Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2022:3

Respiratory disease in Norwegian pig production - with focus on *Actinobacillus pleuropneumoniae*

Luftveissjukdom i den norske grisepopulasjonen med vekt på *Actinobacillus pleuropneumoniae*

Liza Miriam Cohen

Respiratory disease in Norwegian pig production

- with focus on *Actinobacillus pleuropneumoniae*

Luftveissjukdom i den norske grisepopulasjonen med vekt på *Actinobacillus pleuropneumoniae*

Philosophiae Doctor (PhD) Thesis

Liza Miriam Cohen

Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

Ås 2022

Thesis number 2022:3
ISSN 1894-6402
ISBN 978-82-575-1879-0

The project logo of Grisefine lunger. The project name roughly translates to "Really great lungs". This is a play on words, as "gris", which is used for emphasis, is the Norwegian word for pig.

Supervisors and Evaluation Committee

Supervisors

Camilla Kielland
Associate Professor
Department of Production Animal Clinical Sciences,
Faculty of Veterinary Medicine,
Norwegian University of Life Sciences (NMBU), Ås, Norway

Birgit Ranheim
Associate Professor
Department of Production Animal Clinical Sciences,
Faculty of Veterinary Medicine,
Norwegian University of Life Sciences (NMBU), Ås, Norway

Stine Margrethe Gulliksen
Norwegian Pig Health Service,
Norwegian Meat and Poultry Research Centre, Animalia AS, Oslo, Norway

Thea B. Blystad Klem
Section for Terrestrial Animal Health and Welfare
Department of Animal Health, Welfare and Food Safety
Norwegian Veterinary Institute, Ås, Norway

Jens Peter Nielsen
Professor
Department of Veterinary and Animal Science
University of Copenhagen, Denmark

Evaluation Committee

Mari Heinonen
Professor
Department of Production Animal Medicine
University of Helsinki, Finland

Isabel Hennig-Pauka
Professor
Field Station for Epidemiology
University of Veterinary Medicine Hannover, Germany

Henning Sørum
Professor
Department of Paraclinical Sciences
Faculty of Veterinary Medicine,
Norwegian University of Life Sciences (NMBU), Ås, Norway

Acknowledgements

The work presented in this thesis was conducted at the Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine at the Norwegian University of Life Sciences, from June 2017 to December 2021. The thesis was a part of the project "Grisefine Lunger - a cooperation to decrease respiratory disease in pigs in Norway". The project was a collaboration between the following institutions: The Norwegian Veterinary Institute (NVI), The Norwegian Meat and Poultry Research Centre (Animalia AS), Kjøtt- og fjørfebransjens Landsforbund (KLF), Nortura SA, Norsvin SA and the Norwegian University of Life Sciences (NMBU). Funding was provided by the Norwegian Levy on Agricultural Products (FFL), the Agricultural Agreement Research Fund of Norway (JA), Animalia, Nortura, KLF and NVI (grant number NFR-267422).

Writing this thesis has been an adventure. At times, I thought it would be the death of me, but in the end, through the support of my great colleagues, friends, and family, I have been able to produce something that fills me with great pride.

To Camilla Kielland, winner of the world's greatest supervisor award. Words do not do justice to your magnificence. You kept track of me and made sure I did not lose myself along the way. Thanks to you I have been able to believe in myself and in this endeavor. I could count on you to have my back always, be it in personal or professional matters. I am forever thankful for your investment in me.

To Carl Andreas Grøntvedt. You, who time and again have graced me with your seemingly unfathomable wisdom. Thank you.

To Thea Klem, Stine Gulliksen for being good supervisors and role models, and to Birgit Ranheim and Jens Peter Nielsen for also making this document come to life.

To APP gurus Øystein Angen and Janine Bossé, for teaming up with us, and mentoring me so that I could stand a chance, and so this thesis could become what it is today.

To the rest of the Grisefine lunger project group, Odd Magne Karlsen, Peer Ola Hofmo and Karl Kristian Kongsted, thank you for your good ideas, support, and impeccable company in work and socially.

There are many people that have been involved in the sampling and diagnostic work in this study. Thanks to all farmers, field veterinarians, veterinarians and technicians at Animalia, the Food Safety Authority and the abattoirs, that willingly contributed to our research. To Maria Stenklev at Nortura for your aid and relentless support to this project. I admire your knowledge and skill. To Mette Valheim, Øyvor Kolbjørnsen, Helene Wisløff, pathologists at the NVI. I thank you for teaching me and letting my opinions matter. Thanks to the amazingly professional lab technicians at NVI. A big thank you to Marc Stegger at SSI in Copenhagen for your brilliant bioinformatic work and your patience with me. Thanks also to Paul Langford and Yanwen Li at Imperial College in London, who's contributions have been invaluable.

This work could not have been done without the support of family and friends. Thanks to my brother, for being the best little brother a big sister could want. To my dear mother, my number one source of inspiration. Thank you for a happy childhood, filled with lessons in becoming a responsible, hardworking, kind, and honest grown up. Thank you for listening and advising in times of detriment as well as in times of success.

Thank you to my friends, new and old. Thank you for playing DnD with me, thank you for dressing up with me and thanks for supporting my addiction to buns. Thanks for walks, talks, tears, and laughter, and for providing me with the thing I value most in this whole world – my village. A special thanks to Maien and Karin for all the hugs and high fives, for letting me air my dirty laundry and for regular reality checks.

To my beloved Philip, my designated Player 2 in this live action, multiplayer, co-op game known as life. Your endless support has meant everything to me, and your love is my fuel. Thank you for being the best doggo-daddy Luffy could have asked for. Thank you for patiently and tirelessly standing by me, for being my best friend, for giving me something to look forward to in every single day.

In loving memory of my father, a happy, caring, and generous man. You have been with me in spirit in all my work, and inspired me to be kind to all animals and humans. I wish I could have shared this experience with you, knowing you would have been extremely proud.

Ås, December 2021
Liza Miriam Cohen

Table of Contents

1 Abbreviations and definitions

AMR	Antimicrobial resistance
APP	*Actinobacillus pleuropneumoniae*
Apx	Repeat in toxin (RTX) toxins of APP, named ApxI-IV
BEAST	Bayesian Evolutionary Analysis by Sampling Trees
BLAST	Basic Local Alignment Search Tool
CPS	Capsular polysaccharide
DHP	The Norwegian national veterinary treatment database
DNA	Deoxyribonucleic acid
EEA	European Economic Area
EU	European Union
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
NAD	Nicotinamide adenine dinucleotide
NVI	Norwegian Veterinary Institute
OF	Oral fluids
OIE	World Organization for Animal Health
OMP	Outer membrane protein
PCV2	Porcine Circo Virus type 2
PRCV	Porcine Respiratory Corona Virus
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
RT-qPCR	Real-time quantitative polymerase chain reaction
SIV	Swine Influenza Virus
SNP	Single nucleotide polymorphism
SSI	Statens Serum Institut
SPF	Specific Pathogen Free
TMRCA	Time to most recent common ancestor
UK	United Kingdom of Great Britain and Northern Ireland
USR	A Norwegian disease registering system at slaughter
WGS	Whole genome sequencing

2 List of papers

I. Cohen, L.M., Grøntvedt, C.A., Klem, T.B., Gulliksen, S.M., Ranheim, B., Nielsen, J.P., Valheim, M., Kielland, C. (2020). *A descriptive study of acute outbreaks of respiratory disease in Norwegian fattening pig herds.* Acta Vet Scand. 62(1), 35. DOI: 10.1186/s13028-020-00529-z.

II. Cohen, L.M., Bosse, J.T., Stegger, M., Li, Y., Langford, P.L., Kielland, C., Klem, T.B.B., Gulliksen, S.M., Ranheim, B., Grøntvedt, C.A., Angen, Ø. *Comparative genome sequence analysis of Actinobacillus pleuropneumoniae serovar 8 isolates from Norway, Denmark and the United Kingdom indicates distinct phylogenetic lineages and differences in distribution of antimicrobial resistance genes.* Front Microbiol. 12(2558). DOI: 10.3389/fmicb.2021.729637.

III. Cohen, L.M., Grøntvedt, C.A., Gulliksen, S.M., Kielland, C. *A descriptive study of the biosecurity levels in Norwegian fattening pig herds with and without outbreaks of respiratory disease.* Submitted.

Papers I and II are included with permission from the publishers.

3 Abstract

Respiratory disease is considered a major challenge to the porcine health, welfare, and production. To limit the occurrence and impact of clinical respiratory disease, knowledge of the etiologic agent is crucial. Efforts made by the Norwegian pig production sector has resulted in a unique health situation for the Norwegian pig population. *Actinobacillus pleuropneumoniae* (APP) is the suspected agent behind most clinical outbreaks of respiratory disease in pigs in Norway. Due to a complex disease etiology and differences in population structures and production practices, updated knowledge of respiratory disease under the Norwegian conditions was needed.

The aim of this thesis was to increase our understanding of infectious respiratory disease in the Norwegian pig population. The aim was approached through a case/control field study of respiratory disease outbreaks, and a genome study.

Severe clinical characteristics were observed during the outbreaks, and the disease characteristics were typical of APP. Typical acute pathological lesions of porcine pleuropneumonia were present in lungs and pleura of pigs with acute clinical signs, and APP serovar 8 (APP8) was found to be the main etiologic agent. No other respiratory agents were found to contribute to the disease outbreaks. The results confirmed APPs role as a primary infectious respiratory agent.

To further characterize APP8 in the Norwegian pig population, the genomic variability in these bacterial genomes was investigated through whole genome sequencing. Information of the structure of the Norwegian pig population was used to assess its' effect on the dissemination of genetic lines including antimicrobial resistance (AMR) in APP8. A very low within-host variation indicated that APP8 caused monoclonal infections in the pig lungs, yet some within-herd genetic variation was evident. A geographical clustering of APP8 was seen within regions in Norway, and between APP8 in

Norway, the United Kingdom (UK) and Denmark. The time of separation between APP8 isolates from the three national populations was estimated using Bayesian inference. The last common ancestors of Norwegian and Danish isolates were estimated to have occurred around 200 years ago, while a separation of the Norwegian and UK isolates happened even longer ago. The occurrence of AMR genes in the Norwegian APP8 isolates was low, and substantially lower than the UK isolates.

While a high level of national biosecurity in Norway is the most important contribution to the health status of the Norwegian pig population, biosecurity practices at the farm are important for preventing endemic respiratory agents. A study assessing the total, external and internal biosecurity of herds with outbreaks of respiratory disease and non-outbreak herds was performed using Biocheck.UGent™. No difference in biosecurity levels between the herds with and without outbreaks could be detected. Both herd groups scored higher on external than internal biosecurity. However, a lower internal biosecurity score in both outbreak and non-outbreak herds was found, implying there might be a lack of compliance with important biosecurity measures internally at the farm.

In conclusion, APP8 is the dominating serovar of APP in Norway, causing severe outbreaks of clinical respiratory disease in fattening pigs. The structure of the Norwegian pig population has allowed for low variability among APP8 isolates, with clear geographic clustering. International biosecurity considerations allowing for a closed Norwegian pig population has led to a distinct separation between Norwegian isolates and those from neighboring nations Denmark and the UK. More information regarding transmission of APP is needed to assess the influence of biosecurity measures on occurrence of outbreaks with APP. The work described in this thesis has increased our understanding of infectious respiratory disease in the Norwegian pig population and can contribute to improved health of pigs.

4 Norsk sammendrag

Luftveissykdommer er i svineproduksjon ansett som et stort problem med store konsekvenser for grisens helse og velferd. For å begrense forekomsten av klinisk luftveissykdom hos gris må man først komme til bunns i hvilke agens som står bak. Den norske svinenæringa har lagt inn store ressurser i å opprettholde en god helse blant norske griser. *Actinobacillus pleuropneumoniae* (APP) mistenkes å stå bak de fleste utbrudd med luftveissjukdom i den norske grisepopulasjonen i dag. Grunnet komplekse årsaksforhold, og store nasjonale forskjeller i populasjonsstruktur var det behov for å kartlegge luftveissjukdom hos griser ved norske forhold.

Som overordnet mål hadde denne avhandlingen å øke forståelsen av smittsom luftveissykdom i den norske grisepopulasjonen. For å nå målet ble det gjennomført en kasus/kontroll feltstudie av luftveisutbrudd, og en genom-studie.

Under utbruddene ble det observert alvorlige kliniske tegn, og sykdomsbildet var typisk for APP. Typiske akutte forandringer for ondartet lungesyke var til stede i lunger og brysthinne hos griser med akutte kliniske tegn, og APP serovar 8 (APP8) ble diagnostisert som det viktigste etiologiske agenset. Ingen andre luftveisagens bidro til sykdomsutbruddene. Resultatene bekrefter APP som primæragens ved smittsom luftveissykdom.

Gjennom helgenomsekvensering av APP8-isolater fra Norge ble variasjonen i disse bakterienes DNA undersøkt. Informasjon om strukturen i den norske grisepopulasjonen ble brukt til å undersøke effekten av denne på genetisk variasjon og utbredelse av genetiske trekk inkludert antibiotikaresistens i APP8. Det var en beskjeden variasjon mellom APP8 isolater fra Norge. En svært lav variasjon blant isolater fra samme griselunge indikerte at infeksjonene var utgått fra samme bakterieklon. Det ble observert noe variasjon mellom griser innad i den enkelte besetning. Isolatene var tydelig inndelt etter geografisk opphav, både innad i Norge, og mellom APP8 isolater

fra Norge, England og Danmark. Studien indikerte at norske og danske isolater må ha skilt lag for 200 år siden, og at norske og engelske isolater må ha skilt lag betydelig mye før det. Forekomsten av antibiotikaresistens-gener i norske APP8 isolater var lav, og betydelig mye lavere enn i engelske isolater. Strukturen i den norske grisepopulasjonen og nasjonale smitteverntiltak som begrenser forflytningen av griser har antakelig påvirket evolusjonen og utbredelse av APP8 i Norge.

Et solid smittevern nasjonalt er antakelig det viktigste bidraget til den gode helsesituasjonen i den norske grisepopulasjonen. Smittevern på besetningsnivå er allikevel viktig for å forhindre smittsomme sjukdommer som finnes i populasjonen. En studie som kvantifiserte overordnet, eksternt og internt smittevern i besetninger med og uten utbrudd av luftveissjukdom ble gjennomført ved hjelp av verktøyet Biocheck.UGent™. Det kunne ikke påvises noen forskjell i smittevernnivåer mellom besetningene med og uten utbrudd. Begge besetningsgruppene scoret høyere på eksternt enn internt smittevern. Det ble imidlertid funnet en lavere intern biosikkerhetsscore i både utbruddsbesetninger og ikke-utbruddsbesetninger, noe som tyder på at det kan være manglende overholdelse av viktige biosikkerhetstiltak internt på gården.

