Experimental reproduction of bovine *Salmonella* encephalopathy using a norepinephrine-based stress model

Z.P. McCuddin 1,2, S.A. Carlson 1,*, Vijay K. Sharma

Pre-harvest Food Safety and Enteric Disease Research Unit, National Animal Disease Center, Agricultural Research Service, USDA, Ames, IA 50010, USA

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Abstract

Neurological disease represents a sporadic but serious manifestation of bovine salmonellosis that is thought to be related to systemic infection. *Salmonella enterica* serovar Dublin (*S*. Dublin) is the serovar most associated with systemic infection in cattle, although reports of neurological disease associated with *S*. Dublin or any other serovar are rare and usually anecdotal. This study reports the involvement of three strains of *S. enterica*, serovars Saintpaul, Montevideo, and Enteritidis, in *Salmonella* encephalopathies. Encephalopathies were reproduced in calves using a norepinephrine-based stress model. Neurological signs were not observed in calves infected with control strains of *S. enterica*, including *S*. Dublin, or in calves infected with clinical strains in the absence of norepinephrine. Therefore, norepinephrine may play a role in *Salmonella* encephalopathies.

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Keywords: *Salmonella*; Encephalopathy; Bovine; Stress; Norepinephrine

1. Introduction

Neurological manifestations of salmonellosis are usually associated with host-adapted serovars, e.g., *Salmonella enterica* serovar Dublin (*S*. Dublin) in cattle and serovar Choleraesuis (*S*. Choleraesuis) in pigs (Steinbach et al., 2000). Systemic infection, with resultant compromise of the blood-brain barrier, can subsequently lead to encephalopathies in these animals. For non-host-adapted serovars, neurological disease is less likely, since systemic infection is less frequent (Paulin et al., 2002). *S. enterica* serovar Typhimurium (*S*. Typhimurium) can become systemic in cattle (Rasmussen et al., 2005), although there are no specific reports of *S*. Typhimurium-associated bovine neurological disease.

*Salmonella* encephalopathies involving non-host-adapted strains have been sporadically reported in the literature. A report of encephalopathy in pigs involved an outbreak of *S. enterica* serovar Enteritidis (*S*. Enteritidis) meningitis in one-week-old animals that exhibited circling (Reynolds et al., 1968). A fatal *S. enterica* serovar Newport-associated meningitis has been reported in a neonatal alpaca (D’Alterio et al., 2003). Finally, *S*. Enteritidis-associated meningoencephalomyelitis was identified in a neonatal seal (Stroud and Roelke, 1980).

The aim of this study was to evaluate, via in vivo infection, the neuropathogenicity for calves of three strains of *S. enterica* (serovars Enteritidis, Montevideo and Saintpaul) obtained from clinical cases of bovine encephalopathy.

2. Materials and methods

2.1. Bacterial strains

Three bovine isolates of *S. enterica* (serovars Enteritidis, Montevideo and Saintpaul) were obtained from clinical...
cases in which calves displayed neurological signs ranging from head tilt and ataxia to partial blindness. The three isolates were obtained from samples submitted by veterinary practitioners dealing with independent outbreaks of *Salmonella* encephalopathy in Minnesota and Wisconsin in the USA. All three isolates were serotyped by the National Veterinary Services Laboratories in Ames, Iowa. These three strains were given the “NPG” (neuropathogenic) designation for strain identification purposes.

Table 1 summarises the clinical findings in cattle associated with field outbreaks from which the three strains of *S. enterica* reported in this study were isolated. In all three outbreaks, morbidity was moderate (30–60%), while mortality was low (0–5%). Clinical signs included diarrhoea, head tilt, ataxia, auditory hyper-responsiveness and partial blindness. Brain histology was unremarkable. All three strains were isolated from faeces of affected animals.

Salmonella Reference Collection B (SARB) (Pabbaraju et al., 2000) and other control strains were obtained from frozen stock cultures at the National Animal Disease Center, Ames, Iowa (Table 1). All strains, except for *S. Typhimurium* DT104 (98–420) recovered from protozoa, exhibited similar invasive characteristics (data not shown) as determined by a tissue culture invasion assay (Rasmussen et al., 2005). Antibiotic resistance was determined using CLSI breakpoints (CLSI, 2001) by growing cultures in Mueller-Hinton broth (Difco) using twofold serial dilutions.

