Pathological and bacteriological characterization of neonatal porcine diarrhoea of uncertain aetiology

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Neonatal porcine diarrhoea of uncertain aetiology has been reported from a number of countries. This study investigated 50 diarrhoeic and 19 healthy piglets from 10 affected Swedish herds. The piglets were blood-sampled for analysis of serum γ-globulin and necropsied, and the intestines were sampled for histopathology and cultured for Escherichia coli, Clostridium perfringens and Clostridium difficile. Escherichia coli isolates (n=276) were examined by PCR for virulence genes encoding LT, STa, STb, EAST1, VT2e, F4, F5, F6, F18, F41, AIDA-I, intimin, and for the genes aaiC and aggR. Selected isolates were analysed for additional virulence genes by a microarray and subjected to O-typing. Clostridium perfringens isolates (n=152) were examined by PCR for genes encoding major toxins, enterotoxin and beta2-toxin. There was no difference in serum γ-globulin concentration between diarrhoeic and non-diarrhoeic piglets, and pathological lesions in the intestines were generally mild. Porcine enterotoxigenic Escherichia coli, a common cause of piglet diarrhoea, was only found in two piglets. Further, the virulence gene profiling did not suggest involvement of other diarrhoeogenic pathotypes of Escherichia coli. Growth of Clostridium perfringens did not differ between diarrhoeic and non-diarrhoeic piglets. All isolates were type A, all were negative for enterotoxin, and 151 of 152 isolates were beta2-toxin positive. In pigs ≥2 days old, moderate to profuse growth of Clostridium difficile was more common in the controls. In conclusion, it was not possible to relate Escherichia coli, Clostridium perfringens type A and C or Clostridium difficile to neonatal porcine diarrhoea in any of the investigated herds.

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INTRODUCTION

Neonatal porcine diarrhoea (NPD) is a common problem in commercial swine herds. The condition is associated with well-known pathogens such as enterotoxigenic Escherichia coli (ETEC), Clostridium perfringens type C, rotavirus, coronavirus and Cystoisospora suis (Thomson & Friendhip, 2012). During the last decades, NPD has also been associated with Clostridium difficile and Clostridium perfringens type A, but the clinical significance of these bacteria remains to be fully elucidated (Songer & Uzal, 2005; Yaeger et al., 2007). Although most NPD-problems are controllable by vaccination and strict hygiene, some countries, including Sweden, report increased occurrence of disease despite these control measures (Gin et al., 2010; Melin et al., 2010; Svensmark, 2009). Different hypotheses about the underlying causes include the presence of an unknown infectious agent, introduction of new variants of previously acknowledged pathogens (mainly Escherichia coli) or changes in the environment or immune status of the piglets. Kongsted et al. (2013) describe the problem as a new neonatal porcine diarrhoea syndrome (NNPDS), which they define as non-haemorrhagic diarrhoea during the first week of life, without detection of well-known infectious pathogens, and characterized by milk-filled stomachs and flaccid intestines at necropsy. However, systematic microbiological and pathological investigations are few and the results inconclusive (Jonach et al., 2014; Kongsted et al., 2013).
The aim of this study was to characterize the pathology associated with diarrhea in piglets from affected herds and to test for the presence of bacterial enteropathogens commonly associated with neonatal diarrhea. Here we report the gross pathology, histopathology and the prevalence of ETEC, Clostridium perfringens type A and C and Clostridium difficile in diarrheic and healthy piglets from 10 Swedish herds. In addition, we measured serum γ-globulin concentrations and immunostained intestinal sections for Chlamydia spp., and performed PCR and microarray analysis of Escherichia coli virulence genes.

METHODS

The study was approved by the Ethics Committee for Animal Experimentation, Uppsala, Sweden and is part of a series of investigations on NPD of uncertain aetiology. Small intestinal colonization by bacteria identified as Enterococcus hirae has been described previously in this material (Larsson et al., 2014), and viral investigations are ongoing.

Herds and animals. Animals originated from 10 conventional herds (designated A to J) located in the middle of Sweden (within a 3 h drive from the Section of Pathology, Swedish University of Agricultural Sciences, SLU, Uppsala, Sweden). All herds were affected by NPD, despite routine vaccination against ETEC. The herds employed various production systems and varied in size from about 100 to 1000 sows. Two herds (A and E) were satellites in the same sow pool; two others (I and J) were located in proximity to each other and shared the same personnel. Batchwise farrowing with all-in/all-out and washing of the farrowing units between batches was practised in all herds. Farrowing pens had partially slatted concrete floors and supplementary heating for the piglets (heated floor and/or heat lamps). The presence of diarrhea, the floor temperature in the creep area and the cleanliness of the pens were recorded for the farrowing pens in use by each herd. Cleanliness estimates were made on the basis of percentage of the non-slatted floor in the pens that was wet and/or covered with faeces (0–20, >20–60 or >60–100 %). Floor temperatures in the creep area were measured with an infrared thermometer. At each farm, five diarrheic piglets and two healthy controls less than 1 week old were selected. The diarrheic animals had watery to creamy diarrhoea commencing in the previous 24 h. If possible, control pigs were selected from litters with no signs of diarrhea and piglets treated with antibiotics were excluded. Temperatures were measured rectally in all the selected piglets. Age was registered as <1 day old (born the night before the visit), 1 day old (born the day before the visit), etc.