Resultatene fra disse studiene har bekreftet at APP8 forårsaker alvorlige utbrudd av klinisk luftveissykdom hos slaktegriser og er den dominerende serovar av APP i Norge. Strukturen til den norske grisepopulasjonen har bidratt til lav variasjon blant APP8-isolater, og en tydelig geografisk gruppering. Internasjonale smittevernhensyn som tillater for en lukket norsk grisepopulasjon, har ført til et tydelig skille mellom norske, danske og engelske APP8 isolater. Mer informasjon om hvordan APP smitter er nødvendig for å vurdere viktigheten av smitteverntiltak på forekomst av utbrudd med APP. Arbeidet som er beskrevet her har økt vår forståelse av smittsomme luftveissykdommer i den norske grisepopulasjonen og kan bidra til bedre helse hos griser.

5 Synopsis

5.1 Introduction

Modern commercial rearing of pigs (*Sus scrofa domesticus*) is becoming more efficient in order to meet the increased demand for animal dietary protein by a growing global human population (Sørensen *et al.* 2006). The resulting increase in animal density is grounds for a more efficient spread of infectious diseases (Maes *et al.* 2000 , Rose *et al.* 2002). Respiratory disease is considered a major challenge to the porcine health, welfare, and production, and is subject to research globally. To limit the occurrence and impact of clinical respiratory disease in pigs, knowledge of the etiology is crucial (Figure 1).

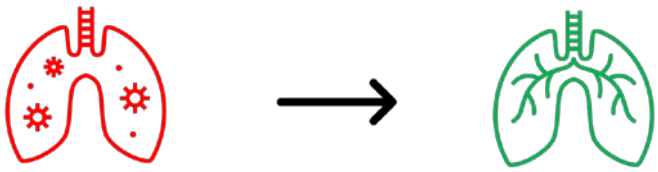

Figure 1. The work described in this thesis can contribute to improve pig health through increased knowledge of respiratory disease in the Norwegian pig population.

Efforts made by the Norwegian pig production sector has resulted in a unique health situation for the Norwegian pig population. *Actinobacillus pleuropneumoniae* (APP) is the suspected agent behind most clinical outbreaks of respiratory disease in pigs in Norway (Animalia 2020b) and the focus of this thesis. A possible association between APP, and/or other agents with respiratory disease outbreaks has not been confirmed. Comprehensive diagnostic investigation of clinical cases could confirm this association. By assessing biosecurity, the risk of infectious diseases in a population could be further understood. Knowledge of the respiratory disease etiology and biosecurity levels in Norwegian pig herds can contribute to prevent future outbreaks.

Genetic methods can help us understand infectious diseases through studying the variability and evolution of agents. Genetic investigations can aid future efforts to prevent respiratory disease in pigs, since genetic methods are frequently used to trace agent transmission. The development of whole genome sequencing (WGS) and bioinformatics are generating new possibilities for studies of transmission and dissemination of genetic traits. The work described in this thesis increased our understanding of the infectious respiratory disease and may contribute to improved health of pigs (Figure 1).

5.1.1 Norwegian pig production

5.1.1.1 Norway in an International perspective

Annual global pork production was estimated to be around 120 million tons (FAO 2020) in 2017, when this study began. The Norwegian contribution to this total was modest, at approx. 0.14 million tons (1.6 million slaughtered pigs) (Statistics Norway 2021b). With a human population of 5.3 million, Norway is self-sufficient in pork, which is the most consumed meat type in Norway (The Norwegian Directorate of Health 2021). Norwegian pig farms have increased in size over the last decade. The total national production of slaughtered pigs increased by 100.000 while the number of registered farms fell from 3379 to 2404 between 2007 and 2017 (Statistics Norway 2021a). This shift towards larger farms is seen in many countries in Europe (Eurostat 2009 , Eurostat 2017) and in the USA (USDA 2017). The national legislation regulates production volume in Norway, which is limited to 105 sows (450 for sow pools) and 2100 finishers per farm (Ministry of Agriculture and Food 2004). Thus, Norwegian pig farms are moderate in size compared to other pig producing countries in Europe, where most sows are being held in large farms with more than 200 sows per farm (Eurostat 2009).

5.1.1.2 Geography, structure, and genetics of the Norwegian pig population

Norway is located to the west on the Scandinavian Peninsula, bordering the ocean in the north, west and south. In the East, Norway borders Sweden,

Finland, and Russia (see Figure 11 for a map of Norway). There are 11 administrative regions in Norway, called counties. Although agricultural production takes place across the whole country, pig production is mainly concentrated in three areas: in the central part of Norway in the county of Trøndelag, in the south-west in the county of Rogaland and in the east surrounding to the capital region of Oslo (Statistics Norway 2021b).

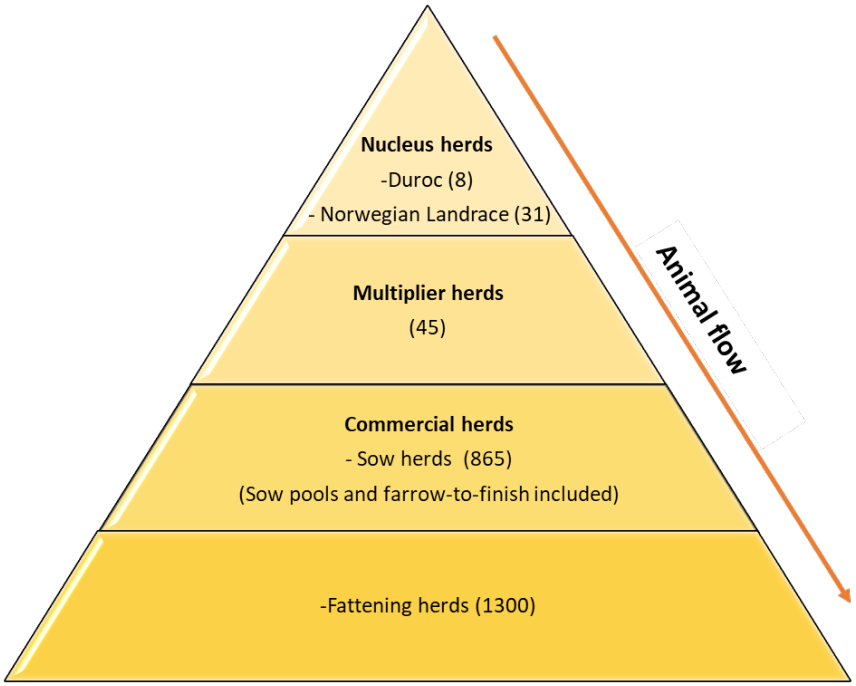

Figure 2. The Norwegian breeding and production pyramid. Purebred Duroc and Norwegian (NO) Landrace nucleus herds at the top and mixed breed multiplier herds in the tier second to the top. Commercial herds that produce pigs for consumption are in the bottom two tiers, with sow herds in the upper bottom tier and fattening herds in the lower bottom. The direction of livestock movement is unidirectionally downwards within this system, as indicated by the arrow (Animal flow). Number of herds per category as of 2017 are included in parentheses (Ingris 2018).

National livestock trade is tied to the structure of the breeding and production system (Figure 2) which is fashioned in a pyramid, with unidirectional downward flow of animals (Svendsby 2013). In the top tier there are nucleus herds (Figure 2), which are purebred Duroc (main boar race in Norway) and Norwegian Landrace breeding herds. The tier next to the top consists of multiplier herds (Figure 2) that cross thoroughbred Landrace sows with semen from Dutch imported Yorkshire (Topigs Z-line) to produce hybrid sows (also known as TN70). The upper bottom tier is made up of commercial sow herds in which hybrid sows are inseminated with Duroc semen (alternatively with Landrace or Hampshire sourced from Norwegian herds) for commercial production of fatteners. Most fattening pigs in Norway is thus a mix of three breeds (Figure 3). In addition to strict piglet producing herds, sow herds includes integrated (farrow-to-finish) herds and sow pools, a system based on leasing of pregnant sows from a central sow pool to several satellite herds until weaning when the sows return to the sow pool (Svendsby 2013). In the bottom tier are the fattening pig herds (Figure 2) that purchase weaner pigs from sow herds at around 10 weeks age or approximately 32 kg body weight (Ingris 2018) and rear the pigs until slaughter (Figure 3). The term fattening pig is thus used here for pigs during the whole rearing stage, as it is not common in Norway to divide this period further.

Main actors in the Norwegian pig production sector include the national cooperative breeding organization (Norsvin SA), the slaughterhouse organizations and The Norwegian Meat and Poultry Research Centre (Animalia AS) (Svendsby 2013). The latter is owned by the slaughterhouse organizations. There are two main slaughterhouse organizations in Norway, namely Nortura SA (cooperative owned by the farmers) and Kjøtt- og fjørfebransjens Landsforbund (union of private slaughterhouses). Livestock trade is usually organized by the slaughterhouses (Nortura 2018).

Figure 3. Norwegian fattening pigs are a mix between three breeds: Duroc, Norwegian Landrace and Dutch Yorkshire (Z-line). This results in differences in phenotypic traits. Photo credit: Liza Miriam Cohen.

5.1.2 The health status of Norwegian pigs

5.1.2.1 Biosecurity for a healthier pig population

Biosecurity is *a set of management and physical measures designed to reduce the risk of introduction, establishment and spread of animal diseases, infections, or infestations to, from and within an animal population* (OIE 2021b). Biosecurity is commonly divided into external and internal biosecurity. External biosecurity is aimed at avoiding the entry of pathogens into a population, and internal biosecurity is aimed at preventing the spread of pathogens to uninfected animals in the population when the pathogen is already present (FAO *et al.* 2010). Decisions regarding biosecurity to protect a defined population (i.e., international, national or herd) are made at

different levels, illustrated in the biosecurity pyramid (Figure 4). All biosecurity strategies are based on knowledge of infection biology and specific disease epidemiology (FAO *et al.* 2010). This way, potential sources of infection and their associated risk can be identified. Surveillance, traceability, prevention, and eradication plans build on the same knowledge foundation, and are together with biosecurity, important components of infectious disease control.

5.1.2.1.1 International and National biosecurity

International and national biosecurity policies have been established (Figure 4) to prevent pig diseases which represent a major threat to production and trade in many countries (FAO *et al.* 2010). To ensure smooth international trade the world trade organization has assigned the World Organization for Animal Health (OIE) to provide guidelines for handling animal disease (WTO 1995). The Terrestrial Animal Health Code was developed (OIE 2021b) to provide generic and disease specific guidance for diagnosing and demonstrating freedom of agents, as well as prevention and surveillance of various diseases (OIE 2021a).

Norway is a member of the European Economic Area (EEA) (Figure 4) and is obligated to implement legislation from the European Union (EU). In alignment with the international standards of OIE, The Animal Health Law (European Parliament and Council 2016b) encourages a unified approach to biosecurity in animal production, and to surveillance and handling of emerging threats like severe infectious disease pathogens.

Restricting the introduction of live susceptible species that endangers the freedom status of a population is a key feature of the national biosecurity (Figure 4). In the years since 1994, *The national surveillance programme for specific viral infections in swine* has been surveilling the Norwegian pig population for occurrence of several viral infections (Grøntvedt 2018b). Norwegian pig herds have continually been free from Aujezky's Disease Virus, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), all strains of Swine Influenza Virus (SIV) except for (H1N1)pdm09, transmissible gastroenteritis and porcine epidemic diarrhea virus,

(Grøntvedt 2018b). Norway's freedom from Aujezky's disease has been recognized in the EEA (ESA 2010). To protect the Norwegian pig population from these agents, import of livestock and genetic material (semen) has been strictly regulated through national legislation and additional regulations provided by the pig production sector (Ministry of Agriculture and Food 1998 , ESA 2010 , KOORIMP *et al.* 2021).

National and local biosecurity measures (Figure 4) limit the occurrence and impact of infections that occur sporadically (FAO *et al.* 2010). National measures to maintain a favorable health status for Norwegian pigs include, but are not limited to legal biosecurity requirements in the *Regulation on keeping of pigs* (Ministry of Agriculture and Food 2003), which are aimed at good hygienic practices and prevention of disease transmission. Regulation of production volume and domestic livestock trade that limit live animal contact points are important to national disease control (Neumann *et al.* 2019).

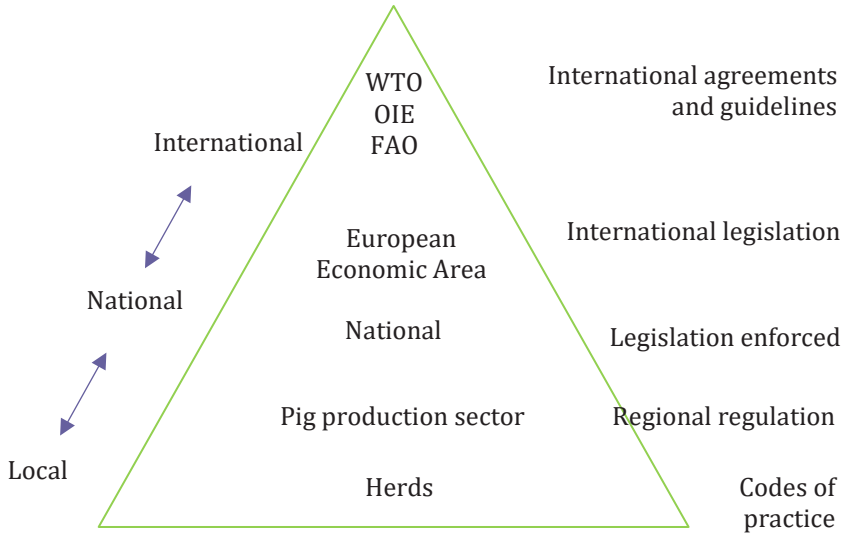

Figure 4. The Biosecurity Pyramid. Decisions regarding biosecurity is made at different levels, from different international levels on top, through national and regional levels down to the individual farms on the bottom. World Trade Organization (WTO), World organization for animal health (OIE), Food and Agriculture Organization (FAO). Figure adapted from Oidtmann et al. (2011).

5.1.2.1.2 Biosecurity assessment

Biosecurity measures for control of diseases are usually targeted at the transmission routes of relevant infective agents (FAO *et al.* 2010). Generally, disease transmission can happen directly via contact between infected and susceptible animals, directly via air, or indirectly via contaminated vehicles. Vehicles for indirect transmission include inanimate objects such as semen, manure, feed, water and fomites, people, or vector animals such as insects, rodents and birds (FAO *et al.* 2010). Direct animal contact is the most common and most efficient mode of transmission (Filippitzi *et al.* 2017). Thus, segregation is listed as the main principle of biosecurity in *Good biosecurity practices in the pig sector* (FAO *et al.* 2010), followed by cleaning and disinfection.

Assessing biosecurity can help us understand at what level a population is protected against the potential threats of infectious disease. In Norway there is a high level of national biosecurity to protect the favourable health status of Norwegian pigs. However, there are no studies describing the current external and internal biosecurity levels of Norwegian fattening pig herds. The biosecurity level in a herd is not easily quantifiable. The overall biosecurity is impacted by many factors, but not all of these are associated with the same risk. A weighted scoring system is therefore useful and can provide better grounds for measuring and comparing biosecurity objectively. Biocheck.UGent™ is an established tool (Ghent University), developed by Ghent University, Belgium (Laanen *et al.* 2010), for mapping biosecurity at herd level in poultry, cattle and pig herds. This tool is comprised of a detailed questionnaire that forms the basis for a quantitative scoring of biosecurity within 12 defined areas of the production. The questionnaire is paired with a knowledge database that provides advice targeted at the areas of improvement. The scoring hence reflects the general prevention of introduction, establishment and spread of infectious disease to, from and within the herd. Biocheck.UGent™ has been utilized for biosecurity assessment in various countries, but is less often used in fattening pig herds, according to published literature.

