *Salmonella*
213 Bovismorbificans on rectal swabs presented with a septicaemic spread of this bacteria
214 (bacteria present in more than one internal organ on culture); similarly one of the three
215 (33.3%) dead pups positive for *Salmonella* Typhimurium on rectal swabs presented
216 with a septicaemic spread of *S.* Typhimurium DT104.

217

218 Table S 4 details the organs from which *Salmonella* spp. were isolated in each of the
219 four septicaemic cases, the most significant lesions/cause of death and any concurrent
220 infections with other species of bacteria. Lesions associated with *S.* Bovismorbificans
221 septicaemia included omphalitis and peritonitis. Concurrent bacterial infections were
222 found in all 3 cases, with a noteworthy presence of *Streptococcus phocae*, *S. agalactiae*
223 and *Arcanobacterium phocae.* Lesions found in the seal pup presenting with *S.*
224 Typhimurium septicaemia included severe fibrino-necrotising interstitial pneumonia
225 and chronic-active encephalitis. In all 4 cases, concurrent bacterial species were isolated
226 from tissues (Table S4).

227

228 **Discussion**

229 **Prevalence and risk factors**

230 The higher prevalence of *Salmonella* spp. in grey seal pups exposed to seawater
231 compared to those not exposed suggests that seawater may be a source of exposure to
232 this pathogen. This finding parallels that in northern elephant seals where a similar

higher prevalence of *Salmonella* spp. was found in stranded pups when compared to pups remaining on their natal beach (Stoddard et al., 2005). Furthermore, *Salmonella* spp. have been shown to survive 8 weeks in seawater with little to no loss of total count (Hernroth et al., 2010). It is, however, important to note that other factors, such as stress from tidal displacement or increased contact due to crowding at high tide, may also play a role as higher stress levels may lead to decreased immune function, and ultimately, increased bacterial colonization/shedding.

Although not supported by statistical analyses, a high number of cases of *Salmonella* in live stranded grey seal pups was found in the South East region of Scotland compared to other areas (Figure 1). It is tempting to speculate that this is a reflection of the proximity to large areas of urbanisation and high human population density bordering the Forth and Tay estuaries. This mirrors the higher prevalence of enteric bacterial pathogens found in sea otters living in the more urbanised coastal regions of California's coastline (Miller et al., 2002). A larger, prospective study could be envisaged to investigate this hypothesis further by comparisons with seal colonies more remote to human habitation and effluent.

The lack of *Salmonella* spp. in samples from yearlings most likely indicates clearance of these bacteria from the gastrointestinal tract by one year of age. However, the possibility that the yearlings had never been exposed to *Salmonella* spp. or that they were infected but simply not shedding the bacterium could not be excluded. This finding parallels that of free-ranging California sea lion pups sampled on the Channel Islands, California, USA which had a 21% prevalence of *Salmonella* spp., compared to

a 0% prevalence in free-ranging adult California sea lions in Puget Sound, Washington, USA (Stoddard, DeLong, et al., 2008). Unfortunately, it was not clear whether this was an effect of age or geographical location as a negative association between *Salmonella* shedding and increasing host age may be possible (Stoddard, DeLong, et al., 2008). To investigate this hypothesis a longitudinal study of tagged animals would be required which would be feasible in grey seals as they return to their natal colony to breed.

Systemic infection

A third of the dead pups harbouring *Salmonella* Bovismorbificans (3/9) and *Salmonella* Typhimurium (1/3) had septicaemic spread of the bacteria. This finding confirms that both these isolates have the potential to cause septicaemia yet the trigger causing a switch between carriage and septicaemia is not clear. In particular, the apparent association of *S.* Bovismorbificans with other phocid pathogens such as *Streptococcus phocae* and *Arcanobacterium phocae*, bacterial species highly correlated with omphalitis (Baily, 2014), could indicate that septicaemic infection with *S.* Bovismorbificans occurs via the umbilicus rather than following systemic spread from an initial intestinal infection. Identifying which lesions are specifically caused by *Salmonella* spp. is challenging given the presence of concurrent bacterial infection in each case but with specific immunohistochemistry this may be possible.

