Del 2: Rapporten (max 10 sidor)

Inledning

Our research efforts into the emergence of neonatal pig diarrhoea (NPD) indicates that there may well be more than one cause. Thorough investigations into the presence of bacterial pathogen in ten affected pig herds showed, unexpectedly, that *Enterococcus hirae* was the probable cause of disease on ca half of the farms (Larsson et al., 2015). In all herds pathogens commonly associated with piglet disease were either absent, i.e. *C. perfringens* type C, or rare, i.e. ETEC (all herds performed routine vaccination against ETEC). However, *C. perfringens* type A and *C. difficile* was found in all herds. These bacteria are commonly found in piglets, and often regarded as disease causing. However, although few studies have been performed on healthy piglets, *C. perfringens* type A and *C. difficile* seem as common on farms unaffected with NPD as affected farms.

The aim of this project is to generate new knowledge of clostridial NPD that can be used to develop diagnostic methods, guide treatment, and be the first step towards the development of effective preventive measures.

Objectives:

1. Determine the diversity of CpA isolates from healthy piglets and piglets with diarrhoea
2. Characterise potential genetic differences in CpA strains isolated from healthy and diarrhoeic piglets
3. Investigate if Cd-toxin can be detected in faeces from healthy neonatal pigs

for Clostridium perfringens
Cp type A (CpA) is part of the normal intestinal flora of neonatal pigs. It is thus not surprising that it often is a major finding when sampling diseased piglets (Songer et al., 2005). Detecting the *cpa* toxin gene has no diagnostic value, so when the beta-2 toxin was discovered several researchers investigate the possibility that this toxin was important for disease development (Bueschel et al., 2003). However, later studies have shown that also this toxin is as common in healthy at disease animals (Farzan et al., 2013). In this study we compared the genetic diversity and difference within a collection of over 200 isolates from disease and healthy piglets, to elucidate wither we could differentiate between CpA from normal flora and CpA associated to disease.

for Clostridium difficile (CD)
CD is claimed to be a common cause of diarrhoea in young piglets, but the clinical signs reported in naturally occurring disease is unspecific (Songer et al., 2005). Since the bacterium also is present in healthy piglets (Hopman et al., 2011; Yaeger et al., 2007) and neonatal piglets (Yaeger et al., 2007; Grześkowiak et al., 22016), the diagnosis is recommended to be based on detection of CD-toxins (TcdA and TcdB) in intestinal contents or faeces (Songer et al., 2005). To evaluate the diagnostic value of toxin detection in new-born piglets, the presence of free Cd toxin and of toxin-producing CD in stool from healthy piglets was investigated.

Materiell och metoder

Ethical approval
The sampling of animals included in this project was approved by the Ethics Committee for Animal Experimentation, Uppsala, Sweden (permission numbers: C120/11 and 5.8.18-00448/2017).

Herds, animals, and laboratory methods for C. perfringens isolation & characterization
In total, 25 diarrhoeic and 25 non-diarrhoeic piglets from ten herds located in the middle of Sweden were subjected to sampling for *C. perfringens* (Cp). All piglets, regardless of their health status, were crossbreds (Landrace x Yorkshire x Hampshire/Duroc), younger than a week of age, and had not been treated with antimicrobials.
The healthy piglets originated from five herds (five piglets per herd) without NPD-problems according to the animal caretakers and their herd veterinarian. The good health status was further confirmed by a veterinarian at the time of sampling. Piglets were selected for sampling ad hoc from four to five litters per herd.
The diarrhoeic piglets originated from another five herds (five piglets per herd) that were sampled in 2011 as a part of a study on infectious causes of NPD (Larsson et al., 2015). No definite diagnosis of the diarrhoea was established in these herds despite thorough investigations (Larsson et al., 2015; Karlsson et al., 2016)

Healthy piglets were sampled by collecting rectal swabs from live animals (one swab per piglet). In contrast, sampling of diarrhoeic piglets was performed post mortem and included one swab from the distal jejunum and one from the rectum. Regardless of the sampling procedure, the swabs were placed immediately in Amies transport medium with charcoal (Copan, Italy) and cultured the same day. The samples were subjected both to direct anaerobic culture, and spore-selection by heat shock followed by anaerobic enrichment in broth as described by (Larsson et al., 2015). Up to eight colonies of presumptive Cp were subcultured per piglet and stored at -70°C.