5.1.2.2 Pathogen freedom in populations

Norway has a tradition for eradicating infectious agents from animal populations. Systematic efforts by the pig production sector have thus led to the eradication of the infectious respiratory agent *Mycoplasma hyopneumoniae* (Gulliksen *et al.* 2021), adding to the list of pathogen freedom in the general pig population. Systematic sanitation is also the reason there are minimal detected pig herds with Methicillin resistant *Staphylococcus aureus* in Norway (Urdahl *et al.* 2021). In addition, Norway has a Specific Pathogen Free (SPF) subpopulation, in which there is also freedom from porcine pleuropneumonia (APP), atrophic rhinitis (toxigenic *Pasteurella multocida*), swine dysentery (*Brachyspira hyodysenteriae*) and sarcoptic mange (*Sarcoptes scabiei* var suis) (Animalia 2021b). In 2016 there were nearly 60 SPF herds in total: six breeding herds, 30 commercial sow herds and 20 fattening herds (Ingris 2017). Norsvin declared in 2016 that a systematic effort to convert all breeding and multiplier herds to SPF will take place in the years to come (Animalia 2021b). Outbreaks of APP have occurred in some SPF herds, causing them to lose their SPF-status. The source of transmission is seldom identified (Animalia 2020b).

5.1.2.3 Health monitoring

Monitoring pig health is performed during the production and at slaughter. Veterinary treatment registration is mandatory for production animals. Dyrehelseportalen (DHP) is the national veterinary treatment database, in which indications for drug use is included. Available recordings of health parameters in the pig herds vary with production type. Fattening pig herds have been less closely monitored through the production stages compared to other herd categories. There are specific requirements to health documentation in herds that supply livestock, i.e. breeding herds (Animalia 2020a) and commercial sow herds (Ingris 2021). Ingris is a voluntary national recording system for Norwegian pig producers, including both health and production data. The Ingris users represented 77.5% of breeding sows and 32% of fattening pigs in Norway in 2020 (Ingris 2021). A new system for documenting health, welfare, and hygiene data from all herds in the production system was established in 2019 (Animalia 2021a).

Health and welfare scorings during routine post-mortem inspections are performed at slaughter by the Norwegian Food safety authority. Scorings within eight relevant lesion categories make up the "Extended disease registration" (USR). The USR data can be a good tool for surveillance of the herd health status. The assessment does, however, not provide information regarding etiology behind the registered lesion.

5.1.2.4 Antimicrobial drug usage

There is a conservative use of antimicrobial drugs in farm animals in Norway (ESVAC 2015 , NORM/NORM-VET 2017). Antimicrobial drugs have not been used as growth promotors in pigs. Drugs for oral treatment of pigs are seldom used compared to injectable formulations, and narrow-spectrum drugs are recommended as first drug of choice (Norwegian Medicines Agency 2012). Group treatments occur mostly as metaphylactic treatment during outbreaks of severe bacterial infections.

Monitoring of antimicrobial usage and resistance in food-producing terrestrial animals is part of a national strategy that has been in place since year 2000 (NORM/NORM-VET 2017). This monitoring is in line with recommendations from the World Health Organization (World Healt Organization 2015) and European efforts (European Comission 2011) to handle emergence of antimicrobial resistance (AMR). A low level of resistance was found in the indicator bacteria *Escherichia coli* and *Enterococcus* spp. in Norwegian pigs (NORM/NORM-VET 2017), compared to 17 other European countries (EFSA and ECDC 2020). Nevertheless, there has lately been an increase in susceptible isolates in most of the European countries tested, including Norway (EFSA and ECDC 2020).

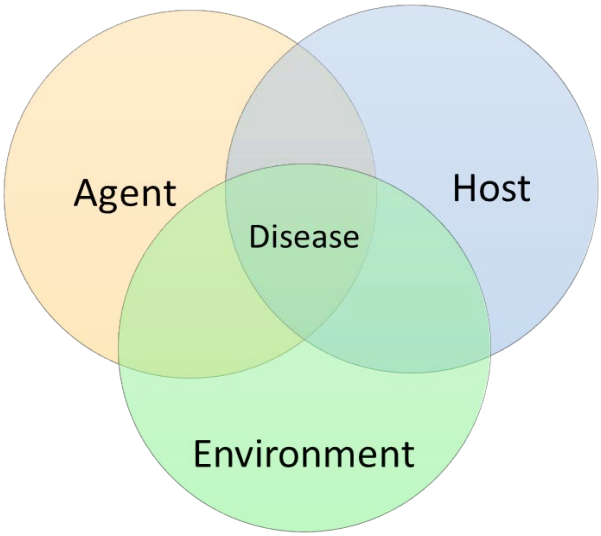

Figure 5. A Venn diagram of the epidemiological triad, with the three aspects of infectious disease, namely the agent, the host, and the environment. Figure adapted from van Seventer et al. (2017).

5.1.3 Respiratory disease in pigs

The understanding of infectious respiratory diseases in pigs can be approached trough the classic model of infectious disease, the epidemiological triad (Figure 5) (CDC 2014 , Robertson 2020). Respiratory disease is a multifactorial disease that results from interactions between a) a variety of environmental factors, b) properties of the host and c) infectious agents (Stärk 2000 , Brockmeier *et al.* 2002 , Opriessnig *et al.* 2011 , van Alstine 2012). The term Porcine respiratory disease complex has been established to encapsulate this intricate nature (Brockmeier *et al.* 2002).

5.1.3.1 The environment

Environmental factors that influence disease occurrence encapsulate geographical and structural traits such as those already mentioned for the Norwegian pig population. Cultural, political, and economic circumstances are also aspects of the environment that may facilitate disease (van Seventer *et al.* 2017). On farm, environmental factors tied to management, climate,

and air-quality are of relevance to respiratory health, as reviewed by Stärk (2000). Examples of management factors include livestock purchase policy, stocking density and the use of sick pens, to mention some. These typically influence the pigs' exposure to infectious agents, and increase stress which compromises the natural defenses of the pig (Stärk 2000). Sub-optimal climatic factors and air quality can also contribute to stress and impairment of the mucociliary apparatus (Yaeger *et al.* 2019). Climatic factors have also been found to alter traits and infective abilities of the infectious agents, exemplified by the effect of different environmental temperatures on the respiratory infectious agent *Bordetella bronchiseptica* (Akerley *et al.* 1992).

5.1.3.2 Host and agent interaction

Interactions between agent and host are central to disease development. The ability to cause disease (pathogenicity), and the severity of the inflicted disease (virulence) are important traits of an infectious agent (CDC 2014). Virulence factors are specific cellular structures or molecules produced by the agent that contribute to agent's ability to cause disease. Like other mammals, pigs have innate respiratory defenses against infections and infestations including a mucociliary apparatus, peripheral immune cells, mucosal immunoglobulins, and a cell-mediated immune response system (Yaeger *et al.* 2019). Respiratory agents depend on virulence factors to colonize the respiratory tract, evading and inhibiting the immune responses of the pig and obtaining nutrients from the host (Yaeger *et al.* 2019).

Respiratory disease typically affects fattening pigs (Thacker 2001), but can also affect younger pigs, depending on the agents involved, maternal and acquired immune status of the pig as demonstrated by Kott (1983). Introduction of new agents into naïve herds will thus affect pigs in all ages. Agent load, virulence factors, and differences in the susceptibility of the host will influence the severity of disease characteristics (van Seventer *et al.* 2017). Severity of respiratory disease can range from a subclinical and mild transient course, to a severe acute or peracute course with a fatal outcome. Clinical signs associated with respiratory disease include sneezing, coughing, respiratory distress (dyspnea), lethargy, fever, reduced general condition, and reduced appetite and weight gain (Loeffen *et al.* 1999 , Bochev 2007).

5.1.3.3 Infectious respiratory agents

Multimicrobial interactions have been central in respiratory disease in pigs (Opriessnig *et al.* 2011). Infectious agents that cause respiratory disease can be divided into primary and secondary invaders (Yaeger *et al.* 2019). While primary invaders possess virulence factors that allow them to cause disease on their own, secondary invaders will only contribute to disease development following a primary infection that reduce the natural respiratory defenses of the pig (Beskow *et al.* 1998 , Yaeger *et al.* 2019). Secondary invaders are often commensals in the respiratory tract, therefore also referred to as opportunistic infectious agents (Brockmeier *et al.* 2002). Large variations in etiologic agents and characteristics of respiratory disease exist between countries, subpopulations, and herds. Comprehensive diagnostic procedures are thus helpful in determining the disease etiology.

5.1.3.3.1 Virus

Primary respiratory viruses include SIV, Porcine Respiratory Coronavirus (PRCV), Porcine Circovirus type 2 (PCV2) and PRRSV (Brockmeier *et al.* 2002). Highly virulent viral strains exist and can cause severe respiratory disease (Opriessnig *et al.* 2011) usually in the form of diffuse interstitial pneumonias (Yaeger *et al.* 2019). Uncomplicated viral infections are often transient; however, these viruses are often involved in multimicrobial interactions with other virus or bacteria. The respiratory viruses impair the natural defenses of the respiratory tract (Opriessnig *et al.* 2011), thus typically precede secondary invaders.

5.1.3.3.2 Bacteria

Prevalent primary bacterial invaders of the lower respiratory tract are *M. hyopneumoniae,* APP, and *B. bronchiseptica* in nursery pigs (Brockmeier *et al.* 2002 , Yaeger *et al.* 2019). Bacteria that are common as secondary invaders in pigs are *P. multocida, Streptococcus suis, Haemophilus parasuis* and *Trueperella pyogenes* (Brockmeier *et al.* 2002 , Yaeger *et al.* 2019). Infectious respiratory bacteria primarily cause bronchopneumonia, with cranioventrally distributed lesions (Yaeger *et al.* 2019), except for APP which is characterized by consolidation and hemorrhage dorsally in the caudal lung lobes (Shope 1964).

5.1.3.3.3 Parasites

Ascarids are enteric parasites that complete their life cycle by larval migration via internal organs, including the lung. *Ascaris suum* infestation and migration therefore causes trauma and inflammation of the lung (Schwartz *et al.* 1932). Secondary bacterial pneumonia succeeding larval migration might also occur (Brewer *et al.* 2019).

5.1.3.4 Porcine respiratory disease globally

Respiratory disease contributes to substantial economic losses and compromised animal welfare. Losses are associated with increased mortality, decreased weight gain, increased feed cost, condemnation at slaughter and increased costs of treatment, vaccination and labor (Yaeger *et al.* 2019). Although some literature focuses on the associated production loss, respiratory disease is also a contributor to animal suffering. In practice, the burden of respiratory disease is easiest to assess in acute manifestations such as outbreaks. It is assumed that other forms of disease also negatively affect production parameters like weight gain (Hill *et al.* 1994), growth rate (Wilson *et al.* 1986) or animal welfare. In USA respiratory disease has been the main cause of deaths in nursery (47.3%) and finishing pigs (75.1%) (USDA 2017). Publications indicating the overall prevalence of clinical respiratory disease are rare (Bochev 2007). In a study of clinical signs in Danish finisher pigs, clinical respiratory signs was found to be the second most prevalent (2.17%) only second to ear necrosis (Petersen *et al.* 2008). Prevalence of pneumonia lesions at slaughter is a widely used metric for occurrence of respiratory disease (Stärk 2000), however the results depend very much on the criteria for scoring (Davies *et al.* 1995). Lung lesions at slaughter do not reflect the clinical significance very well due to a high prevalence of subclinical infections (Stärk 2000), and healing of infections gained early in the fattening period (Wallgren *et al.* 1994).

5.1.3.5 Porcine respiratory disease in Norway

Respiratory diseases have been a major challenge to the pig production in Norway for several decades. A high prevalence of lung lesions at slaughter, caused mainly by *M. hyopneumoniae* (enzootic pneumonia) was found in Norwegian pig herds in the early 1990s *(Lium et al. 1991).* There was strong

evidence of multimicrobial interaction with secondary bacterial invaders (Falk *et al.* 1991a). Although less prevalent, lesions of porcine pleuropneumonia were also identified *(Lium et al. 1991).* Microbiologic investigations of chronic lesions were largely dominated by growth of secondary invaders, mainly *P. multocida* (Høie *et al.* 1991). Serologic investigations indicated that most breeding herds, and thereby most conventional pig herds further down in the breeding and production pyramid (Figure 2), were harboring one or more serovars of APP (Lium 2002).

In 1994 The Board of The Norwegian Pig Health Service (now: Animalia) started a national surveillance and eradication program for *M. hyopneumoniae,* based on partial depopulation and antimicrobial medication in breeding animals. In 2009, the population was declared free (Gulliksen *et al.* 2021). Although no systematic investigation of the respiratory health of Norwegian pigs has been published since 1991, serologic surveillance has continued to confirm the absence of *M. hyopneumoniae* from the population (Gulliksen *et al.* 2021). Furthermore, during 2009 and 2010 the active national serosurveillance documented the first introduction of SIV to the pig population since the surveillance was started in 1997 (Lium 2010). Investigations of SIV in the Norwegian population by Grøntvedt *et al.* (2011) indicated that the virus was of low virulence, as infections in naïve pig herds were mostly subclinical and transient. In 2018 the first serological evidence of PRCV in Norwegian pigs was detected by the surveillance program (Grøntvedt *et al.* 2019b). The PRCV cases have not been associated with clinical disease (Grøntvedt *et al.* 2019a), yet the virus has continued to spread rapidly in the population. In 2019, the national seroprevalence of PRCV was estimated to be 21.7% (Grøntvedt *et al.* 2020).

Postweaning multisystemic wasting syndrome, caused by PCV2, causes pigs to suffer from progressive weight loss (Kim *et al.* 2003) and was diagnosed in Norway for the first time in 2003. Although PCV2 is assumed to be highly prevalent in Norwegian pig herds, little is known about PCV2s relevance to respiratory health in the population. Respiratory infections with PCV2 typically has a low morbidity and high case fatality (Brockmeier *et al.* 2002).

The importance of respiratory disease in Norwegian pigs today can be assessed through available data entries from health registers. Respiratory diseases made out 0.6% of all disease registrations in Ingris in 2017 (Ingris 2018), in which 26,5% of Norway's fattening pigs were represented at the time. Respiratory diseases have been among the 10 most reported diagnoses in pigs the DHP in the years from 2018-2020. The proportion of registered cases of respiratory disease was 6.9% in the DHP in 2018, and 3% on 2019. During the same time there had been a three-time increase in the number of vaccinations against porcine pleuropneumoniae (Gulliksen SM, swine veterinarian, Animalia, personal communication by email, 2021 Oct. 31.). The percentage of pigs with pneumonia lesions at slaughter has been low since 2017, measuring 2.05% on average across all Norwegian slaughterhouses in 2020. Pleuritis was scored in 6.8% of slaughtered pigs in 2020 and has been stable since 2018 (Røtterud O.J., Special Advisor, Animalia, personal communication by email, 2021 Feb. 26.). The criteria for scoring are lung lesions bigger than 5 cm and pleura lesions bigger than 15 cm in diameter.