S. Bovismorbificans

Salmonella Bovismorbificans was present in all study groups of grey seal pups and to the authors' knowledge, has not been reported in any other marine mammal species besides grey seals, harbour seals and a European otter (*Lutra lutra*) (Anderson et al.,

1979; Baker et al., 1980, 1995), all of which were reported in the UK. *S.* Bovismorbificans was first reported in grey seals in 1979 (Anderson et al., 1979) and has since been found in seals presenting with haemorrhagic gastroenteritis, focal hepatitis and sepsis as well as several species of sea birds (Anderson et al., 1979; Baker et al., 1980, 1995). PFGE typing of *Salmonella* Bovismorbificans isolates in the present study demonstrated only two, very similar, pulsotypes indistinguishable from strains isolated from cattle around Scotland. The predominance of one pulsotype, BmoX9 in this study could indicate that BmoX9 is circulating, and possibly maintained, in grey seal populations. However, the presence of identical pulsotypes in cattle and seals strongly suggests that *Salmonella* Bovismorbificans is likely to be circulating between grey seal and cattle populations. This hypothesis is further supported by the higher prevalence of *Salmonella* Bovismorbificans in grey seals exposed to seawater likely reflecting land-sea transfer of this pathogen from cattle.

It is noteworthy that, within Scotland, high densities of cattle farming are located on the East coast of the Scottish mainland and Orkney, areas in close proximity to grey seal populations. A larger, prospective study of *S.* Bovismorbificans in grey seals, fresh water outflow and coastal marine waters would be warranted in order to investigate a potential spatial or temporal correlation between *S.* Bovismorbificans in grey seals and in cattle.

S. Typhimurium

Molecular subtyping of *S.* Typhimurium isolates in seals identified closely related strains in human cases, livestock and wild birds. Reports of *S.* Typhimurium in marine mammals in UK coastal waters are uncommon but include a *S.* Typhimurium phage

type 49 in 3 of 208 (1.4%) free-ranging harbour seals in the Wash, Norfolk at a time when ST49 was a relatively common isolate in human laboratory submissions (Baker et al., 1995) and a DT104 was isolated from a 12 week old stranded grey seal pup, known to have been born on the Isle of May (Foster et al., 1998). These findings prompted debate as to whether this reflected exposure of harbour seals to untreated sewage or whether *S.* Typhimurium was enzootic in harbour seal populations (Baker et al., 1995; Foster et al., 1998). Two separate studies of *Salmonella enterica* from wild birds in Great Britain showed *S.* Typhimurium DT41 and DT40 (Pennycott et al., 2006), and DT40 and DT56 (Pennycott et al., 2002; Lawson et al., 2011, 2014; Horton et al., 2013) circulate widely in wild birds. Given the close interactions between grey seals and seabirds on the Isle of May colony, the contribution of wild birds to the spread of *Salmonella* in this ecosystem warrants further investigation.

S. Haifa

Salmonella Haifa was first described in 1950 in Israel, isolated from a 3 year old child with enteritis (Sapiro and Hirsch, 1950). It has since been isolated from food animals, slaughterhouse personnel and retail meat products worldwide (Tuchili et al., 1996; Zewdu and Cornelius, 2009). The pathogenicity of this bacterium is largely unknown although fatal infection with *S.* Haifa was reported in a 76 year old man in Japan, along with concurrent infection of his 1 year old grandson (Kaibu et al., 2005). Given that the *S.* Haifa isolated in the present study was indistinguishable from that found in a human patient with very close spatial and temporal distribution it is tempting to speculate that one or the other species represented a source of contamination for the other.

Antimicrobial resistance

Very little antimicrobial resistance was found in the *Salmonellae* isolated. This mirrors the study by Stoddard *et al.* in elephant seals and likely indicates a lack of selection pressure in wild animals (Stoddard, DeLong, et al., 2008). In the present study antimicrobial resistance was only recorded in the two isolates of *S.* Typhimurium DT104 which were highly multidrug resistant, as is characteristic of this phage type (Threlfall, 2000).

Conclusion

This study documents the prevalence of *Salmonella enterica* in free-ranging and stranded grey seal pups on a natal colony in Scotland and at a rehabilitation centre during the 2011 breeding season. Molecular typing of bacterial strains revealed close similarities with isolates of terrestrial mammalian origin, raising concerns of anthropogenic microbial environmental pollution from activities such as farming and sewerage discharge, with a strong suspicion of land-sea transfer of *Salmonella* Bovismorbificans from cattle.