The piglets were transported alive to the Section of Pathology, SLU. Upon arrival, they were weighed and blood was collected before euthanasia. Sera were stored at −70 °C until analysed. Total serum protein concentrations were analysed with the Architect c4000 system (Abbott Laboratories) according to the manufacturer’s recommendations. Six protein fractions (albumin, α1, α2, β1, β2 and γ) were determined by semi-automatic gel electrophoresis on a β1−β2 Hydrigel (Sebia) using the Hydrasys system (Sebia) and the Hxrys 2 densitometer (Sebia). Only the γ-protein fraction is presented here.

Pathology. Piglets were anaesthetized with an intramuscular injection of tiletamine and zolazepam, 6 mg kg⁻¹ (Zoletil 50; Virbac Animal Health) and euthanized by intracardiac injection of pentobarbitol sodium, 100 mg kg⁻¹ [pentobarbitol for euthanasia 20 % (w/v) solution for injection; Pharmasol]. Necropsies were performed promptly after death. Tissue specimens were collected from the duodenum (5 cm distal to the pylorus), proximal jejunum (40 cm distal to the pylorus), distal jejunum (40 cm proximal to the ileocecal junction), ileum, cecum, proximal colon (the mid portion of the spiral colon), distal colon (5 cm distal to the spiral colon), extra-intestinal organs, and any visible lesions. Specimens were fixed in 10 % neutral-buffered formalin for 24 h, embedded in paraffin blocks, sectioned at 4 μm and stained with haematoxylin and eosin. All intestinal sections were evaluated by light microscopy by two investigators blinded to the health status of the piglets. Infiltration of inflammatory cells in the intestinal lamina propria was scored semi-quantitatively as mild, moderate or severe in each section.

In addition, paraffin-embedded specimens of the ileum were selected from two diarrheic piglets and one healthy control from each herd for immunohistochemistry for Chlamydia spp. Immunostaining was performed automatically (Dako Autostainer Plus) with a mouse monoclonal antibody against Chlamydia clone AC1 (Progen Biotech) according to Englund et al. (2012). Pig intestine previously demonstrated to be positive for Chlamydia spp. was used as positive control (Englund et al., 2012).

Bacteriology. Bacteriological samples were collected promptly after euthanasia by rubbing a cotton swab against the intestinal mucosa. Swabs were collected from the distal jejunum and rectum for culture of Escherichia coli and Clostridium perfringens, and from the proximal colon and rectum for Clostridium difficile (six swabs per piglet). Samples were immediately put in Amies transport medium with charcoal (Copan) to ensure bacterial viability. All samples were cultured in batches.

For Escherichia coli, swabs were cultured on 5 % horse blood agar plates [blood agar base no. 2, 39.5 g l⁻¹ (LabM); citrated horse blood, 50 ml l⁻¹ (National Veterinary Institute, SVA)], and bromocresol purple–lactose agar plates [balanced peptone, 10 g l⁻¹ (LabM); NaCl, 5 g l⁻¹ (Merck); sodium ammonium phosphate, 1 g l⁻¹ (Merck); Lab LemoCo Powder, 4 g l⁻¹ (Oxoid); agar no. 2, 10 g l⁻¹ (LabM); 20 % lactose solution, 50 ml l⁻¹ (SVA); 1.6 % bromocresol purple solution, 1 ml l⁻¹ (SVA)]. Bacterial growth was examined after 12–24 h incubation at 37 °C. Two colonies per sample were subcultured and isolates were stored at −20 °C for further analysis.

For Clostridium perfringens, swabs were cultured on fastidious anaerobe agar (FAA; LabM) with 5 % defibrinated horse blood. Bacterial growth was examined after 24–48 h anaerobic incubation at 37 °C. Pigs were considered positive for Clostridium perfringens when either of the two samples (distal jejunum/rectum) displayed moderate or profuse growth of double-haemolytic, semi-transparent, irregular colonies with positive lecithinase production on egg yolk agar [tryptone soy agar, 40 g l⁻¹ (Oxoid); egg yolk emulsion, temperature, 100 ml l⁻¹ (Oxoid)] and a micro-morphology consistent with Gram-positive large bacilli. In addition, swabs were put into 9 ml fastidious anaerobe broth (FAB; LabM) that was heated to 65 °C for 30 min for spore selection, incubated anaerobically at 37 °C for 24 h and then cultured on FAA as described above. Growth after spore selection and enrichment was recorded as yes/no. One isolate of Clostridium perfringens per culture was stored at −70 °C for further analysis. Up to five additional isolates were stored from the direct cultures from one diarrheic piglet and one control piglet per herd.

For Clostridium difficile, swabs were cultured on selective CCFAT-agar [proteose peptone no. 3, 40 g l⁻¹ (Difco); disodium hydrogen phosphate, 5 g l⁻¹ (Merck); potassium dihydrogen phosphate, 1 g l⁻¹ (Merck); sodium chloride, 2 g l⁻¹ (Honeywell/Merck); magnesium sulphate heptahydrate, 0.1 g l⁻¹ (Merck); α-fructosidase, 6 g l⁻¹ (Merck); taurocholic acid, 1 g l⁻¹ (Fluka); neutral red 1 %, 3 ml l⁻¹ (SVA); agar no. 1, 12 g l⁻¹ (Oxoid); u-cyclodextrin, 250 mg l⁻¹ (Fluka/Sigma); cefoxitin, 8 mg l⁻¹ (Sigma); egg yolk emulsion, 50 ml l⁻¹ (Oxoid)]. Bacterial growth was examined after 48–96 h anaerobic incubation at
Table 1. Primers and control strains used in virulence gene profiling of *Escherichia coli* isolates from healthy and diarrhoeic piglets by conventional PCR