### 2.2. Experimental design

All in vivo experiments were carried out on *Salmonella*-free Jersey or Holstein Friesian calves 1–10 weeks of age and weighing 30–100 kg. Animals were challenged orally with $10^9$ colony forming units (CFUs) of *S. enterica* grown aerobically for 16 h at 37°C (Carlson et al., 2002).

For each strain, six calves were given daily intramuscular (IM) doses of norepinephrine (noradrenaline, 45 μg/kg; Sigma) starting at the day of infection and continuing until euthanasia, while six calves simultaneously received a placebo (saline). As a separate control, four calves were given norepinephrine without any pathogen. Additionally, three calves were challenged with *S. Saintpaul* NPG and given daily doses of dexamethasone (0.1 mg/kg, IM; Vedco).

Upon observation of neurological signs (4–9 days post-infection), the behaviour of affected calves was photo-documented and subjects were euthanased immediately. Calves that did not display neurological signs were euthanised at 12–14 days post-infection, i.e., 72–96 h after the norepinephrine-treated animals. Euthanasia was performed using xylazine (0.45 mg/kg, IM; Phoenix Laboratories) and pentobarbital (1.2 mg/kg, intravenous; Fort Dodge Laboratories). Animal experiments were approved by the Animal Care and Use Committee at the National Animal Disease Center (Protocol 3462).

### 2.3. Collection of samples

Brain tissue (one cerebral hemisphere) was immediately fixed in 10% buffered formalin for histopathology. The other cerebral hemisphere, cerebrospinal fluid (CSF) taken from the lateral ventricles, blood and faecal samples were collected for isolation of *Salmonella* spp. Blood samples were also collected for haematological examinations (performed by the Clinical Pathology Laboratory at the College of Veterinary Medicine, Iowa State University, Ames, Iowa), including complete blood counts and blood biochemistry analysis.

### Table 1

**Summary of *Salmonella* spp. strains used for inoculations in this study**

<table>
<thead>
<tr>
<th>Strain (Unique antibiogram)</th>
<th>Source or reference</th>
<th>Clinical findings from field cases or laboratory studies</th>
<th>Morbidity and mortality from field cases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. Saintpaul</em> SARB 55 (FKNxT)</td>
<td>SARB collection</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. Montevideo</em> SARB 30 (ANx)</td>
<td>SARB collection</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. Enteritidis</em> SARB 15 (KNx)</td>
<td>SARB collection</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. Saintpaul</em> NPG; Clinical isolate (AFCxT)</td>
<td>4 month old Holstein calf; Wisconsin (1 of 12 calves affected)</td>
<td>Diarrhoea, head tilt, ataxia, auditory hyper-responsiveness, partial blindness</td>
<td>40% morbidity, 0% mortality</td>
</tr>
<tr>
<td><em>S. Montevideo</em> NPG; Clinical isolate (KNx)</td>
<td>1 month old Holstein calf; Minnesota (1 of 6 calves affected)</td>
<td>Diarrhoea, head tilt, ataxia</td>
<td>60% morbidity, 5% mortality</td>
</tr>
<tr>
<td><em>S. Enteritidis</em> NPG; Clinical isolate (G)</td>
<td>3 month old Hereford calf; Minnesota, USA (1 of 4 calves affected)</td>
<td>Diarrhoea, head tilt</td>
<td>30% morbidity, 0% mortality</td>
</tr>
<tr>
<td><em>S. Dublin</em> strain 9276 (AFT)</td>
<td>Helmhut and Seiler (1986)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. Typhimurium</em> DT104 strain 98–420 (AFT)</td>
<td>Rasmussen et al. (2005)</td>
<td>Profuse diarrhoea, bacteraemia, severe pyrexia</td>
<td>NA</td>
</tr>
</tbody>
</table>

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*a* A: Ampicillin; Cx: Ceftriaxone; F: Florfenicol/Chloramphenicol; G: Gentamicin; K: Kanamycin; Nx: Nalidixic acid; T: Tetracycline.