Experimental procedures

Animals and Samples

Over a 6 week period in Autumn 2011, rectal swabs were taken from 50 dead grey seal pups, 90 live, apparently healthy grey seal pups and 19 live yearling grey seals on their natal colony, the Isle of May, Scotland, UK and placed into Amies medium with charcoal (Medical Wire & Equipment, Corsham, UK). Live grey seal pups were sampled from three distinct sites on the Isle of May with different substrate

353 characteristics (tidal boulder beach; muddy/grassy slope and stagnant rocky pools) and
354 at three different time points (early, mid and late pupping season). Three sediment
355 samples were taken also from each of two pupping locations within the colony
356 (muddy/grassy slope and rocky pools). Concurrently, rectal swabs were taken from 26
357 live grey seal pups found stranded along the Scottish coastline (Figure 1) which had
358 been transported to the Scottish Society of Prevention of Cruelty to Animals (SSPCA)
359 National Wildlife Rescue Centre (then located at Dunfermline, Fife, Scotland, UK) for
360 rehabilitation. Pups were sampled within 24 hours of arrival at the rehabilitation centre
361 and were not treated or co-habited until after sampling. Nine grey seal pups that
362 subsequently died or were euthanised on humane grounds were also sampled within 48h
363 of death.
364
365 For all animals the following data were systematically recorded: sex, sampling or
366 stranding location (expressed as decimal degrees longitude and latitude), sampling date,
367 mass (to the nearest 100g) and pup development stage code (as defined previously by
368 Kovacs and Lavigne (1986). A full post-mortem examination was performed on the 59
369 dead pups (colony n=50; rehabilitation n=9). Samples of liver, spleen, brain and lung
370 were systematically collected, frozen at -80°C and submitted to the Scottish Marine
371 Animal Stranding Scheme, SAC Consulting Veterinary Services for bacteriology
372 following routine methods. Formalin fixed samples of 26 organs were collected and
373 processed routinely for histopathology.
374
375 To standardise sampling between field conditions and rehabilitating animals, faecal
376 swabs were placed into Selenite F broth (E and O Laboratories, Bonnybridge, Scotland)

and incubated aerobically at 37 °C for 24 h. The top 1 ml of broth was collected and frozen at -80°C in 20% glycerol (Sigma-Aldrich, Poole, UK) until required. Ten microlitres of each enriched selenite F broth was subsequently cultured on brilliant green agar plates (Oxoid, Basingstoke, UK) and incubated aerobically at 37 °C for 18-24 h. Up to 4 suspect *Salmonella* colonies per case were sub-cultured on MacConkey agar plates (Oxoid) for 18-24 h at 37 °C and resulting growth was assessed visually for purity. Isolates were identified using an API 10S strip (BioMerieux, Basingstoke, UK) and serotyped by the White-Kauffmann-LeMinor classification scheme using specific O and H *Salmonella* antisera (Remel Europe Ltd, Dartford, UK) (Guibourdenche et al., 2010). Positive isolates were frozen on Microbank beads (Pro-lab Diagnostics, Neston, UK) at -80°C while awaiting further classification.

Identification of *Salmonella* isolates

Cultures were submitted to the Scottish *Salmonella*, *Shigella* and *Clostridium difficile* Reference Laboratory (SSSCDRL), Stobhill, Glasgow, Scotland, UK where serotyping was confirmed using commercial antisera (Bioconnections UK, Knypersley, UK; Remel Europe Ltd; Pro-lab diagnostics, Wirral UK and BD Diagnostics, Oxford, UK), plasmid profiling and phage typing of the *Salmonella* Typhimurium isolates were performed using standard procedures (Rabsch, 2007). Antimicrobial susceptibility was determined by breakpoint agar incorporation of antimicrobials for 14 agents, using a predetermined concentration of antimicrobial. The breakpoint for ampicillin was 8 µg/mL, chloramphenicol 1 µg/mL, cefotaxime 1 µg/mL, ciprofloxacin (low dose) 0.125 µg/mL, ciprofloxacin (high dose) 1 µg/mL, furazolidone 8 µg/mL, gentamicin 4 µg/mL, kanamycin 16 µg/mL, nalidixic acid 16 µg/mL, netilmicin 20 µg/mL, spectinomycin 64

µg/mL, streptomycin 16 µg/mL, sulfamethoxazole 64 µg/mL, tetracycline 8 µg/mL and trimethoprim 2 µg/mL. Pulsed-field gel electrophoresis for all *S.* Bovismorbificans and *S.* Typhimurium isolates was carried out as described previously (Ribot et al., 2006), except for *S.* Haifa isolates which had thiourea (VWR, Lutterworth, UK) added to the electrophoresis buffer at a concentration of 200 µM due to their known high susceptibility to genomic DNA degradation (Liesegang and Tschape, 2002).