<table>
<thead>
<tr>
<th>Target gene (product)</th>
<th>Primer sequence (5’–3’)</th>
<th>Primer concentration (pmol µl⁻¹)</th>
<th>Product size (bp)</th>
<th>Positive-control strains*</th>
<th>Reference</th>
</tr>
</thead>
</table>
| aidA (AIDA-I)         | F: ACA GTA TCA TAT GGA GCC A  
R: TGT GCG CCA GAA CTA TTA | 0.1 | 585 | 12-BKT037133 | Ngeleka *et al*. (2003) |
| estA (STa)            | F: TTT CCC CTC TTT TAG TCA GTC AAC TG  
R: GGC AGG ATT ACA ACA AAG TTT ACA G | 0.05 | 160 | 853 (O149); 60 (O141) | Pass *et al*. (2000) |
| estB (STb)            | F: GCC TAT GCA TCT ACA CAA TCA  
R: TGC TCC AGC AGT ACC ATC TCT AAC CC | 0.05 | 114 | 853 (O149) | This study |
| elt (LT)              | F: CCG GAT TGT CTT CTT GTT GTA TGA  
R: TGT TCC TCT GGC GTG AT | 0.3 | 236 | 853 (O149) | This study |
| faeG (F4)             | F: ATC GGT GGT AGT ATC ACT GC  
R: AAC CTG CGA CGT CAA CAA GA | 0.5 | 601 | 853 (O149) | Ojeniyi *et al*. (1994) |
| fasA (F6)             | F: TCT GCT CTT AAA GCT ACT GG  
R: AAC TCC ACC GTT TGT ATC AG | 0.1 | 333 | 853 (O149) | Ojeniyi *et al*. (1994) |
| astA (EAST1)          | F: TGC CAT CAA CAC AGT ATA TC  
R: GAG TGA CGG CTT TGT AGT C | 0.2 | 107 | 853 (O149) | This study |
| stx2e (VT2e)          | F: CCA GAA TGT CAG ATC ACT GCC GAC  
R: GCT GAG CAC TTT GTA ACA ATG GCT | 0.1 | 322 | 60 (O141) | Pass *et al*. (2000) |
| funA (F5)             | F: TGG GAC TAC CAA TGC TTC TG  
R: TAT CCA CCA TTA GAC GCA GC | 0.2 | 450 | Bd 3437 (O101) | Ojeniyi *et al*. (1994) |
| fedA (F18)            | F: GTG AAA AGA CTA GTG TTT ATT TC  
R: CTT GTA AGT AAC CGC GTA AGC | 0.7 | 510 | 60 (O141) | Bosworth *et al*. (1998) |
| fimF41a (F41)         | F: GCA TCA GGC GCA GTA TCT  
R: GTC CCT AGC TCA GTA TTA CCA CCT | 0.2 | 380 | Bd 3437 (O101) | Franck *et al*. (1998) |

*Strains obtained from SVA, Uppsala, Sweden, previously known to be positive for the targeted genes.
37 °C. Piglets were considered culture-positive when either of the two samples (proximal colon/rectum) displayed moderate to profuse growth of yellow colonies with characteristic morphology (large, flat colonies with a filamentous border and ground-glass appearance) and typical smell. In addition, approximately 1 g intestinal content from the proximal colon and rectum was inoculated into 9 ml Clostridium difficile broth (selective medium as above, minus agar, neutral red, egg yolk emulsion, and with 0.1 instead of 1 g 1−1 taurocholic acid) and incubated anaerobically at 37 °C for 7–8 days. Following incubation, the broth was heated to 70 °C for 30 min and then centrifuged at 3800 g for 10 min, and the pellet was spread on CCFAT-plates. The plates were examined for growth (yes/no) after 48 h.

Investigation of virulence genes in Escherichia coli. According to routine diagnostics for ETEC at the SVA, two Escherichia coli isolates per sample (Söderlind & Mölby, 1979) were screened by PCR for eight virulence genes associated with ETEC and six additional genes associated with other Escherichia coli pathotypes. In total, 276 isolates were investigated (four per piglet). Colony material from overnight pure cultures was suspended in 100 µl nuclease-free water (Sigma-Aldrich) and heated to 95 °C for 10 min. Supernatants of the bacterial lysates were used as templates in the PCR systems described below.

Conventional PCR. Single reactions were carried out by AIDA-I (adhesion involved in diffuse adherence I) and multiplex reactions for STs (heat-stable enterotoxin a), STb (heat-stable enterotoxin b), LT (heat-labile enterotoxin), F4, F6 (set 1) and EAST1 (enteroaggregative Escherichia coli) heat-stable enterotoxin 1), VT2e, F5, F18, F41 (set 2). Primers and primer concentrations are listed in Table 1. Each 50 µl multiplex reaction and 25 µl single reaction contained 1 × GeneAmp PCR Buffer II (Life Technologies), 3 mM MgCl₂, 0.2 mM of each deoxynucleotide, 1.5 or 0.5 U, respectively, AmpliTaq Gold DNA Polymerase (Life Technologies), and 2.5 or 1 µl, respectively, template. The amplification conditions were: 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 59 °C for 30 s for multiplex reactions or 60 °C for 30 s for AIDA-I, followed by 72 °C for 30 s with a final extension at 72 °C for 6 min. Control DNA samples from reference strains (Table 1) were included in each run as well as negative controls (blank). Ten microlitres of the product was electrophoresed on a 2 % agarose gel pre-stained with SYBR Safe according to the manufacturer’s instructions (Life Technologies).