After the eradication of *M. hyopneumoniae*, the pig production sector claims the biggest challenge has been related to the occurrence of APP (Animalia 2021b). According to the surveillance of voluntarily submitted specimen for post-mortem diagnostics at the Norwegian Veterinary Institute (NVI), the most common infectious agent involved in respiratory disease in recent years has been APP (Grøntvedt 2017). In addition, bacteria like *P. multocida* and *Glässerella parasuis* were frequently isolated from pneumonic lesions. Although less common, *A. suum* could also play a role in respiratory disease in pigs. *Ascaris suum* has been common in Norwegian pig herds for decades (Norwegian Medicines Agency 2001 , Animalia 2018). Involvement of commonly occurring viruses such as PCV2 and SIV (H1N1)pdm09 in the pathogenesis of porcine respiratory disease in Norway have not been elucidated. An assessment of the etiologic agents behind clinical respiratory disease and to what extent they were the result of multimicrobial interactions, was warranted.

5.1.4 *Actinobacillus pleuropneumoniae*

The bacterium was first isolated from a pneumonic lung of a pig in Great Britain in 1957 and was initially described as a Haemophilus-like organism (Pattison *et al.* 1957). It was later given the names *Haemophilus pleuropneumoniae* (Shope 1964) and *H. parahaemolyticus*, before assigned to the family *Pasteurellaceae* and genus *Actinobacillus* (Pohl 1983). Since its discovery, APP has been associated with infections of the lungs and pleura of pigs, referred to as porcine pleuropneumonia (Shope 1964). APP is a commensal of the porcine upper respiratory tract, residing in tonsillar crypts (Chiers *et al.* 1999), with the potential of causing pneumonia in pigs and wild boar (Reiner *et al.* 2010).

5.1.4.1 Classification and epidemiology

Actinobacillus pleuropneumoniae is a Gram-negative coccobacillary rod (Pattison *et al.* 1957), traditionally classified either by biovar, serovar or toxin profile (Table 1). Classification of biovar (biovar I and II) is based on nicotinamide adenine dinucleotide (NAD) dependency for growth *in vitro*, where biovar I is NAD-dependent (Pohl 1983). Serotyping of APP is based on the composition of capsular polysaccharides (CPS) and the cell wall lipopolysaccharides (LPS), which are usually associated with each other. Discrepancy between CPS (K) and LPS (O) antigens occurs. The result of the serotyping will therefore depend on whether a CPS-based or LPS-based test is used (Gottschalk *et al.* 2000). In traditional LPS based serological assays for serotyping APP, the phenomenon of cross-reacting serovars is well known due to identical LPS in groups of serovars (Table 1) (Dubreuil *et al.* 2000). More specific methods for serovar determination have been pursued. A multiplex PCR that was able to discriminate between all described serovars was published in 2018 (Bossé *et al.* 2018a) and has since become commercially available. Serotype designation has accelerated with increased diagnostic capability. As a result, classification of some previously untypable strains can now be achieved (Bossé *et al.* 2018b), and previously misclassified serovars due to cross-reactions have been reclassified (O'Neill *et al.* 2010 , Gottschalk *et al.* 2015).

Repeats in toxin (RTX) toxins is a group of cytotoxins and cytolysins. Four RTX toxins are produced by APP (ApxI-IV) and have been central in the diagnosis and classification of this bacterium (Frey *et al.* 1993 , Schaller *et al.* 1999). All serovars express the APP specific ApxIV as a surface protein *(Schaller et al. 1999)*. The toxins ApxI-ApxIII are of correspondingly high, moderate, and mild cytotoxicity, expressed in different combinations by different serovars (Table 1) (Frey *et al.* 1993). Although presence of toxins is usually tied to serovar and biovar, many atypical strains have been described (Gottschalk *et al.* 2019).

In Europe, APP serovar 2 (APP2) is a moderately virulent and common clinical serovar (i.e., isolated from clinical cases of porcine pleuropneumonia). As investigations of lung lesions and serum at slaughter in the 1980's showed, APP2 has also been a prevalent serovar in Norwegian pigs (Falk *et al.* 1991b , Høie *et al.* 1991). The serologic investigation of Norwegian breeding herds around the same period found a slightly higher seropositivity to APP2 relative to serovar 6 (APP6) (Falk *et al.* 1990). In later years, diagnostic results from the NVI have concluded that APP serovar 8 (APP8) is the most common serovar isolated from pneumonic lungs, followed by serovar 6 and 2 (Grøntvedt 2018a). As shown in a review by Dubreuil *et al.* (2000), prevalence of the moderately virulent APP8 was only reported from a subset of countries, however, an updated report on the global distribution of serovars is needed. A paper from 2016 stated that APP8 predominates as clinical serovar in the United Kingdom (Li *et al.* 2016).

Table 1. Overview of APP serovars, and their respective biovar, Apx toxin distribution, their associated virulence and LPS group

Serovar	Biovar*[a]	Apx I[a]	Apx II[a]	Apx III[a]	Virulence**[a]	LPS group cross reactivity[b]
1	I	+	+		High	1/9/11
2	I		+	+	Moderate	
3	I		+	+	Low/Moderate	3/6/8/15
4	I		+	+	Moderate	4/7
5a, 5b	I	+	+		High	
6	I		+	+	Moderate	3/6/8/15
7	I		+		Moderate	4/7
8	I		+	+	Moderate	3/6/8/15
9	I	+	+		High	1/9/11
10	I	+			High	
11	I	+	+		High	1/9/11
12	I		+		Moderate	
13	II		+		Moderate	
14	II	+			High	
15	I		+	+	Moderate	3/6/8/15
16[c]	I		+		High	
17[d]	I		+		Moderate	8
18[d]	I		+		Moderate	
19[e]	I		+		Moderate	3/6/8/15 or 4/7

Actinobacillus pleuropneumoniae repeats-in-toxin (Apx), Lipo-polysaccharides (LPS).

*Differences in nicotinamide adenine dinucleotide-dependency among strains of the same serovars exist, so they occur both as biovar I and II.

**Based on cytotoxic/cytolytic abilities of the Apx toxins within the serovar.

Table compiled using data from [a] Frey (2003), [b] Dubreuil *et al.* (2000), [c] Sárközi *et al.* (2015), [d] Bossé *et al*. (2018b), [e] Stringer *et al*. (2021a).

5.1.4.2 Virulence factors and disease pathogenesis

To cause infections in the porcine lung, APP depends on a variety of virulence factors, summed up in reviews by Bossé *et al.* (2002) and Chiers *et al.* (2010). The previously mentioned LPS, embedded in the cell wall, is essential for adhesion to the cells of the respiratory tract (Bossé *et al.* 2002 , Chiers *et al.* 2010). Fimbriae and outer membrane proteins (OMPs) expressed by many strains might also contribute to the adhesive abilities of APP (Overbeke *et al.* 2002). APP thus binds to and colonizes squamous epithelia in the upper respiratory tract, including the cells on the surface and in the crypts of the palatine tonsils (Chiers *et al.* 1999). The ability to take up and utilize iron is vital to the survival and growth of APP in the pig (Bossé *et al.* 2002). As an adaptation to iron deficient environment of the respiratory tract, APP relies on a number mechanisms and has more than 50 genes involved in utilizing iron, as identified in the genome of a Chinese serovar 3 strain (Xu *et al.* 2008).

As APP does not bind well to the ciliated epithelium of the trachea and bronchi, it relies on inhalation to be able to colonize the pneumocytes of the alveoli (Bossé *et al.* 2002). The cytotoxic and hemolytic abilities of the Apx toxins causes significant vascular damage followed by edema, thrombosis, and necrosis within hours of infliction (Auger *et al.* 2009). This mechanism allows the pathogen to obtain necessary nutrients for continued growth (Chiers *et al.* 2010). In addition to its role in adherence, LPS acts to stimulate alveolar macrophages to release inflammatory cytokines, this pro-inflammatory effect is reinforced by activation of phagocytic cells as a response to tissue damage (Fenwick *et al.* 1986). Capsular polysaccharides in the cell wall protects APP from mechanisms of the innate immune response of the pig by preventing phagocytosis and complement binding (Ward *et al.* 1994). Additionally, there are several other molecules, including proteases, ureases, and superoxide dismutase that contribute to the virulence of APP (Bossé *et al.* 2002 , Chiers *et al.* 2010).

Less virulent strains of APP may rely on high bacterial loads or impairment of the pigs' natural defenses to colonize the lower respiratory tract, while

other, strongly virulent strains of APP may not (Sassu *et al.* 2017). Impairment of the natural defenses may be due to prior infection with pathogens that disrupt the mucociliary apparatus, stress related management factors and physical factors such as high levels of dust or ammonia (Stärk 2000). The incubation period and onset of clinical signs is also influenced by factors of the environment and can be shortened considerably if the pigs are subjected to stressful events (Gottschalk *et al.* 2019). Severity of infections with low and moderately virulent strains, including strains of APP8, was observed experimentally to increase in the presence of *M. hyopneumoniae (Marois et al. 2009).* In another study of experimentally infected pigs, many serovars that were less commonly associated with disease, were able to produce severe clinical signs all alone (Costa *et al.* 2011). Due to the complex interactions between factors in the pigs' environment, circumstances of a disease incidence are difficult to capture under field conditions, and hard to simulate in controlled experiments (Stärk 2000). The virulence of APP in Norway has not been evaluated.

5.1.4.3 Pathology and clinical signs

Clinical disease commonly occurs in the form of acute outbreaks, the mechanisms of which have been studied under experimental conditions (Klinkenberg *et al.* 2014). Pigs in all age groups are affected in naïve herds, whereas fatteners have a higher risk of clinical disease in endemically infected herds. This is likely tied both to the immune status of the pigs and the presence of environmental factors that facilitate disease development during the fattening stage (Gottschalk *et al.* 2019).

In cases where the infectious load is high, APP can lead to peracute deaths, typically with a bloody froth from oronasal orifices (Bossé *et al.* 2002 , Sibila *et al.* 2014). Some individuals may develop high fever (>41 °C), severely reduced general condition, cyanotic mucosa/skin, vomitus, and diarrhea for a short period before they succumb. Animals often present in a sitting posture (Gottschalk *et al.* 2019). Gross pathologic changes due to extensive tissue damage include bloody froth in the trachea and bronchi and dark red,

firm lungs with diffuse hemorrhages and necrotic areas on the cut surface, while the pleura can be unremarkable (Gottschalk *et al.* 2019).

During acute illness, hyperemic or cyanotic skin and mucosa can be observed due to venous blood congestion and compromised ventilation (Gottschalk *et al.* 2019). The pigs commonly display reduced general condition, fever, cough, and dyspnea (Vigre *et al.* 2002). Gross pathologic changes in the lungs include firm, rubbery, irregular areas with hyperemia and hemorrhage. A greyish edema is most frequently seen in dorsal parts of the caudal lung lobes (Rosendal *et al.* 1985 , Sibila *et al.* 2014). There is typically fibrin deposited on the pleura. Effects of the LPS endotoxin and hemolytic effects of the Apx toxins will lead to vasculitis, blood stasis, thromboses of blood vessels and hemorrhage and marked fibrinous exudation, which are evident histologically during the acute phase (Bossé *et al.* 2002). Enzyme release from degenerating polymorphonuclear cells will contribute to the considerable vascular damage (Bossé *et al.* 2002). In acute lesions, there are marked demarcation lines between densely packed polymorphonuclear cells in the necrotic tissue and mononuclear cells in the live tissue (Gottschalk *et al.* 2019).

Subclinical manifestation of APP typically occurs in herds that are endemically exposed or vaccinated, and the pigs have responded to the challenge by producing neutralizing antibodies, typically to the antigenic Apx toxins (Nechvatalova *et al.* 2005). In such herds, no clinical signs might be detected, but pleurisy lesions at slaughter can be highly prevalent, depending on the time of infection (Sibila *et al.* 2014). Chronic infections can follow acute or subclinical infections and are characterized by little to no reduction in general condition, and mild to moderate clinical signs from the respiratory tract (Gottschalk *et al.* 2019). Pathologic changes include necrotic abscesses in dorsal parts of the caudal lung lobes. Local chronic, adhesive pleuritis, and sometimes diffuse pleuritis and pericarditis is seen (Merialdi *et al.* 2012). The use of slaughterhouse data is widely used to detect problems with APP in subclinical and chronically infected herds.

5.1.4.4 Transmission and infection dynamics

Direct contact between animals is the main transmission route of APP, commonly from sows to suckling piglets, or by aerosols over short distances (Chiers *et al.* 2002a , Velthuis *et al.* 2003 , Fablet *et al.* 2011 , Tobias 2014 , Tobias *et al.* 2014a). Diseased and infectious pigs can transmit bacteria through aerosols by coughing, and in their saliva and nasal secretions by nose-to-nose contact to susceptible pigs (Gottschalk *et al.* 2019). Indirect transmission of APP in exudates from infective pigs via personnel and equipment is also typical, and was verified in an experimental study by Assavacheep *et al.* (2013). Acute infections and presence of acute clinical signs facilitate the transmission of APP by contributing to an increase in infectious pressure. Yet, healthy carriers that harbor bacteria in their tonsils and in subclinical and chronic lesions are central to the spread of APP. In most modern pig producing countries, herds are endemically infected with APP (Gottschalk 2015 , Sassu *et al.* 2017). Pigs have been shown to carry a variety of APP strains in their tonsils, that can differ in both serovar and virulence (Vigre *et al.* 2002). While the more virulent isolates are able to colonize the lower respiratory tract, other isolates will not (Gottschalk 2015). *Actinobacillus pleuropneumoniae* is known to be a late colonizer in piglets, early transmission from sows to their offspring has been observed from the tenth day of life (Vigre *et al.* 2002). Transmission to suckling piglets has not taken place in all litters by the time they are weaned (Tobias *et al.* 2014b). For this reason, age at weaning influences the carrier rates in piglets.

Between herd transmission occurs with healthy carrier animals, or contaminated tools or materials. Air born transmission is commonly viewed as unlikely (Kristensen *et al.* 2004), however, has on occasion been found to be the most likely explanation for introduction of APP to herds that were previously confirmed free (Desrosiers *et al.* 1998 , Animalia 2020b). The survival of APP in the environment is short but will significantly increase in a cold and humid climate and when protected by organic matter like mucus (Assavacheep *et al.* 2013). In response to stress, APP can form biofilms both in the host where it contributes to increased resistance against immune mechanisms (Bossé *et al.* 2010), and in the environment where it may prolong bacterial survival (Loera-Muro *et al.* 2013). It is acknowledged that

thorough washing and the use of common disinfectants are effective against APP (Gottschalk *et al.* 2019).

Herd factors associated with a higher risk for disease, include large herds, a continuous production and purchase of pigs from multiple suppliers. Mixing pigs of different health status was found by Rosendal *et al.* (1983) to facilitate direct transmission of virulent strains of APP. The way pigs are reared in commercial farms today is quite different from 30 years ago, and the need for updated knowledge regarding risk factors has been addressed by Sassu *et al*. (2017).