Images of the gels were analysed using the software Bionumerics Version 6.6 (Applied Maths, Kortrjk, Belgium) with optimization set at 1.3 % and band tolerance at 1 %. Relationships were determined by Dice correlation and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering. Only restriction fragments of >33.3 kb were included in the analysis. Pulsotypes were compared to those stored in the SSSCDRL database and the PulseNET international database (http://www.pulsenetinternational.org/). The STYMXB nomenclature of PFGE profiles is based on the SalmGene classification (Peters et al., 2003), now superseded by PulseNet International. BmoX (Bovismorbificans isolate X) and HaiX (Haifa isolate X) designations are specific to the Scottish database and were employed when there were no matches for a profile in the PulseNet database.

Salmonella Typhimurium isolates were further characterized using MLVA, by following the standardized procedure established by PulseNet (ECDC and ECDC, 2011). Data were analysed using Bionumerics 6.6 and compared to those stored in the SSSCDRL database. Minimum spanning trees were generated with Bionumerics 6.6 using categorical coefficient and UPGMA clustering.

425

426 **Statistical analysis**

427 Prevalence data were analysed by Fisher's exact tests using the R statistical software
428 package (R Core Team, 2013). Overall prevalence and odds ratios were calculated using
429 a generalized linear model (GLM) with a binomial family and a logit link function. Site,
430 sampling time, pup stage and the interactions between them were used as fixed
431 explanatory factors.

432

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446 was carried out under UK Home Office Project (No. 60/4009) and Personal Licences as
447 issued to the Sea Mammal Research Unit under the Animals (Scientific Procedures)

448 Act, 1986. Stranded grey seal pups were sampled as part of the routine health

449 assessment procedure.

Table and figure legends:

Table 1 *Salmonella* prevalence in different groups. Numbers (% of animals positive for each *Salmonella* spp. within the group of interest); [95% confidence interval of percentage positive animals]; * one seal was positive for 2 isolates; ** The positive sediment sample was taken from the Stagnant rocky pool site.

Table 2 List of the 19 PFGE XbaI patterns (pulsotypes) of *Salmonella* Bovismorbificans in the SSSCDRL database and corresponding host species. The two pulsotypes of *S.* Bovismorbificans found in grey seal pups in this study (BmoX4 and BmoX9) are shaded in grey. Isolates from the present study are not included in this table.

Figure 1 Map of stranding locations of grey seals sampled for *Salmonella* spp. Dots represent stranding location of live grey seal pups before transport to the rehabilitation centre. Red: Isolation of *Salmonella* spp. from rectal swab; Blue: No *Salmonella* isolated from rectal swab.

Figure 2 Map of locations of free ranging grey seals sampled for *Salmonella* spp. on the Isle of May. Individual dots represent locations in which dead pups were found; pie charts represent live seal pups sampled at each of the three different sites (n=30 per sampling site). Red dot or red proportion of pie chart: Isolation of *Salmonella* spp. on rectal swab; Blue dot or blue proportion of pie chart: No *Salmonella* isolated from rectal swab.

Figure 3 Dendrogram and PFGE patterns of 43 *Salmonella* spp. isolates found in grey seals and sediment in this study restricted with XbaI. Cluster analysis was performed with UPGMA using the Dice coefficient, a tolerance level of 1% and an optimisation level of 1.3%. For comparison, 5 isolates originating from grey seal pups sampled in 2010 submitted by the Scottish Marine Animal Stranding Scheme are included in this dendrogram (M274/10/1, M275/10/1, M302/10/1, M302/10/3 and M284/11/1). Serovar, phage type, pulsotype, origin of the sample and case reference/animal reference are listed. The scale at the top indicates the similarity indices (in percentages) between isolates. *A co-culture of *S.* Bovismorbificans and *S.* Typhimurium was isolated from pup A023.

Figure 4 Minimum spanning tree established using MLVA profiles of 921 *Salmonella* Typhimurium isolates (1990 – 2013). Node size is proportional to the number of isolates belonging to each MLVA type. MLVA types differing at a single locus are separated by a thick branch; MLVA types differing at more than one locus are represented by a thin branch. MLVA types of isolate CD016 (DT1) is coloured in dark blue; isolate from CD048 (DT41) is coloured in green and the three DT104 isolates from this study are coloured in red. MLVA types for all previously recorded DT104 isolates in the SSSCDRL database are coloured in pink. MLVA types for all previously recorded DT56, DT40 or DT41 isolates in the SSSCDRL database are coloured in pale blue. MLVA types for all previously recorded DT2 isolates in the SSSCDRL database are coloured in pale yellow. All other isolates remain white.

Supplementary files:

Figure S 1 Dendrogram and PFGE patterns of the 19 *Salmonella* Bovismorbificans pulsotypes recorded in the SSSCDRL database restricted with XbaI. Cluster analysis was performed with UPGMA using the Dice coefficient, a tolerance level of 1% and an optimisation level of 1.3%. The scale at the top indicates the similarity indices (in percentages) between isolates.