Cp isolates were retrieved from -70°C and cultured anaerobically overnight on 5% bovine blood agar at 37°C using the Oxoid, AnaeroGen system (ThermoFisher Scientific). To ensure purity, each sample was subcultured from a single colony DNA extraction was performed using the GeneJet Genomic DNA Purification Kit (ThermoScientific) according to the manufacturers' recommendations.

qPCR for the presence of major toxin genes (*cpa, cpb, etx,* and *iap*) was performed according to (Albini et al., 2008) and presence of the *beta2* toxin was performed according to (Farzan et al., 2013). Reference strains from CCUG (Culture Collection of University of Gothenburg) were used as controls.

PCR for cpn60 was performed according to Das et al., 2018 and PCR products were sent for Sanger sequencing to Macrogen (Amsterdam, the Netherlands) using only the forward primer for sequencing. The cpn60 sequences were analysed and interpreted using CLC Main Workbench 8 software.

For MLVA analysis (Multiple-Locus Variable number tandem repeat Analysis) purified genomic DNA for was amplified using primers and probes described by Chalmers et al., 2008. Foreach MLVA loci obtained alleles were given numbers, and subsequently each allelic profile was

arbitrary assigned resulting in all in all 21 MLVA combinations of allelic variants. Six loci categorical data were then used in further analysis, and were, including data from our strain collection, imported to GrapeTree to generate Minimum Spanning Trees (MST) using default settings (Zhou et al., 2018)
Whole genome sequencing was performed on quality checked DNA, using the Illumina based technology, and performed by SciLifeLab (www.scilifelab.se). Obtained data was processed using an in-house pipeline at the National Veterinary Institute. A SNP (single nucleotide polymorphism) tree was generated by submitting the obtained WGS files to the CSI Phylogeny (Kaas et al., 2014) pipe-line at Centre of Genomic Epidemiology (CGE; www.genomicepidemiology.org), using default settings. The generated Newick tree was imported to Grapetree and a SNP tree was produced, using default settings (Zhou et al., 2018)

Herds, animals, and laboratory methods for C. difficile isolation & characterization
C. difficile (CD) Isolates were collected from five conventional herds (same as above for retrieval of Cp strains from healthy animals) where there was no ongoing or history of diarrhoea. The absence of diarrhoea was determined by a veterinarian at the time of sampling. The piglets were crossbreeds of both sexes and their ages ranged from one to five days old. Rectal swabs were collected from each animal, placed immediately in Amies transport medium with charcoal (Copan), and cultured within eight hours. The samples were subjected both to direct anaerobic culture, and spore-selection by heat shock followed by anaerobic enrichment in broth as described by Larsson et al. 2015. Pure culture isolates were stored at -70°C until further use.

One representative colony per positive sample were subcultured on fastidious anaerobe agar (FAA) with 5% defibrinated horse blood (LabM). Species confirmation of presumptive CD isolates was performed by MALDI-TOF mass spectral fingerprinting using a Microflex LT mass spectrometer according to the manufacturer's recommendations (Bruker Daltonics). Spectra were analysed with the MALDI Biotyper 3.1 software and matched against the MALDI Biotyper database (version 4.0, Bruker Daltonics). Score criteria for identification were those recommended by the manufacturer.

C. diff quik chek complete test (CDqc), was purchased from Abbott (www.globalpointofcare.abbott) and used according to the manufacturer's instructions, both directly on stool samples but also on isolated CD for *in vitro* testing of CD toxin production.

Genomic DNA was extracted using the GeneJET Genomic DNA Purification kit, according to the manufacturer's instructions (Thermo Scientific).

qPCR for the detection of CD toxins *tcdA* and *tcdB*, and binary toxins *cdtA* and *cdtB* was performed as described by Houser et al., 2010.