In the event of an outbreak, infection might not occur in every pen, the within-pen direct transmission has been shown to be 10 times as effective as between pen transmission (Tobias *et al.* 2014a). Proper isolation of sick animals and good hygienic practices between handling sick pigs and contaminated carcasses are implementations that could limit between-pen transmissions. It has been suggested that in endemically infected herds where APP is harbored asymptomatically in the tonsils, environmental triggers (such as stress or co-infection with other respiratory agents) play a larger role in causing disease outbreaks than transmission of a newly introduced virulent strain (Klinkenberg *et al.* 2014). In that case, differences in occurrence of infection between pens could also be explained by presence of the triggering factor. More knowledge regarding the mechanisms behind outbreaks of APP is needed. By assessing the variability of APP isolates within-host during infection, a better understanding of these mechanisms can be gained.

5.1.4.5 Diagnosis

5.1.4.5.1 Detection by bacteriology and molecular methods

The diagnosis of porcine pleuropneumonia differs from that which is applied to detect APP carrier status. Samples from the upper respiratory tract should be evaluated with caution, since APP might reside there without causing disease (Gottschalk 2015). Bronchoalveolar lavage and tonsillar scraping allow the testing of living pigs but are invasive and hard to perform.

Bacteriological examination of tonsils is problematic because APP resides deep in the tonsillar crypts (Overbeke *et al.* 2002), and commensal tonsillar bacterial species tend to overgrow APP in culture (Costa *et al.* 2011 , Gottschalk 2015). Other species of the *Actinobacillus* genus can be found in the upper respiratory tract and tonsils of pigs and are hard to differentiate from APP by traditional bacteriological procedures. Methods to enrich or amplify APP in such samples have been published, as mentioned by Sassu *et al.* in their review (2017). Justification for bacteriological isolation is that it allows for further testing that requires access to live APP, for instance antimicrobial susceptibility or deoxyribonucleic acid (DNA) sequencing, despite the extra time and cost associated with these methods (Gottschalk 2015).

Carrier status is usually assessed serologically. Tonsillar brush or scrape sampling with subsequent detection of APP DNA by polymerase chain reaction (PCR) diagnostics is the most sensitive method to confirm or disprove negative serological test results in negative herds (Chiers *et al.* 2002b). Oral fluid (OF) sampling is much less invasive, but PCR detection on OF was unsuitable for routine testing of subclinical and chronic infections due to differences in sensitivity between serovars (Costa *et al.* 2011). Thus, it is not particularly suited either for diagnosis or verification of carrier state.

Bacteriological examination of affected lung tissue obtained during necropsy is the gold standard for diagnosis of respiratory disease caused by APP, and the successful isolation of APP strains remains the pre-requisite for serovar determination by most reference laboratories (Sassu *et al.* 2017). Testing should be aimed at pigs with acute clinical signs to increase the likelihood of finding typical acute lesions from which the bacteriological samples are taken. Chronic lesions are often overgrown with secondary infectors like *P. multocida* or *T. pyogenes*. They can also be sterile, even so that APP antigen or DNA cannot be detected in the lesions (Gottschalk *et al.* 2019). Although standard bacteriological typing is still widely practiced, Matrix-assisted laser desorption-ionization mass spectrometry-Time Of Flight Mass Spectrometry (MALDI-TOF MS) can be used for identification and classification of APP.

5.1.4.5.2 Serovar determination

A variety of methods are utilized for serovar determination of bacteria based on antigen detection via serotype specific antibodies (Mittal *et al.* 1992). Co-agglutination test, agar gel diffusion and indirect hemagglutination are all prone to cross-reaction between serovars (Gottschalk *et al.* 2014). In addition, these tests are dependent on the use of serum-derived antibodies from live animals, and the access to these are becoming limited out of animal welfare considerations. Thus, molecular classification methods will probably become increasingly relevant. The most reliable PCR assays for serovar determination are CPS locus based. Although there are commercially available PCR assays that discriminate well between the 19 described serovars (Stringer *et al.* 2021a), atypical K:O variants can be challenging. As an alternative, genetic serovar determination can be performed by sequencing the isolate and comparing them to serovar reference isolates (Christensen *et al.* 2020). This method has never been applied in Norway.

5.1.4.5.3 Antibody detection in serum

Serologic investigations are commonly used in field practice, in diagnostic work on herd level and for screening purposes. Serologic tests are generally either designed to detect all APP by detection of ApxIV (Dreyfus *et al.* 2004), which is often used for screening purposes, or serovar specific to discriminate against serovars (Gottschalk 2015).

Circulating antibodies can be detected approximately 7-14 days post infection. They reach a maximum level within 4-6 weeks, and may persist for many months, seen both experimentally (Bossé *et al.* 1992) and in field trials (Gardner *et al.* 1991). Colonization of the tonsils by some strains of APP might induce production of antibodies that lack cross-protectivity for neutralization of other strains, which is why pigs can be seropositive to APP even at the start of the infection (Gottschalk 2015). Colonization of the tonsils may also occur without induction of an immune response which makes detection of subclinically infected animals challenging (Chiers *et al.* 2002a). To avoid ambiguous diagnostic results, necropsies and bacterial cultivation should accompany serologic testing during outbreak investigations.

5.1.4.6 Treatment and prevention

Alternatives to control disease in a herd include antimicrobial treatment in acute stages, or prevention by vaccination or strategies of eradication, in combination with improved management and implementation of biosecurity measures (Neumann *et al.* 2019).

5.1.4.6.1 Treatment

Pleuropneumonia is a common indication for antimicrobial therapy. Use of antibiotics increases the risk for the emerging antimicrobial-resistant bacterial strains (FAO 2016). Prudent use of antimicrobial drugs require that the first drug of choice should be of a narrow spectrum. The recommended drug of choice for acute pleuropneumonia in Norway is benzylpenicillin procaine (Norwegian Medicines Agency 2012) in the form of intramuscular injections that is re-administered daily for three to five days. The efficacy of benzylpenicillin is believed to be good in practice, despite the poor efficacy results in an inoculation experiment by Sjölund *et al.* (2009). Metaphylactic administration of the drug to all animals in a compartment to reduce the infectious load, is sometimes recommended and practiced in severely affected herds as per recommendation from the Norwegian Medicines Agency (2012). Early initiation of treatment can be crucial to reduce case fatalities.

5.1.4.6.2 Antimicrobial resistance status

To keep treatment recommendations up to date, knowledge of the AMR status of relevant pathogens is vital. Resistance to a variety of antimicrobial substances have been reported for strains of APP, and an increasing level of AMR has been observed in APP in major pig producing countries like Spain (Gutiérrez-Martín *et al.* 2006) and Italy (Vanni *et al.* 2012). It is common to include APP in national surveillance of AMR in pigs. The first characterization of the AMR status of APP in Norway was completed in 2021. Clinical isolates collected since 2004 were evaluated by minimum inhibitory concentrations (MIC) to 19 antimicrobial substances. Although epidemiological cut-off values were unavailable for several of the substances, the report concluded that there was negligible resistance to the first-choice drug benzylpenicillin (NORM/NORM-VET 2020 2021).

5.1.4.6.3 Vaccination

Actinobacillus pleuropneumoniae is toxigenic, and protection from disease comes from neutralizing both the bacteria and the toxins. Toxin neutralization occurs when antibodies prevent toxin from binding to receptors on target cells (Cruijsen *et al.* 1995). Ensuring cross-protection against all major serotypes of APP has been important to the development of vaccines. Cross-protection has been achieved for instance in vaccines targeted at an APP OMP and ApxI-III. Once a pig has acquired immunity towards APP, it will respond more rapidly and efficiently compared to a naïve encounter. However, no vaccine currently in use can prevent animals from being colonized by APP (Ramjeet *et al.* 2008).

In Norway vaccination against APP occurs to some extent, most commonly in herds having reoccurring problems with acute pleuropneumonia. Piglets are being vaccinated before expected exposure during the fattening stage, as described in the summary of product characteristics of a commercially available vaccine on the Norwegian market, Porcilis APP vet. (Norwegian Medicines Agency 2019). At that age, tonsillar colonization has likely happened in most pigs already (Vigre *et al.* 2002).

5.1.4.6.4 Eradication

Actinobacillus pleuropneumoniae has been eradicated in the Norwegian SPF sub population, by rearing of caesarian derived piglets in already established SPF sow herds, which is the most reliable method to sanitize a farm (Sassu *et al.* 2017). Establishment of new SPF herds then relies on total depopulation and restocking with SPF animals. Alternatively, eradication was pursued by partial depopulation and antimicrobial medication using enrofloxacin (Kaspersen *et al.* 2020). There is a lack of documented successful clearing of APP with antimicrobials from colonized animals (Angen et al. 2008). Also, the occurrence of quinolone resistant *E.coli* in pigs was significantly increased several years after a single treatment effect with enrofloxacin (Kaspersen *et al.* 2020), indicating the detrimental long-term effect of this control strategy.

5.1.5 The use of genome sequencing

Genetic traits of an infectious agent helps us understand disease mechanisms. (Kao *et al.* 2014). Whole genome sequencing is a method to determine the entire nucleotide sequence of an organism's genome, and thus provides comprehensive insight into the organisms' genetic traits. Data from WGS can be applied in a variety of ways (Figure 6). The first complete genome sequence of APP was published in 2007 (Foote *et al.* 2008). In 2017 there were four published genomes (Genomes OnLine Database). Some additional draft genomes have also been published; most genomes published are reference genomes for the different serovars, for instance used for serovar determination of isolates. This illustrates how WGS is used for identification and classification of organisms (Figure 6). Nucleotide sequence analysis across the capsule gene clusters can also solve serologic non-typeability in APP (Ito *et al.* 2016).

Common applications of WGS also include answering biological questions e.g., related to phenotypic traits (Figure 6). With increased knowledge of genetic markers for phenotypic traits, the use of sequence analysis will be useful in characterization of clinical isolates. For APP, AMR genotype have been shown to correlate nearly 100% with the phenotype for many antimicrobial agents (Bossé et al., 2017), which goes to show that WGS is a sensitive method for detecting known AMR genes in bacteria (Anjum et al., 2017).

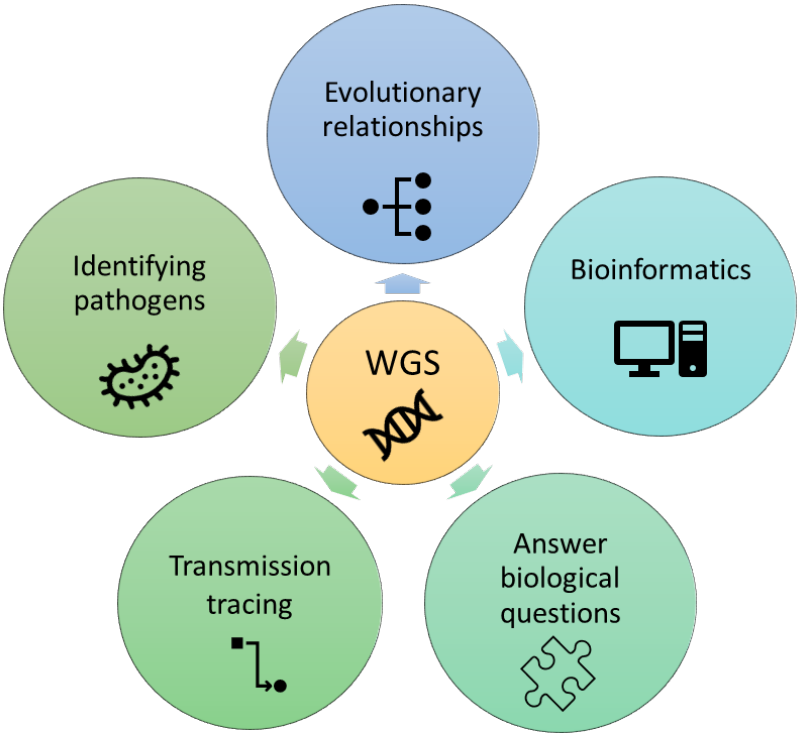

Figure 6. Applications of whole genome sequencing (WGS).

Genome composition can be analyzed by using WGS variation identification techniques such as Single Nucleotide Polymorphisms (SNPs). Phylogenies are models of evolutionary relationships (Figure 6), based on genetic variability between sequences (Vandamme 2009), and a visualization of genetic variation. Evolutionary relationships and dissemination of genetic lines or traits in the population also increase our understanding of disease mechanisms and transmission patterns of infectious agents (Vandamme 2009 , Kao *et al.* 2014). A close genetic relationship is the basis for agent transmission tracing, for which WGS can be very helpful (Figure 6).

Changes in the genome accumulate over time, however not at a constant rate. Genetic variability in infectious agents is influenced by the host population structures, management practices, and patterns of animal movements that affect the transmission and selection pressure for specific traits (Vandamme 2009). For instance, antimicrobial drug use drives the prevalence of AMR genes (FAO, 2016) due to increased selection pressure. Population studies of relevant agents, including AMR patterns, can teach us about the effects of previous implementations of disease control.

5.1.5.1.1 Population studies

The advances of molecular and genomic methods have allowed us to effectively study larger collections of bacterial isolates. Some previous studies of APP populations have been performed using multilocus enzyme electrophoresis (Møller et al., 1992; Hampson et al., 1993), ribotyping (Fussing *et al.* 1998) or amplified fragment length polymorphism (Kokotovic and Angen, 2007), which have indicated that the species is divided into groups mostly consistent with serovar. A degree of variation within populations was also shown by Yee *et al*. in 2018, where APP isolates of serovars 1, 7, and 15 in were analyzed with an enterobacterial repetitive intergenic consensus-based PCR (Yee et al., 2018). In addition to the contribution of random mutations, genetic variation in APP has been shown to occur due to prophages and other mobilizable genetic elements (Prado et al., 2020), which are segments of DNA encoding molecules that mediate movement of that whole segment. The dissemination of genetic variants among large collections of APP isolates can be interpreted with the application of relevant demographic metadata and might be important in future research of mechanisms of disease to achieve disease control. So far, few population studies of single serovars of APP using WGS are published.

5.1.6 Knowledge gaps

Respiratory disease is considered a major challenge to the porcine health and welfare. To prevent outbreaks of respiratory disease in pigs, knowledge of the etiology is crucial. *Actinobacillus pleuropneumoniae* was assumed to play an important role in respiratory disease outbreaks under Norwegian conditions. However, there is a need to confirm the role of APP and

investigate the nature of these outbreaks. The following research question is to be addressed in this thesis:

1. What are the clinical characteristics of respiratory disease outbreaks in Norwegian fattening pigs, and what is the etiologic agent behind these?