Table S 1 Serovars, plasmid profiles, phage types, antimicrobial resistance characteristics, MLVA profile, origins and numbers of *Salmonella enterica* ssp. *enterica* isolates identified from grey seal rectal swabs, visceral organs and sediment in this study. Antimicrobials: A: Ampicillin, C: Chloramphenicol, Na: Nalidixic acid, Sp: Spectinomycin, St: Streptomycin, Su: Sulphamethoxazole, Tc: Tetracycline, CpL: Ciprofloxacin low dose. NA: None applicable.

Table S 2 Categorical risk factors, using univariate analysis, for grey seals that are harbouring *Salmonella* spp.. N: number of animals per group; OR: odds ratio; 95% CI: 95% confidence interval of OR; Sign: Statistical significance of results; NS: non-significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Table S 3 Multivariate logistic regression analysis showing the factors associated with risk of carrying *Salmonella* Bovismorbificans in free-ranging live grey seal pups. S.E.: standard error or coefficient; 95% CI: 95% confidence interval; OR: odds ratio; Sign:

521 Statistical significance of results; NS: not significant; *: $p<0.05$; **: $p<0.01$; ***:

522 $p<0.001$.

523

524 Table S 4 Pathological findings, concurrent bacteriology results in 4 seals with

525 septicaemic spread of *Salmonella* spp. Br: Brain, Li: Liver, Lu: Lung, Sp: Spleen.

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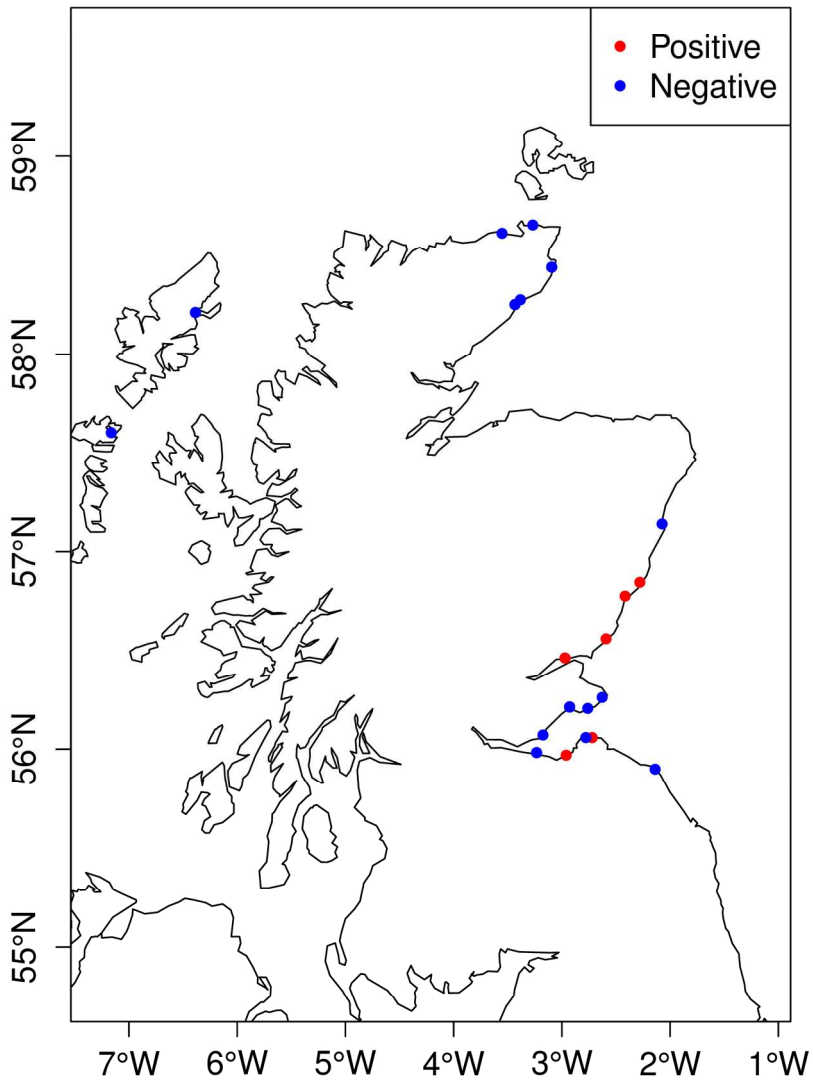
Table 1 *Salmonella* prevalence in different groups. Numbers (% of animals positive for each *Salmonella* spp. within the group of interest); [95% confidence interval of percentage positive animals]; * one seal was positive for 2 isolates; ** The positive sediment sample was taken from the Stagnant rocky pool site.