TaqMan real-time PCR. TaqMan real-time PCR for intimin (eaeA) was performed with probe and primers described by Nielsen & Andersen (2003) using the strain EDL933 O157 : H7 as a positive control. The total reaction volume of 15 µl contained 1 × PerfeCTa qPCR FastMix, UNG, Low Rox (Quanta Biosciences), 0.3 µM of each primer, 0.1 µM TaqMan probe and 2 µl template DNA. The PCR amplification was performed using Applied Biosystems 7500 Fast Real-Time PCR System with an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s, then analysed using the automatic threshold setting in the 7500 software (v.2.0.6).

Presence of the genes aggR and aaiC was analysed by a TaqMan real-time duplex PCR according to the EU-RL VTEC Method 05 Rev 1 protocol issued by the EU reference laboratory for Escherichia coli (EU–RL VTEC, 2013). The positive control was D4116 strain C679-12 obtained from EU-RL.

Microarray analysis. One astA (EAST1)-positive Escherichia coli isolate per piglet from herds C (n = 5) and F (n = 4) was selected for further characterization by a microarray designed for genotyping of pathogenic Escherichia coli (Identibac Ec v.3; Identibac). Another four isolates from control piglets from the same herds were included for comparison. Isolates were grown overnight on 5 % horse blood agar, suspended in 250 µl nuclease-free water (Sigma-Aldrich), heated to 98 °C for 20 min, chilled on ice for 10 min, and centrifuged. DNA was extracted from the supernatant using BioRobot EZ1 (Qiagen). Identibac Ec v.3 microarrays (Identibac) containing 124 Escherichia coli virulence gene probes were used according to the manufacturer’s recommendations. IconoClust 3.0 (Clondiag) was used for image analysis using the positive control gapA for normalization and cut-offs as recommended by the manufacturer. Only results from probes yielding positive signals are presented.

Escherichia coli O-typing. Based on results from the PCR-screening of virulence factors in Escherichia coli, 22 isolates were selected for serotyping using O-antisera produced in rabbits (SVA). Selected isolates included Escherichia coli positive for EAST1 and intimin, and isolates positive for enterotoxins in combination with fimbrial adhesins or AIDAl. The test panel included the following O-groups: O6, O8, O9, O45, O46, O98, O101, O115, O138, O139, O140, O141, O147, O149, O157 and O179 (SVA). Serogroupingclut was carried out in 96-well microtitre plates with rabbit O-antisera as previously described (Söderlind, 1971). EAST1-positive isolates from herds C and F were also analysed by real-time PCR for the wblI gene associated with serogroup O111, using the probe and primers described by Perelle et al. (2004). Each 15 µl PCR mixture contained 1 × PerfeCTa qPCR FastMix, UNG, Low Rox (Quanta Biosciences), 0.5 µM of each primer, 0.1 µM TaqMan probe, an internal amplification control system, 1 × Exo IPC /VIC Mix, 1 × Exo IPC DNA (Applied Biosystems) and 2 µl of template DNA. The PCR amplification was performed using Applied Biosystems 7500 Fast Real-Time PCR System with an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. DNA extracted from an intimin-positive Escherichia coli O111 strain (obtained from EU-RL as a O111-positive control in a proficiency test performed at the SVA during 2007, EU-RL ID A07) served as a positive control. Fluorescence signals were analysed using the automatic threshold setting in the 7500 software (v.2.0.6).

Toxinoyping of Clostridium perfringens by PCR. From all jejunal samples positive for Clostridium perfringens, one to six isolates per piglet were subjected to toxinoyping by PCR. In total, 98 isolates from diarrhoeic piglets and 54 from healthy controls were typed. From each isolate, 50 µl of pure culture suspension stored at −70 °C was thawed and then heated for 15 min at 98 °C. The samples were centrifuged and the supernatants stored at −20 °C until used as templates in the PCRs. PCR for major toxins (alpha-, beta-, epsilon- and iota-toxin), enterotoxin, and beta2-toxin genes was performed as previously described (Engström et al., 2003).

Statistics. The γ-globulin concentration (g l−1) was analysed by a linear regression model with health status and age (classified in four categories; <1 day old, 1 day old, 2 days old and >2 days old) as explanatory variables. Associations between bacteriological findings and health status were evaluated by Fisher’s exact test. P-values <0.05 were considered significant. The analyses were performed in R version 2.15.3 (R Core Team, 2014).

RESULTS

Herds and animals

A total of 50 diarrhoeic piglets and 19 healthy controls were included in the study (one control piglet from herd I was excluded as the piglet had diarrhoea upon arrival at the laboratory). All piglets (45 females and 24 males) were cross-bred and originated from 60 litters (three to seven per herd). None had been treated with antibiotics. Seventeen
of the non-diarrhoeic piglets were from litters where none of the piglets showed signs of disease. In herd J, diarrhoea was present in all litters matching the inclusion criteria but two healthy controls could be identified. Diarrhoeic piglets ranged in age from <1 day to 6 days (median age, 1 day) and the controls from 1 to 5 days (median age, 2 days). The weight range was 0.8 to 2.2 kg with a median weight of 1.6 kg in diarrhoeic piglets (interquartile range, IQR=0.6 kg) and 1.8 kg in controls (IQR=0.4 kg). Rectal temperatures varied between 36.2 and 39.9 °C with a median temperature in diarrhoeic piglets of 38.8 (IQR=0.5 °C) and 39 °C (IQR=0.65 °C) in the controls. The diarrhoea was non-haemorrhagic in all cases. Cleanliness of the pens and floor temperatures in the creep area were recorded for a total of 256 pens, of which 97 had diarrhoeic piglets (missing data from six pens). The percentage of diarrhoeic litters in the herds at the time of the visit varied from 17 (herd F, total n=24) to 67 % (herd I, total n=21). The percentage of the non-slatted floor that was wet and/or covered with faeces was 0–20 % in 94 of 97 pens with diarrhoeic piglets and in 145 of 153 pens with non-diarrhoeic piglets. The median temperature in the creep area in each herd varied between 22.4 and 33.6 °C. Median temperatures below 28 °C were recorded for herds A (median 23 °C, IQR=1.8 °C), D (median 26 °C, IQR=1.6 °C) and E (median 22.4 °C, IQR=1.4 °C). There were no obvious differences in median temperatures of the creep area in pens with diarrhoeic and non-diarrhoeic piglets in any herd. The serum γ-globulin concentration was $21.8 \pm 7.7$ g l$^{-1}$ (mean ± SD) in diarrhoeic piglets and $19.8 \pm 9.1$ g l$^{-1}$ in non-diarrhoeic piglets (see Fig. 1) ($P>0.05$).