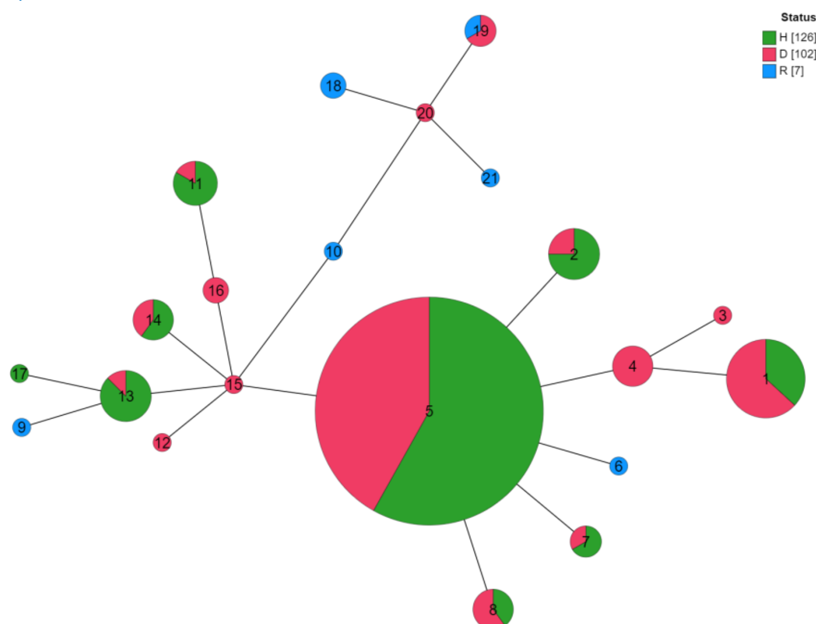
Whole genome sequencing was performed on quality checked DNA, using the Illumina based technology, and performed by SciLifeLab (www.scilifelab.se). Obtained data was processed using an in-house pipeline at the National Veterinary Institute. 7-loci MLST profiles were obtained by blasting PubMLST (www.pubmlst.org) database for CD, and core genome MLST (cgMLST) profiles from Centre of Genomic Epidemiology (CGE; www.genomicepidemiology.org)

Resultat och diskussion

Clostridium perfringens (Cp)
126 isolates from healthy and 100 from diseased piglets and eight different control strains were toxin typed by qPCR. All but one was qPCR positive for the *cpa* gene, and are therefore of toxin type A, and all but one carried the gene for the *beta2* toxin. These two isolates were not aberrant to related isolates from respective farm (two different farms with healthy animals) on MLVA typing; the *beta2* negative strains being of the major MLVA type 5 and the *cpa* negative strains of type 11.

All strains were subjected to cpn60 (data not shown) and MLVA (Figure Cp1) subtyping for molecular epidemiological studies. Both methods indicate that Cp isolated from Swedish piglets are quite homogenous, that one MLVA type (type 5), divided in to two cpn60 types, dominates in both healthy and diseased herds, and that all herds but one also had other minor MLVA / cpn60 types present. Thus, there is some diversity in this Cp population, but there is no evident genetic difference between isolates from healthy and diseased piglets or farms with different history of NPD. If a pathogenic clone of Cp would be present one would expect this clone to dominate in diseased piglets, however this was not the case, as the dominating subtype (MLVA type 5) was present in both kinds of herds, and the genetic diversity was similar or even higher in isolates from farms with cases of diarrhoea (Figure Cp1).

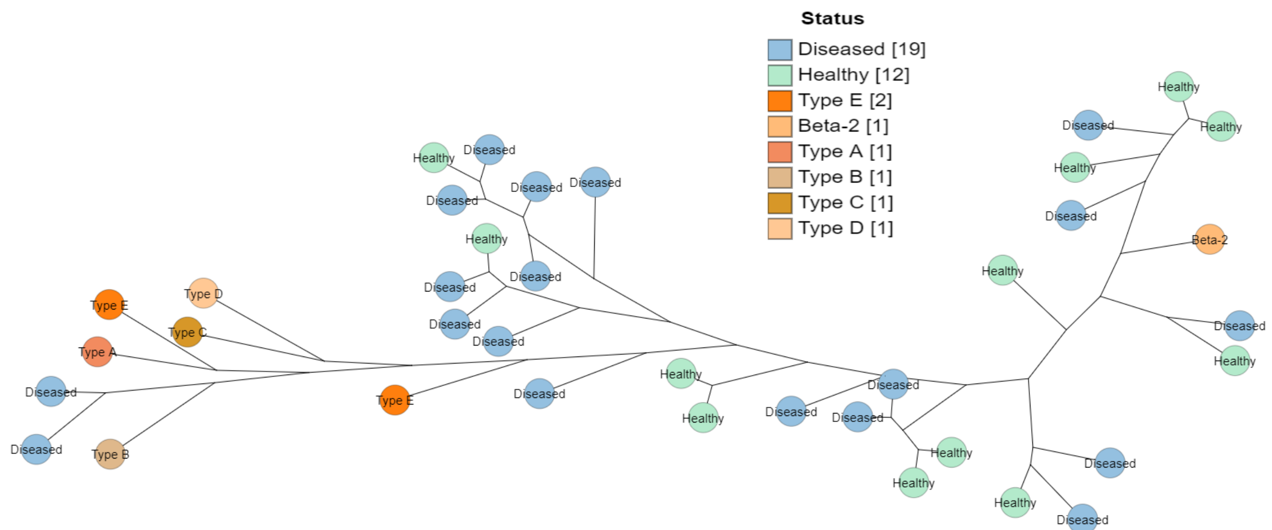
Figure Cp1
Relationships between isolates of *C perfringes* from healthy and diseased piglets as assessed by 6-loci MLVA analysis. Green represent isolates from healthy piglets, red isolates from diseased piglets, and blue reference strains.