*b* Clinical findings from field outbreaks (*S. Saintpaul*, *S. Montevideo* or *S. Enteritidis*) or in vivo studies (DT104); NA, Not applicable.

*c* NA, Not applicable.
2.4. Neurohistopathological examinations

Following formalin fixation, brain tissues were processed for routine histopathology (Hamir et al., 2006), embedded in paraffin wax and sectioned at 5 μm. The sections were stained with haematoxylin and eosin (H&E). Ten to twelve different representative sections were microscopically examined for pathological changes, as described previously (Stroud and Roelke, 1980).

2.5. Qualitative isolation of Salmonella spp.

Salmonella spp. were selectively cultured by inoculating 3–5 g samples into 100 mL GN Hajna broth (Difco), incubating overnight at 37°C aerobically, then transferring 100 μL of the inoculum into 5 mL Rappaport-Vassiliadis R10 broth (Difco) which was also grown aerobically at 37°C overnight. Cultures were then transferred to Brilliant Green Sulpha (BGS) agar (Difco) plates which were incubated at 37°C overnight. Individual colonies recovered from selective plates were grown overnight in Lennox L broth (Difco) and identified using Salmonella-specific polymerase chain reaction (PCR) targeting the sipB-sipC junction (Carlson et al., 1999). All media included antibiotics specific to the individual isolate’s antibiogram.

2.6. Quantification of Salmonella spp. isolated from brain, blood and faeces

Since there were relatively low numbers of Salmonella spp. in blood and brain, these samples were directly plated onto BGS agar. For blood samples, 10 mL was plated onto 10 separate BGS plates. For brain samples, a 50–75 g sample was homogenised, filtered with sterile cheesecloth and then plated onto 10 BGS plates. For faecal samples, a 1 g sample was inoculated into 100 mL phosphate buffered saline and then 100 μL aliquots were plated onto 10 BGS plates.

Colonies were enumerated the following day. The identity of Salmonella strains was confirmed using antisera/agglutination-based serogrouping (Difco), along with confirmation of the original antibiogram for each isolate.

2.7. Statistical analysis

Statistical analysis was performed using an analysis of variance (ANOVA) with Scheffe’s F test for multiple comparisons (StatView, SAS). Comparisons were made between strains and between norepinephrine-treated and untreated animals.

3. Results

3.1. Neurological signs in calves infected with NPG strains of Salmonella spp.

Clinical and post mortem findings for in vivo studies are reported in Table 2 and in the supplemental video. The onset of neurological signs ranged from 4 days in S. Saintpaul NPG infection to 9 days for S. enterica serovar Montevideo (S. Montevideo) NPG infection. The range of neurological signs varied between strains (Fig. 1). The greatest number of signs was observed in calves infected with S. Montevideo NPG, which had the latest onset of signs. Neurological signs were evident in 18 of 18 calves that were infected with one of the clinical isolates and given norepinephrine. Only one of three dexamethasone-treated calves displayed neurological signs. Control calves, which were infected with one of the three clinical strains but did not receive norepinephrine, did not exhibit neurological signs (Table 2).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Drug</th>
<th>Number of animals displaying neurological signs and types of signs</th>
<th>Blood culture</th>
<th>Brain culture</th>
<th>Fecal culture</th>
<th>Brain pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NE</td>
<td>0 of 4</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>S. Saintpaul NPG</td>
<td>NE</td>
<td>6 of 6; Tonic–clonic seizures, PPD, ATX, ear fluttering</td>
<td>+ (1 of 3)</td>
<td>+ (1 of 3)</td>
<td>+ (all 3)</td>
<td>None</td>
</tr>
<tr>
<td>S. Montevideo NPG</td>
<td>Dex</td>
<td>6 of 6; PPD, hyperaesthesia, opisthotonus, ear fluttering, head tilt, ATX</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>S. Enteritidis NPG</td>
<td>NE</td>
<td>6 of 6; ATX, ear fluttering, rigors, hyperaesthesia, tonic–clonic seizures, PPD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>Three NPG strains</td>
<td>PL</td>
<td>0 of 6 for each strain</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>Three SARB strains</td>
<td>NE</td>
<td>0 of 6 for each strain</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>Three SARB strains</td>
<td>PL</td>
<td>0 of 6 for each strain</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>DT104 (98–420) NPG</td>
<td>NE</td>
<td>0 of 3</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>DT104 (98–420) PL</td>
<td>PL</td>
<td>0 of 3</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>S. Dublin 9276 NPG</td>
<td>NE</td>
<td>0 of 3</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>S. Dublin 9276 PL</td>
<td>PL</td>
<td>0 of 3</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>None</td>
</tr>
</tbody>
</table>