### Pathology

Major gross findings are presented in Table 2. All diarrhoeic piglets had liquid to creamy intestinal contents in the distal colon/rectum. In 30 of the 50 diarrhoeic piglets, the small intestine was dilated and flaccid, and 19 piglets had congested blood vessels in the small intestine (Fig. 2b). Dilation and flaccidity of the large intestine were present in 29 diarrhoeic piglets. Macroscopic mucosal lesions were absent in all piglets. Mesenteric oedema was observed in the spiral colon of 22 diarrhoeic piglets and 10 of 19 controls. Of the total 69 piglets, the oedema was graded as mild in 25, moderate in 5, and severe in 1 diarrhoeic and 1 control piglet. In all piglets except for one non-diarrhoeic control, the stomachs were filled with milk curd.

Major histopathological findings are listed in Table 3. Villous epithelial damage ($n=13$) and villous atrophy ($n=5$) in the small intestine were only observed in diarrhoeic piglets. Small intestinal colonization by Gram-positive cocci identified as *Enterococcus hirae* was seen in 18 diarrhoeic piglets of which 10 displayed epithelial damage and 4 villous atrophy, as detailed previously (Larsson *et al.*, 2014); see Fig. 3(c). Small intestinal epithelial damage in the three piglets not colonized by *Enterococcus hirae* was subtle and was associated with villous atrophy in one piglet. Both diarrhoeic and non-diarrhoeic piglets had a mild to moderate infiltration with neutrophils in the small intestinal lamina propria, most frequently in the distal jejunum and ileum. Severe infiltrates were present in 10 of 50 diarrhoeic piglets but in none of the controls. Notably, in seven of the diarrhoeic piglets, the severe neutrophilic infiltrates were present adjacent to ileal lymphoid tissue, with no signs of epithelial damage and no or scanty transmigrination of cells to the lumen.

### Table 2. Major gross intestinal lesions observed at necropsy of diarrhoeic ($n=50$) and non-diarrhoeic ($n=19$) piglets from 10 herds

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Diarrhoeic pigs</th>
<th>Non-diarrhoeic pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congested blood vessels in the small intestine*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Dilated small intestine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Dilated large intestine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Oedema in mesocolon</td>
<td>22</td>
<td>10</td>
</tr>
</tbody>
</table>

*Data missing for one diarrhoeic piglet.

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**Fig. 1.** Boxplot showing serum γ-globulin concentration. Shown is the distribution of individual measurements of serum γ-globulin (g l$^{-1}$) from 50 diarrhoeic and 19 healthy piglets originating from 10 herds experiencing problems with neonatal diarrhoea. No difference was detected as analysed by a linear regression model including health status and age ($P=0.74$).
Epithelial lesions in the caecum and/or colon were noted in seven diarrhoeic and one non-diarrhoeic piglet. These lesions consisted of mild epithelial hyperplasia and sloughing enterocytes \( (n = 2) \), non-specific superficial erosions \( (n = 4) \) and minute erosions associated with attached coccoïd micro-organisms \( (n = 2) \). Infiltration of inflammatory cells in the large intestinal lamina propria was similar in the diarrhoeic piglets and the controls.

A large proportion (19 out of 50) of the diarrhoeic piglets had no or very mild histopathological changes in the intestine (Fig. 3b). Notably this was more common in diarrhoeic piglets \( \leq 2 \) days of age than in older piglets (14 of 30 diarrhoeic piglets \( \leq 2 \) days had no or mild changes compared with 5 of 20 diarrhoeic piglets \( \geq 2 \) days of age). Further, no protozoa were noted in the intestinal mucosa in any of the piglets. In all piglets investigated for Chlamydia spp., the immunostaining was negative. Table 4 details the distribution of gross and histopathological findings and median age of diarrhoeic piglets per herd.

**Bacteriology**

All piglets had moderate to profuse growth of non-haemolytic *Escherichia coli*. Sparse to moderate growth of haemolytic *Escherichia coli* in mixed culture was observed in four piglets (all diarrhoeic). Most isolates from diarrhoeic and non-diarrhoeic piglets were negative for the virulence factors examined by PCR (68.5 and 84.2 %, respectively; see Table 5 for detected virotypes). Classic porcine ETEC positive for STa : F5 : F41 was isolated from two diarrhoeic piglets from different herds (A and E). STb : EAST1 : AIDA-I-positive *Escherichia coli* was found in two diarrhoeic and one control piglet from different herds (A, B and G). In two diarrhoeic piglets and six controls, intimin was the only virulence factor demonstrated. Isolates positive

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**Table 3.** Major histopathological lesions in intestinal sections from diarrhoeic \( (n = 50) \) and non-diarrhoeic piglets \( (n = 19) \) from 10 herds