A subset of strains, i.e. 31 Cp isolates, were chosen based on the MLVA results and to represent different herds and disease status, for whole genome sequencing. Also, the type strains were included in this analysis (Figure Cp2). The results confirm the intermingling of isolates from healthy and diseased animals, with one exception, also seen in that MLVA analysis. The

exception being two isolates from one farm, of MLVA type 19, cluster closer to the type strain CCUG 2035 of toxin type B. However, this type was not dominant on the farm, four other MLVA types were identified, including MLVA type 5, also being the most common on this farm.

Figure Cp2
Relationships between isolates of *C perfringes* from healthy and diseased piglets as assessed by single nucleotide polymorphism analysis from whole genome data



Clostridium difficile (CD*)*

From the five farms, with no previous history of NPD, five pigs from each farm were sampled, and out of these 25 pigs, 19 were positive for the presence of CD-antigen in stool by CDqc; stools from three piglets were clearly positive for CD-toxins and another three showed weak positive signals. On direct culture, 16 pigs were positive for CD and after enrichment and spore selection, CD could be detected in 21 pigs; from two pigs spore selection was not performed.

All in all, 36 isolates of CD were obtained from 22 out of 25 healthy pigs tested. In addition (obtained after spore selection) one isolate was found to be *C. symbiosum* as assessed by Maldi-tof species determination. Notably, the *C. symbiosum* tested positive in the CDqc antigen test that is to be specific for CD.
For quality control, two reference strains of CD were included in the further analysis, CCUG 4938 and CCUG 20309, CCUG 4938 contains *tcdA* and *tcdB* genes, but not *cdtA* or *cdtB,* while CCUG 20309 do harbour the *cdtA* and *cdtB* genes.
At least one isolate from all piglets produced CD toxins as assessed by CDqc performed on the isolates *in vitro.* All isolates possessed the toxin genes *tcdA* and *tcdB* as assessed by qPCR. For the qPCR of the binary toxins, the results were more variable, with three farms having high Cq scores for both *cdtA* and *cdtB,* similar to CCUG 4938, and should thus probably be regarded as negative for the binary toxins. Also, on one farm, with healthy animals, all isolates were clearly qPCR positive for the two binary toxins, but one.