NPG, Neuropathogenic strain; PPD, Proprioceptive placing deficit; ATX, Ataxia; ND, Not determined.
3.2. Absence of neurological signs in calves infected with the SARB strains, DT104 (98–420) or S. Dublin 9276

Neurological signs were absent in calves inoculated with the corresponding SARB strains, regardless of norepinephrine treatment. These calves were euthanased at 12–14 days post-infection and only faecal cultures were positive for Salmonella spp.

Additionally, calves were infected with S. Typhimurium DT104 (98–420) recovered from protozoa. As we have shown recently, DT104 is more virulent after exposure to protozoa, whereby more of the pathogen is recovered from spleen and lymph nodes (Rasmussen et al., 2005). Table 2 illustrates a lack of Salmonella encephalopathy in calves infected with DT104 recovered from protozoa.

The stress model was also applied to calves infected with the bovine-adapted strain S. Dublin 9276. As shown in Table 2, neurological signs were not manifested.

3.3. Qualitative bacterial isolation from blood, brain and faeces

For all norepinephrine-treated calves infected with clinical (NPG) isolates and for one dexamethasone-treated calf infected with S. Saintpaul NPG, cultures from faeces, blood and brain samples were positive for Salmonella spp. (Table 2), although very few organisms were isolated from the brain (Fig. 2). CSF was negative for Salmonella spp. in all calves (data not shown). Control calves, which were infected with one of the three clinical NPG strains but did not receive norepinephrine or dexamethasone, only had faecal cultures that were positive for Salmonella spp. The same results were observed for the SARB strains (S. Saintpaul SARB 55, S. Montevideo SARB 30 and S. Enteritidis SARB 15), regardless of norepinephrine treatment.

In S. Typhimurium DT104 infections, calves treated with norepinephrine had positive blood cultures, whereas calves that did not receive norepinephrine had negative

![Observations of neurological signs in calves infected with clinical isolates of Salmonella spp.](image)
blood cultures. DT104 (98–420) was also isolated from faeces but not from the brain.

S. Dublin was isolated from the blood and faeces of all six calves (i.e., norepinephrine-treated and untreated calves). However, brain cultures were negative (Table 2).

3.4. Brain pathology

Histologically, the brain was unremarkable in calves displaying neurological signs. Minor intracerebral hemorrhage was observed in both treated and control animals (data not shown) and was considered likely to be an artefact from euthanasia.

3.5. Haematological examinations

Haematological data were determined for the initial set of calves infected with S. Saintpaul NPG. All three norepinephrine-treated calves showed an inflammatory leukogram with a left shift, while blood biochemistries were unremarkable (data not shown).

3.6. Enumeration of Salmonella spp. isolated from brain, blood and faeces

The clinical isolates of Salmonella spp. were recovered from the blood of calves treated with norepinephrine in quantities much lower than S. Dublin 9276 and DT104 98–420 (norepinephrine-treated and untreated). For brain tissues yielding the clinical NPG isolates, one CFU was recovered from approximately each 10 g of tissue (Fig. 2).

As shown in Fig. 3, faecal counts of Salmonella spp. fell within the range of 10^3–10^4 CFU/g, with norepinephrine-treated S. Saintpaul NPG-infected calves representing the highest shedding group. Some differences in shedding patterns were noted, e.g. the SARB strains of S. Saintpaul and S. Enteritidis were shed less than that of their corresponding NPG isostrains. Conversely, the SARB strain of S. Montevideo exceeded that of S. Montevideo NPG, S. Enteritidis SARB and S. Dublin 9276 demonstrated decreases in faecal shedding associated with norepinephrine treatment.