Although APP is a well-known infectious respiratory agent, there are few studies describing genetic variability in large collections of clinical isolates. Genetic composition, genetic variability, AMR status, and associations between strains of relevant agents can now be studied with WGS and bioinformatic analysis. Application of metadata can help us understand the dissemination of genetic lines. Also, a population phylogeny of APP may be valuable in future monitoring of APP. The following research question was framed:

2. How does the structure of the pig population influence the genetic variability and the AMR status of APP in Norway?

Biosecurity measures have been important in reducing the risk of infectious disease through practices at international, national and herd level. Much is known about the national biosecurity level in Norway, however, the biosecurity levels in herds that have suffered respiratory disease outbreaks has, to the author's knowledge, not been described. There are few studies describing the biosecurity levels in fattening pig herds, let alone in relation to respiratory disease. The final research question was thus framed:

3. What is the biosecurity level in Norwegian fattening pig herds with and without outbreaks of respiratory disease?

5.2 Aims of the thesis

The overall aim of this thesis was to increase our understanding of the infectious respiratory disease in the Norwegian pig population. The main aim was approached through these specific aims:

1. Describe the clinical characteristics and etiologic agent of respiratory disease outbreaks in Norwegian fattening pigs through comprehensive diagnostic procedures (Paper I)

2. Characterize genetic variability in Norwegian clinical isolates of APP using WGS through serovar determination, phylogenetic reconstruction, and assessment of AMR, in light of the pig population structure (Paper II)

3. Describe the biosecurity levels in Norwegian fattening pig herds with and without outbreaks of respiratory disease (Paper III)

5.3 Material and methods

This section describes the material of the studies described in this thesis. This section also contains a brief description of the methods used. A visual description of the methodology is included below (Figure 7). Methodological considerations are included in the Discussion section. A detailed description of material and methods is given within each paper, attached as Paper I-III.

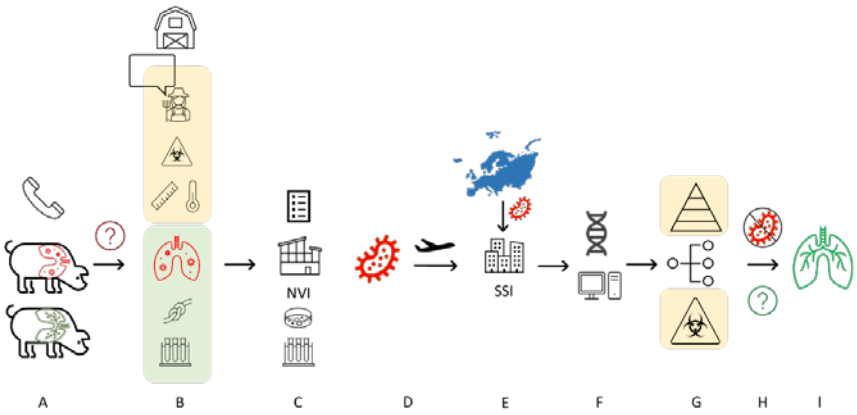

Figure 7. The different phases of the research presented in this thesis. ***A.*** *Farmers facing outbreaks of respiratory disease got in touch. Non-outbreak control herds were assigned.* ***B.*** *Comprehensive diagnostic procedures were performed in outbreak and non-outbreak herds. A custom-made diagnostic kit (green box) contained equipment for necessary diagnostic sampling. Herd data (yellow box) was collected through management interviews, biosecurity assessments and environmental registrations.* ***C.*** *Samples were analyzed at The Norwegian Veterinary institute (NVI).* ***D****. After isolating Actinobacillus pleuropneumoniae (APP) from pig lungs, the bacterial isolates were brought from the NVI to Statens Serum Institut (SSI).* ***E.*** *Isolates and sequences of APP from Denmark and the UK were included in the study.* ***F****. The genomes of APP isolates were whole genome sequenced.* ***G****. Genomic data of APP was analyzed to produce genetic phylogenies and assess genetic variability. Information from these analyses was paired with metadata of the Norwegian pig population (yellow boxes).* ***H.*** *The knowledge from these studies can contribute to prevent APP in Norway and has besides identified areas that require further investigation.* ***I.*** *Preventing APP in Norway will lead to healthier pigs.*

5.3.1 Study samples

In the field study of Paper I and III, the study samples, i.e., the included herds, were identical (Figure 8). This field study yielded samples for the genome study in Paper II, in which a large collection of sequenced isolated of APP8 were analyzed (Figure 8). An overview of the study samples and their source is presented in Table 2.

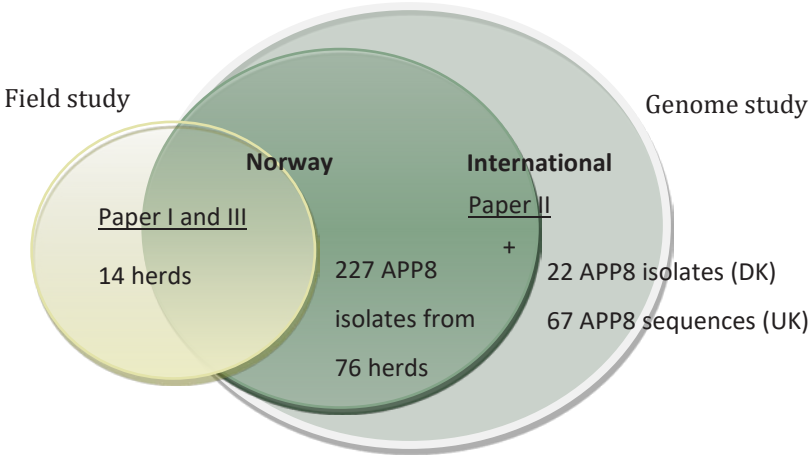

Figure 8. The origin and relationship between study samples of Paper I, II and III illustrated in a Venn diagram. A field study (left) including 14 Norwegian pig herds, gave rise to a subset of samples for the genome study (right) of clinical isolates of Actinobacillus pleuropneumoniae serovar 8 (APP8) from Norway, Denmark (DK) and the United Kingdom (UK).

Table 2. An overview of the number and type of study units, their source and year of collection in Paper I, II and III, respectively.

Paper	Study unit (n)	Source	Year
I	Fattening pig herds (14)	Field study	2017-2018
II	APP8 isolates (135)	Project sampling at NVI	2017-2020
	APP8 isolates (93)	NVI diagnostic archive	2004-2020
	APP8 isolates (22)	SVSL diagnostic archive	1983-2009
	APP8 sequences (67)	Imperial College sequence repository	2003-2011
III	Fattening pig herds (14)	Field study	2017-2018

Actinobacillus pleuropneumoniae serovar 8 (APP8), Norwegian Veterinary Institute (NVI), State Veterinary Serum Laboratory (SVSL), Denmark. Bayesian Evolutionary Analysis by Sampling Trees (BEAST).

5.3.2 Field study

Papers I and III are descriptive and based on a case/control sampling in a field study of respiratory disease outbreaks in Norwegian fattening pigs. In Paper I, the aim was to investigate clinical outbreaks of respiratory disease in Norwegian fattening pig herds (hereby referred to as outbreak herds). In Paper III, the aim was to describe the biosecurity levels in Norwegian fattening pig herds with and without outbreaks of respiratory disease. The case/control approach was used to identify differences in biosecurity practices between the herd groups to address the use of biosecurity to control respiratory disease. The case/control approach was also a direct consequence of the study design of Paper I. Due to small sample sizes and lack of statistical power, a risk factor analysis was not performed.

5.3.2.1 Enrollment of herds

A clear definition of respiratory disease outbreaks had to be made. The inclusion criteria for outbreak herds were three or more pigs displaying acute signs of respiratory disease including fever and coughing and/or dyspnea, and/or otherwise reduced general condition e.g., lethargy or inappetence. The study population included Norwegian fattening pigs in farrow-to-finish or fattening herds. Between September 2017 and October 2018, seven outbreak herds and seven non-outbreak herds were enrolled.

Pig farmers, farm advisors and veterinarians were encouraged to contact the project group at immediate notice of an ongoing respiratory disease outbreak. Convenience-sampling of non-outbreak herds was based on geographic proximity. Upon contact, the field veterinarians were asked to suggest a non-outbreak herd from their practice area, on the condition that there was no ongoing outbreak of respiratory disease in that herd.

5.3.2.2 Herd visits

Each outbreak herd was visited on three occasions (Figure 9). The first visit was done by the field veterinarian for sampling and clinical examination (Visit phase 1) before initiating medical treatment. Shortly after, the herds were revisited by the PhD candidate (Visit phase 2) for herd data sampling and biosecurity assessment. In non-outbreak herds, visit phase 1 and 2 were performed concurrently by the PhD candidate. The follow-up sampling (Visit phase 3), although organized by the project, was performed by the field veterinarians in all herds. This regime resulted from a matter of urgency and convenience, since the initial sampling would have to take place sooner than what was achievable by the PhD candidate. The interval between visit phases was planned out of consideration to the detection of circulating antibodies following an infection.

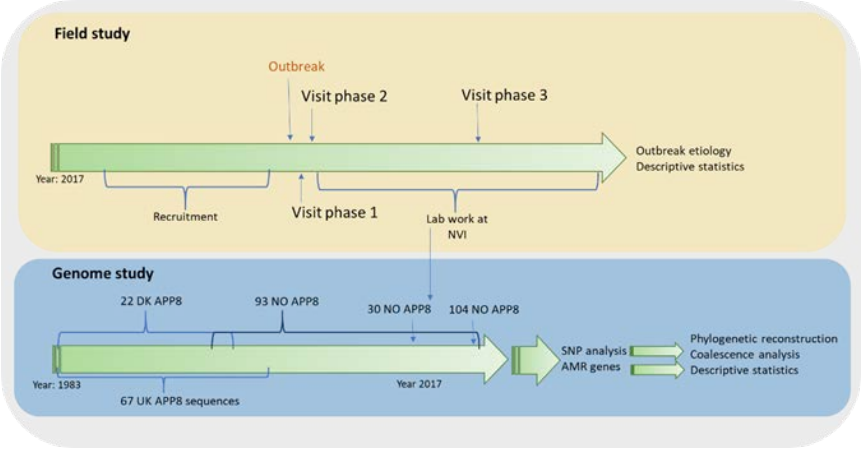

Figure 9. Timelines overviewing of the events and study samples of the field study (yellow box) and the genome study (blue box).
The Norwegian Veterinary Institute (NVI), isolates from Denmark (DK), isolates from the United Kingdom (UK), isolates from Norway (NO), whole genome sequencing (WGS), single nucleotide polymorphism (SNP), antimicrobial resistance (AMR).

5.3.2.3 Selection of animals and diagnostic sampling

Based on the sample requirements, general sampling recommendations from the NVI and transport alternatives in Norway, customized equipment kits for sampling, packing, and shipping were produced and made available to the field veterinarians.

Three to five untreated pigs with clinical signs of respiratory disease were required for lung sampling. This number was chosen in case of inconclusive diagnostic test results. Ten pigs from individual herds were requested for clinical examination and paired serum sampling. Individual identification by ear tagging the pigs was required. In one farm, paired serum samples were excluded from the analysis due to missing identification. The sample size of 10 was based on an estimate of one positive animal at a disease prevalence of 25% and a 95% Confidence Interval. A pooled oral fluid (OF) sample from pigs in two pens in each herd was collected.

5.3.2.4 Diagnostic procedures

The diagnostic procedures described in Paper I were based on the NVIs established protocols and was targeted mostly at major respiratory agents. The procedure included post-mortem evaluation of pigs' lungs and bacteriological sampling from lungs and pleura. Bacterial sampling took place after shipping the lungs to the NVI, and APP was successfully isolated from submitted samples (Paper I). The performed analyses also included serological analysis for antibodies to major respiratory agents, and OF real-time quantitative polymerase chain reaction (RT-qPCR) for selected respiratory viruses. Whether the diagnostic protocols were able to verify the etiologic agents behind the outbreaks should be discussed.

Seroconversion rates for APP were calculated as incidence proportions, defined as proportion of seronegative pigs to seroconvert during the time at risk. The time at risk was the interval between samplings. Additionally, the relative risk ratios for seroconversion following initial sampling were compared in the outbreak and non-outbreak groups to assess the contribution of serological investigations for outbreak diagnostics. The relative risk ratio was estimated from a two-by-two contingency table.

5.3.2.4.1 Serovar determination

According to former NVI protocols, serovar determination of APP has been performed serologically by antibody agglutination using activated rabbit serum. In Paper I APP serovar determination was performed both serologically and genetically based on presence of serovar specific CPS. The genetic reference sequences for these CPS have been published (Bossé *et al.* 2018a , Stringer *et al.* 2021a) and were available in the National Center for Biotechnology Information (NCBI) for comparison using The Basic Local Alignment Search Tool (BLAST).

5.3.2.5 Herd data sampling

5.3.2.5.1 Disease characteristics

A description of clinical signs during the herd visit phase 1 (Figure 9) was provided by the referring field veterinarian. They were also instructed to perform a simple clinical examination and measure rectal temperature of pigs during blood collection. Individually identification of these pigs was necessary to use each pig as individual control for rectal temperature when reassessed during follow up sampling at visit phase 3 (Figure 9). To assess the body temperature change as a clinical characteristic of the outbreaks, a variable "fever" was defined as temperatures above 39.5°C, and Odds ratios (OR) for fever during the outbreak were estimated from a two-by-two contingency table.

The other disease characteristics that were addressed in Paper I, including morbidity, case fatality and mortality were based on information about the outbreak provided by the farmer during visit phase 2. Although no systematic grading of clinical characteristics was performed, the clinical signs were evaluated to infer disease severity.

5.3.2.5.2 Farmer interview and on-farm environmental registrations

An extensive mapping of management and farm environment factors was performed due to the aim of the research project "Grisefine lunger" to

investigate possible risk factors for respiratory disease outbreaks. A questionnaire comprised of 120 questions about the farm, management routines and details about the disease outbreak was made for the field study. The questionnaire was performed as an interview either at the farm or over telephone shortly after the visit phase 2 (Figure 9). A subset of the data provided by this interview was published in Paper I, partly to contribute to descriptive herd demographics and partly to characterize the outbreaks.

A form for on-farm environmental registrations comprised of 74 inquiries was made for the field study. Measurements and registrations for this form were performed during the herd visit phase 2 (Figure 9). A subset of these registrations was published in Paper I and III as descriptive herd demographics, in accordance with the aim of this thesis.

5.3.2.5.3 Slaughterhouse records

Slaughterhouse records on livestock supply and production volume were included as herd demographic data in Paper I and II. A main aim of the research project "Grisefine lunger" was to investigate possible associations between outbreaks of respiratory disease and health and production parameters registered at slaughter. Carcass weight and quality, and USR scorings for the animals included in the study, and historical data from the 12 months prior were collected, but not analyzed for this thesis. Further, the herds studied were affiliated with eight different slaughterhouses, and USR scorings are prone to inter observer variation despite efforts by the food safety authorities to standardize carcass inspection.

5.3.2.6 Biosecurity assessment

In Paper III the aim was to describe the biosecurity levels in outbreak herds and in non-outbreak herds. To be able to compare levels of biosecurity in the two herd groups, an objective and quantifiable assessment of biosecurity was chosen. In partnership with the Biocheck.UGent™ developers, the Biocheck.UGent™ questionnaire was translated to Norwegian for the purpose of this study. The Biocheck.UGent™ translation is accessible on the official web pages (https://biocheck.ugent.be/en/surveys). The questionnaire was performed as an interview in visit phase 2 (Figure 9) by a single interviewer

to reduce bias. The subcategory scores from the questionnaire were the basis for weighted subtotal (internal and external) and total biosecurity scores. The subcategories represent areas of the production covering known transmission routes of infectious agents, which are weighted based on the associated risk of each area, assigned by the developers of Biocheck.UGent™.