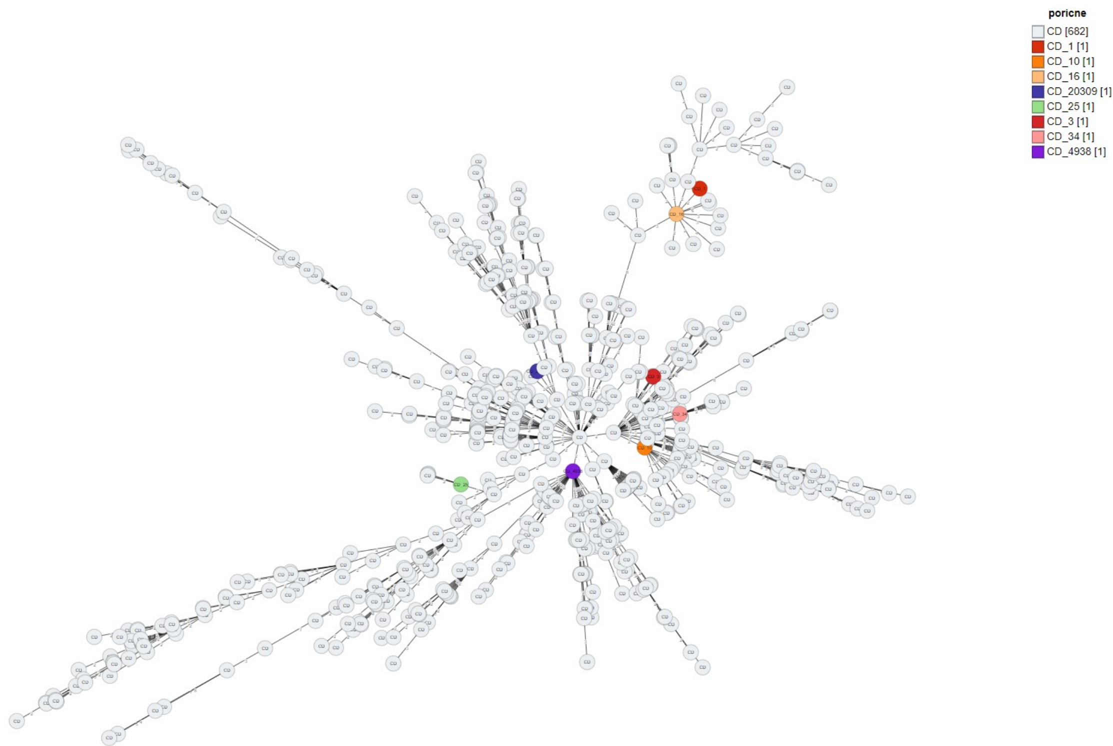
Six isolates, one from each farm, except the farm mentioned above, were two isolates with different qPCR results for the binary toxins, were chosen for whole genome sequencing, to better characterize the isolate collected. The results of this deeper investigation into a subset of the isolates reveal that each farm seemingly has its own variant of CD, or, as seen on one farm, were two isolates were analyse, two different variants on the same farm. The most used typing system for CD from human clinics in Sweden today is ribotyping, while many international laboratories are currently choosing to use WGS as a more reliable and portable method for isolate typing. Ribotypes can not be extracted from WGS data, but many publications include both methods so cross referencing between them is possible. As reported by Rizzardi et al., 2018, the most commonly found ribotypes during the past eight years in human clinical cases in Sweden includes: RT1, 2, 3, 5, 10, 11, 12, 14, 17, 20, 23, 29, 45, 46, 70, 78, 81, 220, 231; of which, if the cross-referencing between 7-gene MLST and ribotyping holds true, RT2, 5, 46 and 78 are found in our small isolate collection from healthy piglets.

Using 7-loci categorical MLST data, our isolate collection can be compared to a larger database of international CD strains (www.pubmlst.org). This comparison can be used to access whether isolates in local or national studies are closely related or represent a broad perspective of the CD strain community. The database contains over 1700 entries, divided into almost 700 different 7-loci MLST variants (STs). In Figure CD1, a dendrogram where isolates from this study are highlighted in colour shows that they are not a cluster on their own, but well integrated in the CD strain web.

The 7-loci MLST scheme reflexes difference in the core genome (genes present in all isolates of the species studied) of isolates of this species. It has been shown that the pan genome (total number of genes found in any isolate of the species studied) is about ten time larger than the core genome (Scaria et al., 2010). Thus, although CD was equally common in diarrhetic piglets (Larsson et al., 2015) as in this set of healthy animals, we hypothesize that CD is not a causative agent of NPD, but it cannot be ruled out that a there are clones that have acquired certain pathogenicity traits that may cause disease in piglets. With the advent of whole genome sequencing, we foresee that this will be possible to investigate in the future.

Figure CD1

7-loci MLST dendrogram, including one representative of each the different STs in the PubMLST CD database, and also isolates from the present study, these highlighted in different colours.,



cgMLST has so far mostly been used for detailed studies of relationships between strains within ribotypes, or to study the progression of clones within one patient or in a hospital ward. Therefore, there is not so much data to compare this small collection of isolates with, by the use of cgMLST. We predict that this will rapidly change in the future as more researchers are using WGS and cgMLST for epidemiological studies.