4. Discussion

In this study, we provide preliminary experimental evidence for three strains of Salmonella enterica involved in the phenomenon of Salmonella encephalopathy in calves. Neurological disease could not be reproduced in preliminary in vivo studies. Since all three field outbreaks of Salmonella encephalopathy involved animals that were recently stressed (i.e., transportation, co-mingling or weaning), we postulated that a neuroendocrine component may underlie the encephalopathy. Therefore, calves were challenged with Salmonella spp. and given daily doses of norepinephrine in a pseudo-stress model. Dexamethasone was also evaluated as a mediator of Salmonella encephalopathy for one NPG strain.

Bovine subjects were challenged with all three putative NPG strains of S. enterica and neurological signs were observed in 18 of 18 calves receiving norepinephrine and in one of three calves receiving dexamethasone. Although previously it has been suggested that Jersey calves may be more susceptible to salmonellosis (Wray and Sojka, 1978), in this study there were no differences between animals of different ages or breeds. Therefore, physiological responses to norepinephrine appear to play a significant role in bovine Salmonella encephalopathy for these three strains. The role of the immune system may also be involved, given that stress can modulate immune responses (Kehrl et al., 1999). The stress component of bovine Salmonella encephalopathy is, unfortunately, not easy to mitigate, since transportation and co-mingling are inherent facets of livestock production.

It is interesting that norepinephrine was needed for the clinical isolates to gain access to the systemic circulation and to induce encephalopathy. This could be due to a catecholamine receptor-driven host response or to a direct effect of norepinephrine on the pathogen. Norepinephrine has been shown to enhance the systemic spread of S. Typhimurium in mice (Williams et al., 2005), which could account for some of the observed effects, as evident for DT104 (98–420) in this study. Also, norepinephrine can enhance the growth of Salmonella spp. (Bailey et al., 1999).

Our findings are in contrast to a study with S. Choleraesuis in mice, where the course of disease was not influenced.
by norepinephrine (Nietfeld et al., 1999). However, an alternative study revealed that norepinephrine can elevate Salmonella enterotoxin production (Rahman et al., 2000). Nonetheless, it appears that norepinephrine may promote passage across the intestinal-portal barrier in these instances of Salmonellaencephalopathy. Although there were differences in faecal shedding between NPG and SAR B strains, norepinephrine had no effect upon faecal shedding of the NPG strains.

It is also interesting that dexamethasone could mediate this phenomenon in vivo. While only three calves and one strain were used, this part of the study suggests that Salmonellaencephalopathy has a neuroimmunological component. Combined with the norepinephrine data, the neuronal-endocrine-immune triad seems to govern this disease.

Neither of the control strains, hypervirulent DT104 (98–420) or bovine-adapted S. Dublin 9276, induced neurological disease in experimentally inoculated calves, thus indicating unique features of the clinical isolates. The neuropathogenic phenotype could be related to specific genetic differences that are present in the NPG clinical isolates but absent in the other strains examined. This could be either through the acquisition or loss of genetic material or specific polymorphisms present in the genome. Future work aimed at identifying the presence and nature of genetic factors involved in Salmonellaencephalopathy will aid in elucidating the pathogenesis of this syndrome.

The absence of observable neurohistopathological changes is consistent with certain manifestations of encephalopathy; i.e., a lack of neurohistopathological changes has been reported in some Salmonellaencephalopathies in humans (Fraimow et al., 1990; Kostiala et al., 1992; Martin et al., 1994; Arii et al., 2002). Thus, it is possible that the neurological effects may be due to alterations in neuronal second messengers and not visible using standard neurohistopathological examinations.

5. Conclusion

This study is the first to experimentally reproduce Salmonellaencephalopathy in cattle. The syndrome was reproduced using three unique strains of S. enterica, serovars Saintpaul, Montevideo and Enteritidis, and required concurrent administration of a stress hormone. Future studies will be aimed at identifying pathogenic mechanisms and treatments related to this phenomenon.

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Appendix A. Supplementary data


References


serotype Gallinarum correlates with bacterial dissemination from mesenteric lymph nodes and persistence in vivo. Infection and Immunity 70, 6788–6797.