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Diarrhoeic piglets</th>
<th>Non-diarrhoeic piglets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td><strong>Small intestine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial lesions*</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>villus : crypt ratio ( \leq 3 : 1 )†</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Enterococcal adherence cocci( d )</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>Inflammatory cell infiltrate*,( § )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>Moderate</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td>Severe</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td><strong>Large intestine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial lesions*†</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Inflammatory cell infiltrate( § )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Severe</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Not evaluated in the duodenum of one diarrhoeic piglet in herd C owing to missing tissue specimen.
†The lowest villus : crypt ratio estimated in the small intestine for each piglet. Owing to poor tissue orientation, the villus : crypt ratio was not determined in the proximal small intestine in nine case piglets and three controls nor in the distal small intestine in two case piglets and two controls.
§Inflammatory cell infiltrates of the lamina propria scored as mild, moderate or severe in each intestinal section. The results are based on the highest score registered in the small and large intestine respectively, for each piglet.
*Not evaluated in one control piglet in herd A owing to poor specimen quality.

Epithelial lesions in the caecum and/or colon were noted in seven diarrhoeic and one non-diarrhoeic piglet. These lesions consisted of mild epithelial hyperplasia and sloughing enterocytes \( (n = 2) \), non-specific superficial erosions \( (n = 4) \) and minute erosions associated with attached coccoïd micro-organisms \( (n = 2) \). Infiltration of inflammatory cells in the large intestinal lamina propria was similar in the diarrhoeic piglets and the controls.

A large proportion (19 out of 50) of the diarrhoeic piglets had no or very mild histopathological changes in the intestine (Fig. 3b). Notably this was more common in diarrhoeic piglets \( \leq 2 \) days of age than in older piglets (14 of 30 diarrhoeic piglets \( \leq 2 \) days had no or mild changes compared with 5 of 20 diarrhoeic piglets \( \geq 2 \) days of age). Further, no protozoa were noted in the intestinal mucosa in any of the piglets. In all piglets investigated for *Chlamydia* spp., the immunostaining was negative. Table 4 details the distribution of gross and histopathological findings and median age of diarrhoeic piglets per herd.

**Bacteriology**

All piglets had moderate to profuse growth of non-haemolytic *Escherichia coli*. Sparse to moderate growth of haemolytic *Escherichia coli* in mixed culture was observed in four piglets (all diarrhoeic). Most isolates from diarrhoeic and non-diarrhoeic piglets were negative for the virulence factors examined by PCR (68.5 and 84.2 %, respectively; see Table 5 for detected virotypes). Classic porcine ETEC positive for STa : F5 : F41 was isolated from two diarrhoeic piglets from different herds (A and E). STb : EAST1 : AIDA-I-positive *Escherichia coli* was found in two diarrhoeic and one control piglet from different herds (A, B and G). In two diarrhoeic piglets and six controls, intimin was the only virulence factor demonstrated. Isolates positive
for EAST1 only were present from 22 diarrhoeic piglets and one control piglet. EAST1-positive isolates were particularly common in diarrhoeic piglets from herds C and F (29 EAST1-positive isolates from 9 of the 10 diarrhoeic piglets). These piglets were also colonized by *Enterococcus hirae*. The genes *aaIC* and *aggR*, associated with entero-aggregative *Escherichia coli*, were not detected. On microarray analysis, EAST1-positive *Escherichia coli* isolates from herd C were positive for the genes *iroN* (5/5) and *tsh* (2/5) and isolates from herd F were positive for *IpfA*

### Table 4. Median age and major gross and histopathological intestinal lesions in 50 diarrhoeic piglets presented by herd (A–J, five piglets per herd)

<table>
<thead>
<tr>
<th>No. piglets</th>
<th>Herd</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (days)</td>
<td></td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Gross pathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congested blood vessels in small intestine*</td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Dilated small intestine</td>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Dilated large intestine</td>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Histopathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus : crypt ratio = 3 : 1†</td>
<td></td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Epithelial lesions, small intestine‡</td>
<td></td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Enteroadherent cocci§</td>
<td></td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Severe neutrophil infiltration, small intestine¶</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Epithelial lesions, large intestine</td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

*Not assessed for one piglet from herd C.
†The lowest villus : crypt ratio estimated in the small intestine for each piglet. Owing to poor tissue orientation, the villus : crypt ratio was not determined in the proximal small intestine in nine piglets and in the distal small intestine in two piglets.
‡Not evaluated in the duodenum of one diarrhoeic piglet in herd C owing to missing tissue specimen.
§Previously described in detail (Larsson et al., 2014).
¶Inflammatory cell infiltrates of the lamina propria scored as severe in any of the small intestinal sections investigated.
||Not evaluated in one control piglet in herd A owing to poor specimen quality.
(4/4) and iss (4/4). Isolates from control piglets were positive for lpfA and tccP or iss in herd C and lpfA or iss in herd F. All isolates were positive for the control genes gad and ihfA.

Isolates subjected to O-grouping by seroagglutination included EAST1-positive isolates analysed by the microarray (n=9) and one Escherichia coli isolate per piglet positive for intimin (n=8) or enterotoxin in combination with fimbrial antigens (n=2) or AIDA-I (n=3). EAST1-positive isolates generated indistinctive agglutination with O-antisera against O45, O139 and O141 concurrently, and were hence considered non-typable by the O-antisera used. None of the other isolates agglutinated with the O-antisera applied. Additional PCR O-typing for O111 on the EAST1-positive isolates was negative.