Slutsatser

As stated in the introduction the project had three main objectives, and the conclusion for each of these objectives are presented below:

Objectives:

1. *Determine the diversity of CpA isolates from healthy piglets and piglets with diarrhoea &*
2. *Characterise potential genetic differences in CpA strains isolated from healthy and diarrhoeic piglets*

The genetic diversity within the CpA isolate collection from healthy and diseased piglets was found to be generally low, using cpn60 and 6-loci MLVA typing. Based on these two methods and, in addition, on whole genome sequencing followed by single nucleoid polymorphism analysis of a subset of isolates, no genetic difference between healthy and diarrhoeic piglets could be shown.

These results indicate that CpA as a cause of NPD in Sweden is quite unlikely. However, it is still possible that, although the isolates are highly similar, they may differ in their pathogenic potential, by having acquired different virulence gene repertoires. Before publishing our results,

we will therefore look more deeply into the obtained whole genome sequence data and mine this for different virulence and adhesion factors, that may differ even within genetic subtypes.

3. Investigate if Cd-toxin can be detected in faeces from healthy neonatal pigs

The presence of CD in stools of healthy piglets could be confirmed with the C. diff quik chek complete test (CDqc), and production of toxins could also be shown in some of the stool samples with this antigenic method.

As Cd-toxins can be detected in faeces from healthy neonatal pigs from farms with no history of piglet diarrhoea, using antigenic tests such as CDqc as a tool to diagnose CD as the causative agent of piglet disease is questionable.

From a majority of the piglets, CD could be isolated. By the same antigen test as was used for the stool samples (CDqc), all, but one, isolates were shown to be able to produce CD toxins *in vitro*. These isolates were also shown to carry the CD toxin genes by qPCR.

Nytta för näringen och rekommendationer

Our research efforts into the emergence of neonatal pig diarrhoea (NPD) indicates that there may well be more than one cause.

To efficiently develop guidelines for antimicrobial treatment, specific and sensitive diagnostic methods, and effective preventive measures, the causative agent must be known. Thus, this project aimed at elucidating the role of Cp and CD in NPD in Sweden. Our results indicate that Cp and CD are unlikely causes of diarrhea in piglets in Sweden. Both bacteria were equally common in healthy as diseased animals. As for Cp there was low genetic diversity between isolates, and no apparent genetic difference between isolates from these two groups of animals. Therefore, vaccination and / or antibiotic treatment against clostridia (primarily CpA) is most probably unnecessary.

However, for both bacterial species the acquisition of virulence traits regardless of genetic subtype is possible. The new genomic tools now available, such as long-read whole genome sequencing, open the possibilities to further characterize our isolate collection.
Notably, by whole genome sequencing we could show that each farm had a different variant of CD, variants that have previously been associated with human disease. This knowledge is important for farms, to prevent CD infection in farmers or staff.

Referenser

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Del 3: Resultatförmedling

Ange resultatförmedling av projektet, inklusive titel, referens, datum, författare/talare, och länk till presentation eller publikation om tillämpligt. Planerade publiceringar (med preliminära titlar) ska ingå i tabellen. Ytterligare rader kan läggas till i tabellen.

Vetenskapliga publiceringar	**Comparative genomic analyses of *Clostridium perfringens* type A isolated from diarrhoeic and healthy newborn piglets** Anna Aspán, Sofia Lindström, Magdalena Jacobson, Jenny Larsson (*manuscript in preparation*)
	Questionable diagnostic value of Clostridium difficile toxin detection in new-born piglets Anna Aspán, Sofia Lindström, Magdalena Jacobson, Jenny Larsson (*short communication in preparation*)
Övriga publiceringar	**Questionable diagnostic value of Clostridium difficile toxin detection in new-born piglets** Anna Aspán, Sofia Lindström, Magdalena Jacobson, Jenny Larsson ESPHM 2019 poster abstract
Studentarbete	**Neonatal Porcine Diarrhoea; Subtyping of Porcine *Clostridium perfringens* Type A Isolates Using Cpn60 Sequencing** Sophie Masterson (2018) BSc (Hons) in Biomedical Science School of Biological Sciences, Dublin Institute of Technology & Department of Clinical Sciences, Swedish University of Agricultural Sciences
	A Study of the Diversity of *Clostridium perfringens* type A in Neonatal Porcine Diarrhoea Alison Nolan (2018) BSc (Hons) in Biomedical Science School of Biological Sciences, Dublin Institute of Technology & Department of Clinical Sciences, Swedish University of Agricultural Sciences