			Yearlings	Pups					Sediment
	N Isolates	All seals combined (n=194)	Isle of May Live (n=19)	All pups (n=175)	Isle of May Dead (n=50)	Isle of May Live (n=90)	Rehabilitation Dead (n=9)	Rehabilitation Live (n=26)	Isle of May (n=5)
***Salmonella* spp.**	**39**	**37 (19.1%)***	**0 (0%)**	**37 (21.1%)**	**13 (26%)**	**16 (17.8%)**	**1 (11.1%)**	**7 (26.9%)**	**1****
95% CI		[14.2, 25.2%]	[0, 16.8%]	[15.7, 27.8%]	[15.9, 39.6%]	[11.2,39.6%]	[0.5, 43.5%]	[13.7,46.1%]	
***Salmonella* Bovismorbificans**	**33**	**32 (16.5%)**	**0 (0%)**	**32 (18.3%)**	**9 (18%)**	**15 (16.6%)**	**1 (11.1%)**	**7 (26.9%)**	**1****
95% CI		[11.9, 22.4%]	[0, 16.8%]	[13.3, 24.7%]	[9.7, 30.8%]	[10.4, 25.7%]	[0.5, 43.5%]	[13.7,46.1%]	
***Salmonella* Typhimurium**	**4**	**4 (2.1%)**	**0 (0%)**	**4 (2.3%)**	**3 (6%)**	**1 (1.1%)**	**0 (0%)**	**0 (0%)**	**0**
95% CI		[0.8, 5.2%]	[0, 16.8%]	[0.8,5.7%]	[2.1, 16.2%]	[0.05, 6%]	[0, 29.9%]	[0, 12.9%]	
***Salmonella* Haifa**	**2**	**2 (1%)**	**0 (0%)**	**2 (1.1%)**	**1 (2%)**	**0 (0%)**	**0 (0%)**	**1 (3.8%)**	**0**
95% CI		[0.3, 4.1%]	[0, 16.8%]	[0.3, 4.1%]	[0.1, 10.5%]	[0, 4.1%]	[0, 29.9%]	[0.02,18.9%]	

Table 1 List of the 19 PFGE XbaI patterns (pulsotypes) of *Salmonella* Bovismorbificans in the SSSCDRL database and corresponding host species. The two pulsotypes of *S.* Bovismorbificans found in grey seal pups in this study (BmoX4 and BmoX9) are shaded in grey. Isolates from the present study are not included in this table.

PFGE-XbaI-Pattern	Host species (number of isolates)	Additional comments
BmoX1	Human (1)	Recent travel: Kenya
BmoX2	Grey seal (1)	Nasal swab
BmoX3	Human (2)	
BmoX4	Grey seal (1), Bovine (5)	
BmoX5	Human (1)	
BmoX6	Human (1)	Recent foreign travel*
BmoX7	Human (1)	
BmoX8	Human (1)	Recent travel: Lebanon
BmoX9	Grey seal (4), Cattle (10), Ovine (1)	
BmoX10	Human (1)	
BmoX11	Human (1)	
BmoX12	Human (2), Canine (2)	
BmoX13	Human (1)	Recent travel: Thailand
BmoX14	Human (1)	Recent foreign travel
BmoX15	Human (1)	Recent travel: Sri Lanka
BmoX16	Human (3)	
BmoX17	Human (1)	Recent travel: Malaysia
BmoX18	Human (1)	
BmoX19	Human (1)	
BmoX20	Human (1)	