To illustrate that there was indeed no detectable difference between the outbreak herds and non-outbreak herds, a statistical comparison between the outbreak and non-outbreak groups was included in Paper III. A two-sample Wilcoxon rank-sum (Mann-Whitney) test was used to compare the group scores. This is a non-parametric test for small sample sizes that tests the hypothesis that there is no difference between the distribution of scores in the groups, which was indicated by a p-value of 0.05.

5.3.3 Genome study

The genome study was an in-depth analysis of a large collection of Norwegian clinical isolates of APP (Figure 10). In the study, WGS was used to characterize the genetic variability of the isolates by assessing their serovar, phylogenetic relationships, and presence of AMR genes. The isolates were sequenced at Statens Serum Institut (SSI), Copenhagen (Figure 7) and turned out to be mainly APP8 (Figure 10). During the visit to SSI, the large international comparison study of APP8 (Paper II) was initiated, involving bioinformatic research groups at the SSI and Imperial College in London. A collection of Danish (n=22) isolates and UK sequences (n=67) (European Nucleotide Archive project PRJEB2343) were used for the comparison. Genetic phylogenies and AMR gene profiles were compared to assess differences between the populations.

5.3.3.1 The Norwegian APP isolate repository

Isolates of APP from 30 lungs were obtained from the diagnostic sampling in Paper I (Figure 10) and included in the genome study (Paper II). At the NVI, an additional 104 bacterial isolates from routine diagnostics (diagnosed cases of APP) since 2004 were made available for the genome study (Figure 10). The frozen isolates were revived through bacteriological procedures recommended for cultivation of APP (Gottschalk *et al.* 2019). In the end, 128 isolates (Figure 10) from 81 herds were confirmed as being APP. Following genetic serovar determination based on comparison to respective serovar reference strains, 123 isolates were confirmed as serovar 8 (Figure 10). The APP8 isolates originated from a total of 76 herds, of which 23 herds had given rise to multiple (range 2-6) isolates.

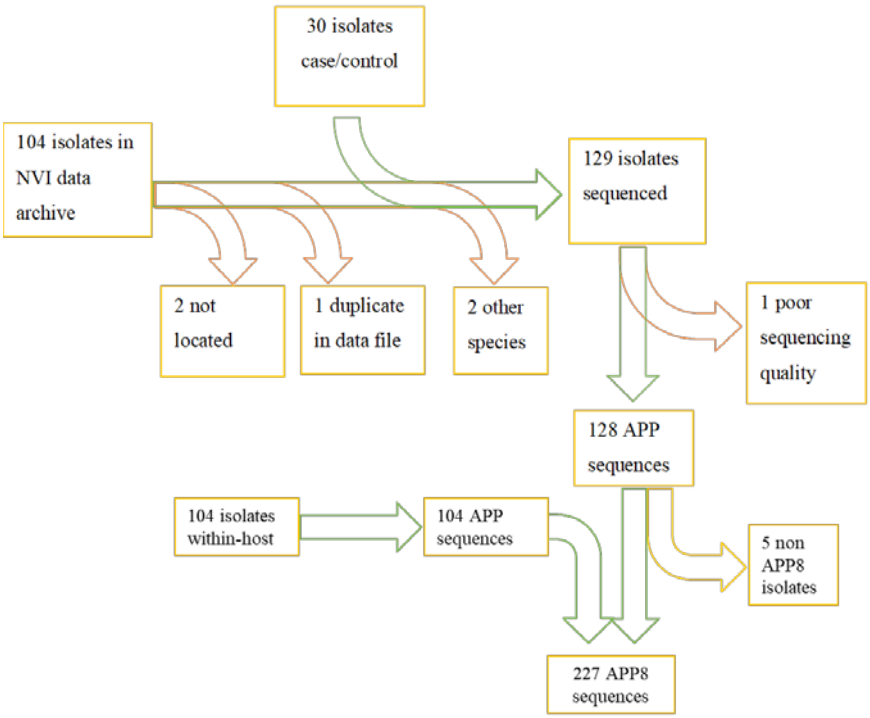

Figure 10. A schematic overview of the retrieval of isolates of Actinobacillus pleuropneumoniae serovar 8 (APP8) for sequencing, from the isolate repository and additional sampling (within-host) at the Norwegian Veterinary Institute (NVI).

To elucidate within-host variability of APP infections, additional isolates were collected (Figure 11). Six infected pig lungs, from five geographically unrelated herds were sampled after being issued to NVI for diagnostic purposes in 2019 and 2020. This sampling resulted in 104 isolates of APP (Figure 10), all confirmed to be serovar 8 by genomic serovar determination.

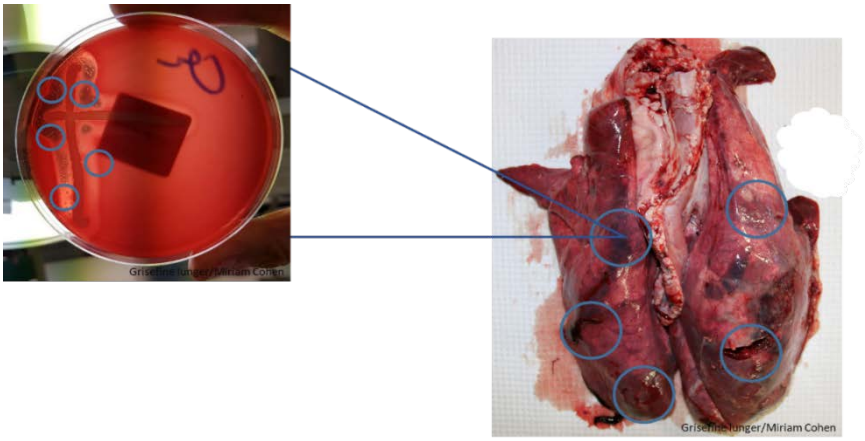

Figure 11. Sampling of isolates for within-host variability assessment. Two to five lesions within each set of lungs, in six lungs, were swabbed. Each swab was cultured on individual culture plates. From each plate five to eight colonies were sampled and purified for whole genome sequencing, resulting in 104 isolates, all confirmed to be Actinobacillus pleuropneumoniae serovar 8. Photo credit: Liza Miriam Cohen.

5.3.3.2 Nucleotide extraction and whole genome sequencing

Purified bacterial isolates, confirmed as APP by MALDI-TOF MS at the NVI were sent on transport medium to SSI, where genomic DNA was extracted following their routine procedures using commercial kits. The DNA was then pair-end sequenced using Illumina technology.

5.3.3.3 Determination of serovar from whole genome sequences

The serovar was determined based on the presence of the serovar specific CPS as previously described. All non-APP8 isolates were excluded from the study in Paper II (Figure 10). Five isolates from the Norwegian isolate repository were non-APP8, visualized in the maximum likelihood phylogeny of the whole Norwegian APP collection (n=128) in Figure 12 alongside the respective reference strains (n=18) of APP serovar 1-18.

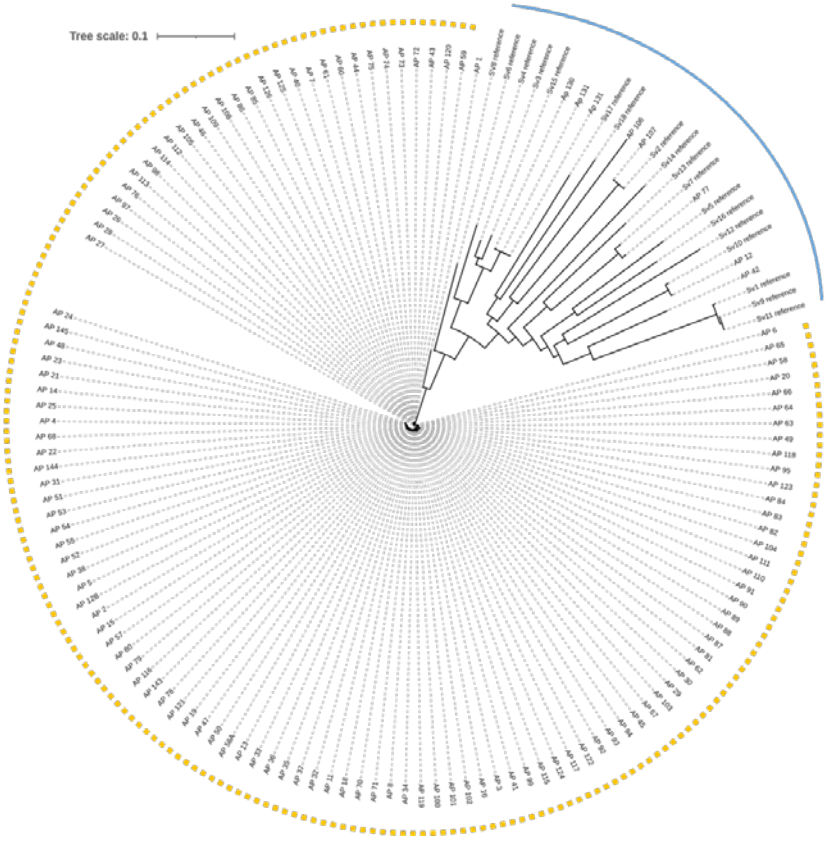

Figure 12. A maximum likelihood phylogeny of 128 isolates of Actinobacillus pleuropneumoniae collected from Norwegian pigs' lungs between 2004 and 2020. While 123 isolates belong to serovar 8 (yellow curve), the remaining five isolates were serovar 2, 7 (n=2), 10 and 12 (blue curve). The respective reference strains for serovar 1-18 are included (blue curve). Tree scale indicates number of substitutions per site as a measure of genetic variability.

5.3.3.4 Single nucleotide polymorphism analysis

Single nucleotide polymorphism analysis was performed in the shared "core genome" of nucleotide sequences that were compared. Duplicated regions in the sequences were removed before SNP calling was performed by comparing the isolate sequences to that of an APP8 reference strain, whose closed genome has been published (GenBank accession number LN908249.1). Sequenced areas with low sequencing depth and ambiguous variant calls were removed, and high-density SNP regions were also identified and removed. Sequencing depth, or coverage, refers to the number of times a given nucleotide in the genome was read, determining with what confidence the nucleotide was observed in that position. Illumina recommends a coverage between 30 x and 50 x for WGS. Variant calling is concluding that there is a difference in the nucleotides at a given position, and another term for SNP. An ambiguous call means that the conclusion is unclear or could not be made. High density SNP regions can stem from recombination, an event where genes are acquired from an external source. Although an important source to genetic variation, such events can complicate the phylogenetic reconstruction as it masks the true relationship between sequences, such regions were thus removed. This resulted in a core genome of 1.67 Megabase pairs, equal to 71.6% of the reference chromosome, as a basis of the comparison.

5.3.3.5 Clonality

Clonality was determined based on the results of the SNP analysis, but no strict cut-off was established. Single nucleotide polymorphism distances (number of SNPs) between the 104 isolates from the within-host sampling were analyzed to assess clonality. The number of SNPs between isolates from the same pigs in the within-host variation study were ≤2, indicative of clonality. The lowest number of SNPs between isolates from different pigs that were also from different herds was 18, which were considered non-clonal.

5.3.3.6 Phylogenetic reconstruction

Pairwise comparison and determination of SNP distances between isolates is the basis for the phylogenetic reconstruction, which was done by

maximum likelihood approach, using software with a ModelFinder function (Kalyaanamoorthy *et al.* 2017). Likelihood is the probability of observing the data given the model (Lemey *et al.* 2009). With the maximum likelihood approach, all trees (models) that could explain our observations are compared to find the tree with the highest probability. Robustness was assessed with bootstrap analysis with 100 replicates to evaluate the strength of the estimated phylogenetic relationship in each branch.

5.3.3.7 Metadata annotation

The farm location was used for geographic annotation and visual mapping of the phylogenies. Also relevant to the host population structure, herd category (level in pyramid) and slaughterhouse affiliation was requested from production records at Animalia and used for phylogenetic annotation. Year of sampling of the isolates was necessary for the estimation of time to most recent common ancestor (TMRCA). Year of sampling was thus drawn from the diagnostic records of the Norwegian and Danish isolates, and from the annotation data of the UK sequences (European Nucleotide Archive project PRJEB2343).

Geographic visualization was performed using the freely available Microreact (http://microreact.org), a hierarchical and geographical analysis tool (Argimón *et al.* 2016). A map has been included here (Figure 13) to illustrate that isolates originated mainly from the three areas with the most concentrated pig production in Norway. The map includes the five non-serovar 8 isolates in the Norwegian isolate collection (Figure 13). Phylogenetic relationships are not illustrated in this figure but was demonstrated in Paper II.

Visualization and annotation of a maximum likelihood phylogeny displaying the whole Norwegian APP collection, including the respective reference genomes (Figure 12) was performed using iTol v4.314 (https://itol.embl.de)(Letunic *et al.* 2019). A more detailed phylogeny of the relationships between the APP8 isolates was published in Paper II.

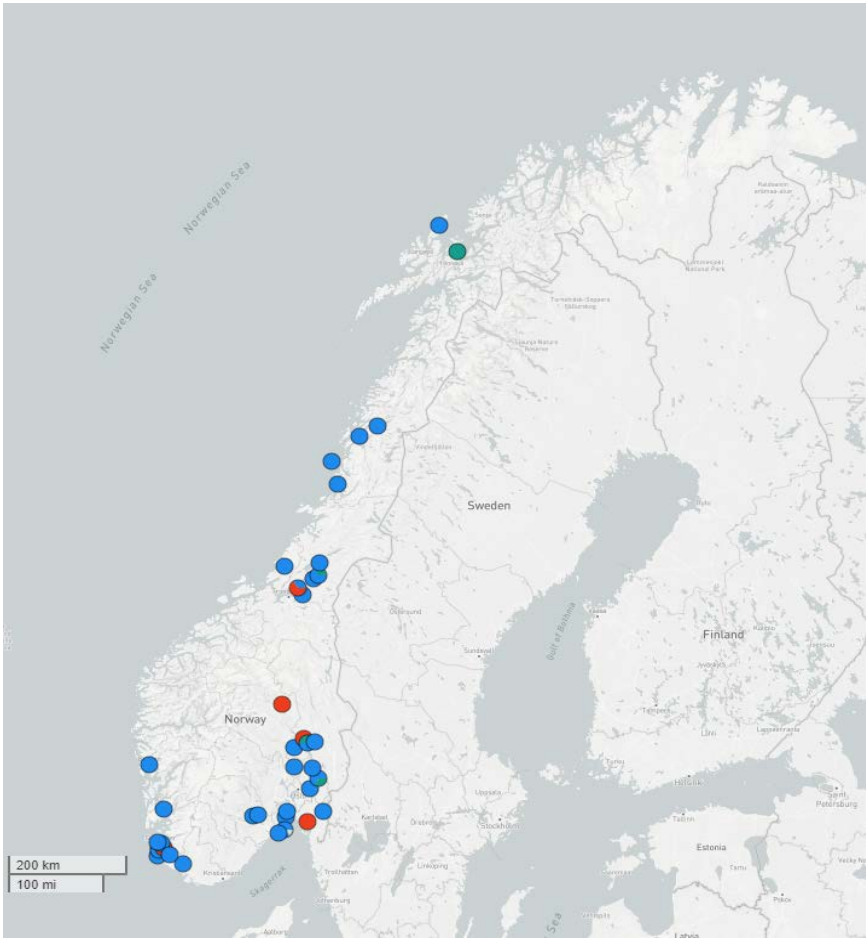

Figure 13. Geographic distribution of Norwegian Actinobacillus pleuropneumoniae isolates in the genome study. Green circles are non-serovar 8 isolates. Red and blue circles are serovar 8 isolates, red circles also indicates that the isolate was collected during the field study of respiratory disease outbreaks in 2017 and 2018.