Positive direct culture of Clostridium perfringens (moderate to profuse growth) was demonstrated in 35 of 69 piglets and 68 of 69 were positive after spore selection and enrichment. There was no difference in Clostridium perfringens growth on direct culture from diarrhoeic and healthy piglets (38/50 positive versus 13/19 positive, P>0.05). All isolates were Clostridium perfringens type A, and 151 of 152 carried the beta2-toxin gene, whereas the gene for enterotoxin was not detected.

Positive direct culture of Clostridium difficile (moderate to profuse growth) was demonstrated in 35 of 69 piglets. Following enrichment and spore selection of samples, Clostridium difficile could be isolated from all piglets. Positive direct culture was more common in piglets ≥2 days than in those <2 days old (22/32 versus 13/37 positive, P<0.01). Among the piglets ≥2 days old, Clostridium difficile was more prevalent in controls (12/12 positive) than in diarrhoeic piglets (10/20 positive, P<0.01), whereas in those <2 days old no difference could be recognized (5/7 positive controls versus 8/30 positive diarrhoeic piglets, P>0.07).

DISCUSSION

During the last decade a number of countries have reported problems with NPD despite the use of previously effective control measures (Gin et al., 2010; Melin et al., 2010; Svensmark, 2009). The existence of an NNPDS unassociated with previously acknowledged enteropathogens has therefore been suggested (Kongsted et al., 2013). Our investigation of NPD in 10 Swedish herds indicated that bacteria commonly associated with piglet diarrhoea were rare (ETEC), absent (Clostridium perfringens type C) or apparently unrelated to the diarrhoea (Clostridium perfringens type A and Clostridium difficile). Pathological lesions were generally mild and the previously described colonization by Enterococcus hirae (Larsson et al., 2014) remained the most prominent feature.

Flaccidity and dilation of the intestine were common among the diarrhoeic piglets, a gross pathology consistent with NNPDS (Kongsted et al., 2013). However, these findings, as well as the hyperaemia of the small intestine in some piglets, are common in neonatal diarrhoea irrespective of its cause (Brown et al., 2007). Another frequent finding was the presence of an oedematous mesocolon (present in 22/50 diarrhoeic versus 10/19 healthy piglets). Mesocolonic oedema has been previously described as characteristic for NPD caused by Clostridium difficile (Songer & Uzal, 2005) but the current study and that of others have encountered it also in healthy piglets (Yaeger et al., 2007).

Light microscopical lesions in the small intestine were mainly observed in piglets colonized by Enterococcus hirae [36% of the diarrhoeic piglets (Larsson et al., 2014)]. However, some of the youngest diarrhoeic piglets (seven piglets ≤1 day of age) had severe neutrophilic infiltration of the ileal lamina propria without any evident bacterial colonization or epithelial lesions. The significance of this infiltrate is unclear but the proximity to ileal lymphoid

Table 5. Virulence factors in 267 Escherichia coli isolates from 50 diarrhoeic and 19 non-diarrhoeic piglets from 10 herds

<table>
<thead>
<tr>
<th>Virotypes</th>
<th>Diarrhoeic piglets</th>
<th>Non-diarrhoeic piglets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isolates</td>
<td>Animals</td>
</tr>
<tr>
<td>EAST1</td>
<td>46</td>
<td>23</td>
</tr>
<tr>
<td>Intimin</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>STb : EAST1 : AIDA-I</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td>STa : F5 : F41</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>AIDA-I</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>VT2e</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>F41</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>None</td>
<td>137</td>
<td>68.5</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>100</td>
</tr>
</tbody>
</table>

*Two additional piglets also had isolates positive for intimin and VT2e, respectively, and are included under these categories.
tissue and limited transepithelial migration of neutrophils suggest that the infiltrate could be a result from a physiological immunological event rather than a sign of inflammation (Fournier & Parkos, 2012). Further, the few and miscellaneous lesions in the large intestine were likely of minor importance and therefore the identity of the colonizing agent in two piglets was not investigated.

Despite the fact that all diarrhoeic piglets were euthanized within 24 h from the onset of diarrhoea and had creamy to liquid contents in the distal large intestine at necropsy, absence of histological lesions in the intestine was common, especially among piglets younger than 2 days. Interestingly, a study of the clinical course of NNPDS showed that diarrhoea only on the day of birth was common (13 % of 874 piglets) and that it had no adverse effect on daily weight gain (Kongsted et al., 2014), indicating mild disease and quick recovery. Hence, one could speculate that presence of only mild lesions may have passed undetected. Alternatively, the diarrhoeal cause did not affect the intestinal micro-morphology.

Chlamydia spp. is commonly found in Swedish pig herds (Englund et al., 2012) and has previously been suggested to be a cause of NPD (Guscetti et al., 2009) but this was not demonstrated in the current study. Moreover, protozoa were on no occasion observed on histopathology of the intestinal mucosa.