*Destination unknown



Map of stranding locations of grey seals sampled for Salmonella spp. Dots represent stranding location of live grey seal pups before transport to the rehabilitation centre. Red: Isolation of Salmonella spp. from rectal swab; Blue: No Salmonella isolated from rectal swab.
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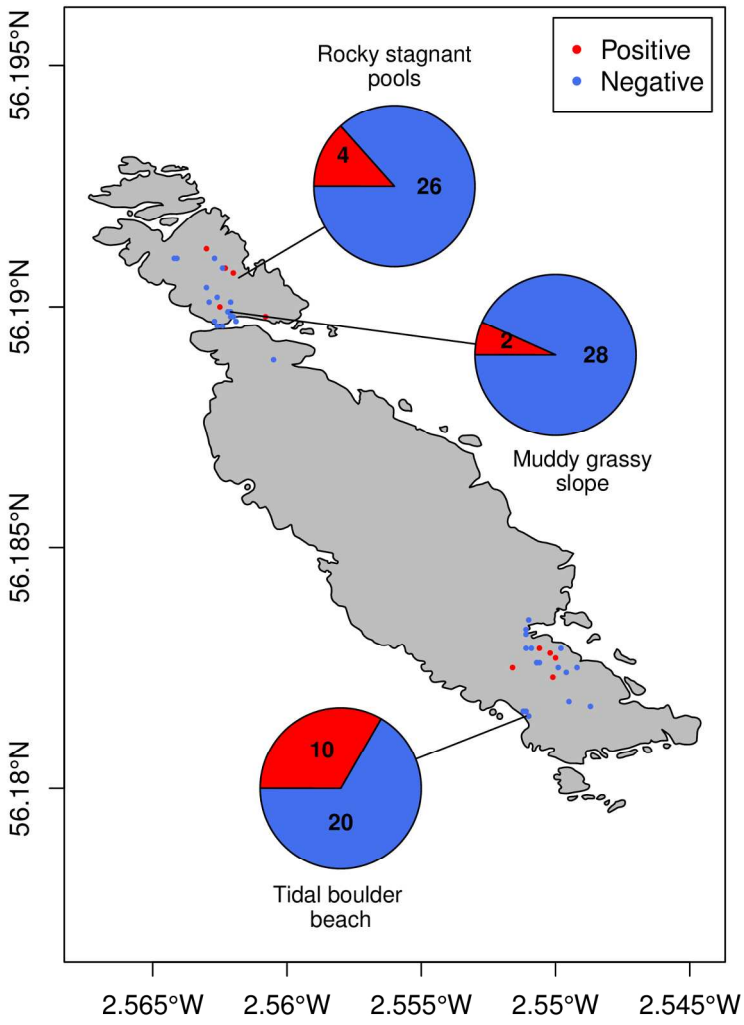

Map of locations of free ranging grey seals sampled for Salmonella spp. on the Isle of May. Individual dots represent locations in which dead pups were found; pie charts represent live seal pups sampled at each of the three different sites (n=30 per sampling site). Red dot or red proportion of pie chart: Isolation of Salmonella spp. on rectal swab; Blue dot or blue proportion of pie chart: No Salmonella isolated from rectal swab.

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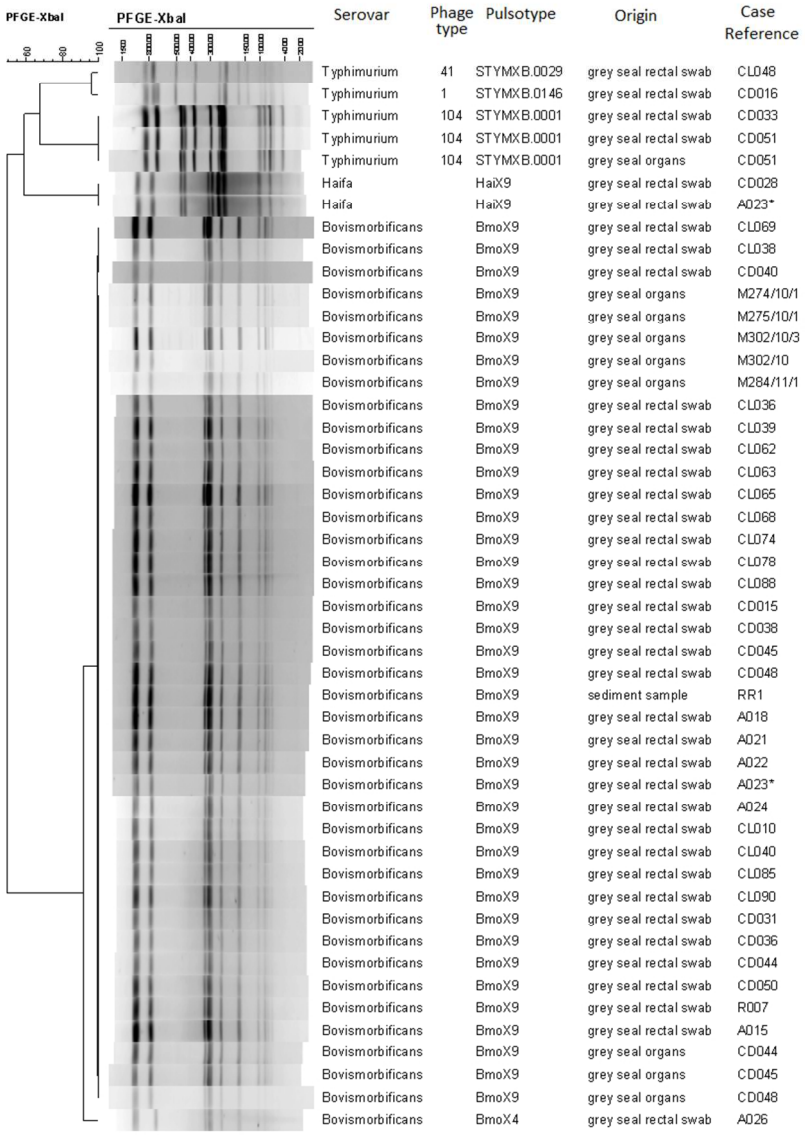
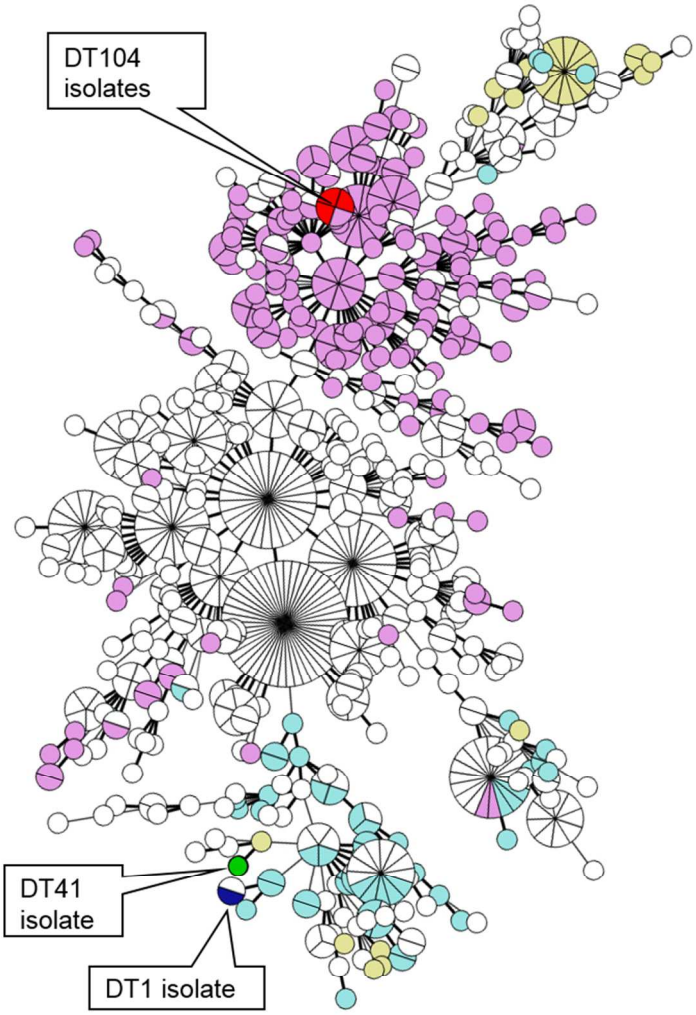


Dendrogram and PFGE patterns of 43 Salmonella spp. isolates found in grey seals and sediment in this study restricted with XbaI. Cluster analysis was performed with UPGMA using the Dice coefficient, a tolerance level of 1% and an optimisation level of 1.3%. For comparison, 5 isolates originating from grey seal pups sampled in 2010 submitted by the Scottish Marine Animal Stranding Scheme are included in this dendrogram (M274/10/1, M275/10/1, M302/10/1, M302/10/3 and M284/11/1). Serovar, phage type, pulsotype, origin of the sample and case reference/animal reference are listed. The scale at the top indicates the similarity indices (in percentages) between isolates. *A co-culture of S. Bovismorbificans and S. Typhimurium was isolated from pup A023.
183x256mm (120 x 120 DPI)



Minimum spanning tree established using MLVA profiles of 921 Salmonella Typhimurium isolates (1990 – 2013). Node size is proportional to the number of isolates belonging to each MLVA type. MLVA types differing at a single locus are separated by a thick branch; MLVA types differing at more than one locus are represented by a thin branch. MLVA types of isolate CD016 (DT1) is coloured in dark blue; isolate from CD048 (DT41) is coloured in green and the three DT104 isolates from this study are coloured in red. MLVA types for all previously recorded DT104 isolates in the SSSCDRL database are coloured in pink. MLVA types for all previously recorded DT56, DT40 or DT41 isolates in the SSSCDRL database are coloured in pale blue. MLVA types for all previously recorded DT2 isolates in the SSSCDRL database are coloured in pale yellow. All other isolates remain white.
201x245mm (120 x 120 DPI)