Classic porcine ETEC is considered to be one of the most common causes of NPD, but was rarely demonstrated (2/50 diarrhoeic piglets). In addition, our results did not suggest involvement of other diarrhoeagenic pathotypes of Escherichia coli, previously suggested to be associated with NPD [i.e. attaching-effacing Escherichia coli (Helie et al., 1991; Higgins et al., 1997; Janke et al., 1989), enteropathogenic Escherichia coli (Choi et al., 2001; Penteado et al., 2001; Tzipori et al., 1992) and Escherichia coli positive for STb : EAST1 : AIDA-I (Ngleka, 2002; Ngeleka et al., 2003)]. Escherichia coli positive for the adhesin intimin, associated with attaching and effacing Escherichia coli (Jerse et al., 1990; Zhu et al., 1994), was more common in healthy than in diarrhoeic piglets (6/19 versus 2/50), whereas all isolates were negative for genes associated with enteropathogenic Escherichia coli (aaiC and aggR) that cause diarrhoea in humans. Isolates positive for STb : EAST1 : AIDA-I were only found occasionally (6/276 isolates from two diarrhoeic and one healthy piglet). In contrast, the enterotoxin EAST1 was prevalent among diarrhoeic piglets (22/50 versus 1/19), especially in two herds where piglets also showed intestinal colonization by Enterococcus hirae. Interestingly, recent investigations of NNPDS suggest that simultaneous intestinal colonization by Enterococcus spp. and non-ETEC O111a Escherichia coli plays a role in the pathogenesis (Hermann-Bank, 2014; Jonach et al., 2014). However, the present investigations of EAST1-positive isolates from piglets simultaneously colonized by Enterococcus hirae did not demonstrate virulence gene profiles previously associated with enteropathogenicity, and none of the isolates belonged to serotype O111a. The importance of EAST1 for piglet diarrhoea is questionable since Escherichia coli solely positive for EAST1 has not been shown to induce diarrhoea experimentally (Nglerka et al., 2003; Ruan et al., 2012). Hence, the EAST1-positive isolates in this study may be of lesser importance and a pathogenic role of Escherichia coli in the investigated diarrhoea seems unlikely.

Clostridium perfringens type C was not detected in the present study whereas Clostridium perfringens type A was detected in both diarrhoeic and healthy piglets. Beta2-toxin and enterotoxin are described as potential virulence factors for Clostridium perfringens type A (Bueschel et al., 2003; Collins et al., 1989; Gibert et al., 1997; Popoff & Jestin, 1985) and we found that the gene for beta2-toxin was highly prevalent whereas that for enterotoxin was absent. These results agree with other studies, implying that these genes are of little value for distinguishing commensal Clostridium perfringens type A from strains allegedly associated with disease (Farzan et al., 2013; van Dammen-Jongsten et al., 1990). Thus, the diagnosis of Clostridium perfringens type A enteritis is ambiguous and beta2-toxin-positive Clostridium perfringens type A may reflect the normal intestinal microbiota.

The isolation of Clostridium difficile from all investigated piglets corresponds with previously reported high detection rates of Clostridium difficile in neonatal piglets (Hopman et al., 2011; Weese et al., 2010; Yaeger et al., 2007). Moreover, lesions characteristic of Clostridium difficile-induced NPD, including mesocolonic oedema and microscopic typhlocolitis (Songer & Uzal, 2005), could not be related to diarrhoea in the present study. Hence, a pathogenic role of Clostridium difficile in the present material seems unlikely. Interestingly, in piglets ≥2 days old, growth of Clostridium difficile on direct culture was more prevalent in controls than in diarrhoeic piglets, possibly reflecting an alteration of the intestinal microbiota in the latter.

Serum γ-globulin concentrations were similar in diarrhoeic and healthy piglets and comparable with those previously reported for healthy piglets of the same age group (Svendsen et al., 1972), suggesting a sufficient colostrum supply. All herds also practised all-in/all-out, washed the stables between farrowing batches, and showed a satisfactory level of cleanliness in the farrowing pens, indicating suitable environmental conditions (Fairbrother & Gyles, 2012). However, the low median temperatures in the creep areas in herds A, D and E should be noted; low ambient temperature is a risk factor for disease in neonatal piglets (Blecha & Kelley, 1981; Malmkvist et al., 2006). Interestingly, the two piglets positive for ETEC originated from herd A and E, respectively.

An unexpected association between NPD and small intestinal colonization by enteroadherent Enterococcus hirae in diarrhoeic piglets from 6 of the 10 herds in this study has previously been reported (Larsson et al., 2014). This association was strengthened by the present results, which
demonstrated that bacteria commonly associated with NPD were rare (ETEC), absent (*Clostridium perfringens* type C) or apparently unrelated to diarrhoea in these piglets (*Clostridium perfringens* type A and *C. difficile*). Recent studies of NNPDs in Denmark also report the presence of enteroadherent enterococci in 27% of 51 diarrhoeic piglets compared with 2% of 50 healthy controls, and the association with villous atrophy and epithelial lesions (Jonach et al., 2014). The intestinal microbiota of the NNPD piglets also had an abundance of the genus *Enterococcus* (Hermann-Bank, 2014). Similarly, Gin et al. (2010) described enteroadherent *Enterococcus durans* as an emergent pathogen in neonatal diarrhea in France in 2010. Although our investigation was carried out on a small number (10) of non-randomly selected herds, the similarities with previous studies on piglet diarrhoea in Denmark and France are intriguing. The results may reflect a shift in the relative importance of the enteropathogens involved in NPD in Europe. This needs to be further investigated by prevalence studies of intestinal pathogens in neonatal piglets and studies of the potential pathogenicity of enteroadherent enterococci.

In diarrhoeic piglets not colonized by *Enterococcus hirae*, pathological and bacteriological findings were few (except for the two with ETEC) and the aetiology remains obscure. The present study focused on bacterial enteropathogens commonly associated with diarrhoea during the neonatal period. However, the possibility that the diarrhoea is of viral origin cannot be ruled out. Metagenomic analysis of the intestinal virome is ongoing.

In conclusion, the current study demonstrated the occurrence of neonatal porcine diarrhoea where well-known bacterial enteropathogens found in the Swedish pig population were of minor or no aetiological importance. Neither were any associations with *Clostridium perfringens* type A nor *Clostridium difficile* demonstrable. Hence, the results of this study support the hypothesis of a diarrhoeic condition in newborn piglets not caused by the enteropathogens commonly associated with NPD.

**ACKNOWLEDGEMENTS**

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