5.3.3.8 Chi squared test

Categorical data variables regarding the isolates were analyzed using a cross table and the chi squared test to assess the relationship between geographical location or slaughterhouse affiliation and phylogenetic clade. The test assesses the distribution within the cross table and compares the result to a null hypothesis of equal distribution of observations in the cells.

5.3.3.9 Antimicrobial resistance genes

The bacterial genomes were searched for genes that have been annotated in the ResFinder database (Zankari *et al.* 2012) to be associated with phenotypic antimicrobial resistance, by sequence alignment using BLAST. The isolates in which AMR genes were identified were not tested for phenotypic resistance, the clinical relevance of these genes was therefore assumed from literature. The effect of national differences between Norway, Denmark and the UK in antimicrobial drug use and restricted animal movement on the occurrence of resistance was assessed.

5.3.3.10 Coalescence analysis

Time of separation from TMRCA, in this case between the Norwegian and Danish APP8 isolates was estimated using Bayesian Evolutionary Analysis by Sampling Trees (BEAST) (Suchard *et al.* 2018 , Bouckaert *et al.* 2019). Bayesian inference combines the prior probability of the phylogeny P(A) with the likelihood of the data (B). The analysis produces a posterior probability distribution on trees P(A|B), which is the probability that the phylogeny is correct, given the prior, the data and the correctness of the likelihood model:

$$P(A|B) = \frac{P(B|A)\,P(A)}{P(B)}$$

Different coalescent and clock models were applied to allow both constant and variable mutation rates across branches.

5.4 Synopsis of papers

A summary of the Paper I-III is provided below. The detailed results can be found in the attached Papers I-III.

5.4.1 Paper I

A descriptive study of acute outbreaks of respiratory disease in Norwegian fattening pig herds

The main aim of this study was to investigate outbreaks of respiratory disease in conventional Norwegian fattening pig herds. The aim was pursued in a case/control field study including 14 herds. In seven herds with reported outbreaks of acute respiratory disease (case), herd demographic data and clinical signs were recorded. Diagnostic protocols were compared by parallel analysis of clinically healthy pigs from seven non-outbreak (control) herds. Diagnostic samples for laboratory examination included lungs from three to five pigs, pooled oral fluid (OF) samples from two pens, and paired serum samples from 10 pigs per herd.

Non-outbreak herds were smaller, had fewer livestock suppliers and more air space per pig than the outbreak herds. Onset of the outbreaks were 35 days after arrival in the compartment. Number of days from first discovered symptom to calling the field veterinarian was one day. The most reported clinical signs were sudden deaths and dyspnea. Odds ratio for fever during the outbreak was 2.8 compared to after recovery. An average compartment morbidity of 60%, mortality of 4% and case fatality of 9% was recorded in the outbreak herds. All outbreak herds treated sick pigs with procaine benzylpenicillin and reported that this effectively reduced the clinical signs and stopped the outbreak. Post-mortem examinations revealed acute lesions resembling porcine pleuropneumonia in all 28 pigs investigated from the outbreak herds. The lesions were distributed in all lung lobes, but the caudal lobes were the most affected. Acute porcine pleuropneumoniae-like lesions were also present in two of the 24 (8%) pigs from the non-outbreak herds, while chronic lesions were recorded in another two of these pigs (8%). Diffuse interstitial pneumonias, typical viral infections, was not observed. *Actinobacillus pleuropneumoniae* serovar 8 was isolated from lungs and/or

pleura from all (100%) pigs (n=28) in the outbreak herds, and from two out of 24 pigs (8%) in the non-outbreak herds. No other significant bacterial findings were made. Seroconversion to APP antibodies was detectable in all outbreak herds analyzed and in six out of seven non-outbreak herds, but the risk ratio for seroconversion of individual pigs was higher (risk ratio 2.3 [1.50- 3.43 95% CI; P<0.001]) in the outbreak herds. Antibodies to SIV was only detected in serum in one outbreak herd, in which it was not found to be linked to the outbreak. All herds tested positive for PCV2 and negative for influenza A viruses in OF using RT-qPRC.

The main etiological pathogen found during outbreaks of respiratory disease was APP8. All pigs from outbreak herds had typical lesions of acute porcine pleuropneumonia. Co-infections were not found to impact disease development. Disease characteristics were typical of APP and caused severe clinical signs.

5.4.2 Paper II

Comparative genome sequence analysis of *Actinobacillus pleuropneumoniae* serovar 8 isolates from Norway, Denmark and the United Kingdom indicates distinct phylogenetic lineages and differences in distribution of antimicrobial resistance genes

The primary objective of this study was to characterize the genetic variability of isolates of APP8 in the Norwegian population. The secondary objectives were to determine the within-host variability of APP8, and to compare the APP8 bacterial populations in Norway, Denmark, and the United Kingdom (UK). Serovar 8 is the predominant clinical serovar of APP in Norway and the UK and has been isolated from clinical cases in Denmark. Isolates of APP8 from the UK (n=67), Denmark (n=22) and Norway (n=123) collected between 1983- 2020 were compared using WGS. In this material each isolate represented one pig. By including an additional 104 APP8 isolates from the lungs of six Norwegian pigs, genetic variability within individual infected pigs was analyzed. Genetic variability was defined by SNPs in the core genome. The comparison also included AMR gene profiles to assess the effect of national differences in antimicrobial drug use and

restricted animal movement on the occurrence of resistance genes. In this study we combined bacterial genome data (the genetic variability and dissemination of APP8 isolates, including their AMR profiles) and metadata of the structure of the pig population to assess its effect on the dissemination of APP8 in Norway.

The study confirmed APP8 as the dominating clinical serovar in Norway. Isolates that had previously been classified as APP6 serologically were confirmed to be APP8 by genomic serovar determination and were thus reclassified. Other serovars detected in the collection of clinical isolates include 2, 7, 10 and 12, and these were excluded from the study. Very low within-host variation was observed (≤ 2 SNPs), indicative of monoclonal infections with APP8. The phylogeny of 123 Norwegian APP8 isolates from 76 herds revealed some within-herd genetic variation. Norwegian isolates formed three phylogenetic clades. Substantial geographical clustering was observed within these clades, meaning that isolates from the same geographic region were more genetically similar. Slaughterhouse affiliation and herd category did not show significant clustering in the phylogeny. Persistence of genetic lines over time was signs of a slow genetic divergence, indicating that the bacterium is under low selective pressure in Norway.

In the phylogeny of the three international APP8 collections, we found two distinct monophyletic branches characterized by the Norwegian and UK isolates, respectively. Three Danish isolates were scattered across the UK branch, whereas the remaining 19 Danish isolates clustered in two monophyletic groups within the Norwegian branch. Coalescence analysis estimating the separation of Norwegian and Danish isolates as TMRCA, indicated that this separation happened around 200 years ago. A separation of the Norwegian and UK isolates thus happened even longer ago. The phylogenetic analyses also revealed striking differences in occurrence of AMR genes, as these were 23-times more prevalent among the UK isolates than among the Norwegian isolates. The occurrence of AMR in Danish isolates was intermediate. Across populations, the sulfonamide resistance gene *sul2* was the most occurring. Only four Norwegian APP8 isolates had

AMR genes. Three of these isolates carry the resistance gene *tet*(y) which has previously not been identified in species within the *Pasteurellaceae* family.

International biosecurity considerations allowing for a closed Norwegian pig population has allowed for a distinct separation between Norwegian isolates and those from neighboring nations. Regional livestock trading patterns has led to limited variation among APP8 isolates in Norway, with clear geographic clustering. The overall genetic variability in isolates of APP8 from Norway is generally low, and genetically persistent over time.

5.4.3 Paper III

A descriptive study of the biosecurity levels in Norwegian fattening pig herds with and without outbreaks of respiratory disease

The objective of this study was to describe the biosecurity levels in Norwegian fattening pig herds with outbreaks of respiratory disease (case) caused by APP and in non-outbreak (control) herds. The aim was pursued in a case/control field study including 14 herds. Biocheck.UGent™ was used for objective soring of biosecurity at the herd level. The scores of the total, external and internal biosecurity were given in indexes between 0-100 where 0 is the lowest possible score, indicative of poor biosecurity implementation.

The total, subtotal external and subtotal internal Biocheck.UGent™ scores for the outbreak herds (n=7) were 61, 64 and 57, respectively. The total, subtotal external and subtotal internal Biocheck.UGent™ scores for the non-outbreak herds (n=7) were 60, 69 and 61, respectively. Both herd groups scored higher on external than internal biosecurity. Both herd groups had implemented measures to reduce the risk of introduction and spread of infectious disease through purchase of livestock, and management implementation in the finishing unit, while measures between compartments were poorly implemented. Objective scoring of biosecurity in Norwegian fattening pig herds showed no difference between herds with outbreaks of respiratory disease and in non-outbreak herds.

5.5 Discussion

This section provides a discussion of the main results, methodological considerations, limitations, and some future perspectives. The understanding of multifactorial infectious respiratory diseases in pigs has been approached with regards to the epidemiological triad (Figure 5), investigating the infectious agents, a variety of environmental factors and properties of the host.

5.5.1 Agent

Based on the comprehensive diagnostic procedures performed in Paper I the main etiologic agent behind outbreaks of respiratory disease was APP8. Results of Paper II indicate that lung infections with APP8 were monoclonal.

5.5.1.1 Disease severity

The clinical signs found during outbreaks in Norway where in line with typical clinical signs reported from acute infections with APP, such as reduced general condition and appetite, fever, dyspnea, and coughing (Vigre *et al.* 2002 , Gottschalk *et al.* 2019). Regarding the virulence, the results of Paper I indicated that APP8 harbors virulence factors that surpass the natural resistance mechanisms of the pigs. The outbreak morbidity and a case fatality described in Paper I were somewhat moderate compared to descriptions of porcine pleuropneumonia in literature reviewed by Klinkenberg *et al.* (2014b). They found morbidity to range from 10-100% and mortality of 1% 10%. Case fatality rate is rarely included in published literature, but is regarded as a measure of virulence (van Seventer *et al.* 2017). Case fatality rate is a more robust measurement of disease lethality than mortality, due to being less subjected to the confounding effect such as that of other illnesses occurring simultaneously in the herd. However, it requires information regarding the cause of death to be accurate. Since multimicrobial interactions are common for respiratory disease, it can be hard to assess virulence of single infective agents under field conditions. In this study, however, the clinical characteristics are representative of the traits of APP8. Morbidity and case fatality rate may be influenced further by the time of treatment initiation and susceptibility to drug of choice. As described in Paper I, the sampling and initiation of antimicrobial treatment

occurred one day after the first clinical signs were noticed, and the treatment was reported to stop the outbreak and further development of clinical signs. The time and effect of treatment should be included when assessing the disease characteristics as a measure for virulence.

In lungs and pleura of pigs in outbreak herds there were typical acute macroscopic lesions of porcine pleuropneumonia (Gottschalk *et al.* 2019), from which APP8 was isolated (Paper I). High severity of the infections was indicated by the extensive lesion distribution (Paper I). The whole lung may be affected in severe cases (Sibila *et al.* 2014). This distribution of lesions could have occurred from inhaling high doses of bacteria due to a high infectious pressure in the environment, or extensive growth and dissemination post inhalation. The lesions were indicative of profuse tissue damage, even as short as 1 day after the first clinical signs were noticed by the farmer (Paper I). The tissue damage was likely caused by actions of the bacterial virulence factors, including Apx-toxins. Isolates of APP8 are traditionally associated with the expression of ApxII and ApxIII (Table 1) (Frey 2003). No diagnostic test was performed in the current study to assess virulence in the isolates of APP. The severity of clinical signs and pathology caused by infections with APP8 in Norway were indicative of high virulence.

Since variation in virulence within serovars is well known, updated information about the relevant serovars and virulence should be attained within the population, as argued by Sassu *et al.* (2017). While this study is a contribution towards a database of relevant clinical serovars, there is a lack of such databases from populations around the world.

5.5.1.2 Diagnosis

In paper I, the diagnosis was determined both by the presence of APP, and the seeming absence of other major respiratory agents. Multimicrobial interactions have been central in respiratory disease in pigs (Opriessnig *et al.* 2011), but were not fount to contribute to the development of outbreaks in Norway (Paper I). It is possible that some minor pathogens were missed. For instance, the procedure did not include selective *Mycoplasma spp*. culturing. The presence of *M. hyopneumoniae* was assessed serologically, but

there are other mycoplasmas that could be involved in respiratory infections (Falk *et al.* 1991a). Future investigations of the lung microbiota using molecular methods are likely to increase our insight into microbial interactions in the pig lung.

Collection of OF allows for efficiently sampling for respiratory pathogens that are excreted in oronasal fluids during the prolific stage (Hernandez-Garcia *et al.* 2017 , OIE 2021a). Although OF collection is done with minimal intervention, a weakness of this procedure, when used for outbreak diagnostics, is that pigs with reduced general condition are reluctant to chew on the ropes and contribute to the pooled sample. The virology results in Paper I suggested that neither SIV nor PCV2 contributed to the disease outbreaks. The absence of SIV in all OF samples was supported by the lack of typical pathological lesions and absence of SIV antibodies in serum. No difference was detected in PCV2 levels between the outbreak- and the non-outbreak herds. Reluctancy of sick pigs to chew on the ropes thus seemed to have minimal influence on the results in the present study.

Currently, there are no internationally acknowledged protocol for diagnosing APP, such as in the Terrestrial Animal Health Code (OIE 2021a). A case/control approach was used in Paper I so that the diagnostic procedures to verify the etiology behind the outbreaks could be compared to a control group (non-outbreak herds). The protocol used in Paper I, showed that collecting lungs on farm works well for diagnosing APP during an ongoing outbreak. The results indicate that in the presence of typical acute clinical signs, one lung is sufficient to demonstrate the presence of APP (Paper I). Since APP was also found to cause monoclonal infections (Paper II), one bacteriologic sample from one clinically affected pig will suffice for the diagnosis of APP during outbreaks.

Even if APP was successfully isolated from submitted samples, post-mortem changes in the lung could alter the microbiologic composition (Brooks 2016). Sampling bacteriologic swabs in field, prior to sending the lungs for pathologic evaluation, was a feasible alternative. However, field swabbing could be sensitive to contamination that might influence the results. In 2021

a method for sampling lesions in field to detect APP by PCR was published (Stringer *et al.* 2021b), which could reduce the possibility of contamination and altered microbiological composition. However, this method does not allow for isolation of APP for further testing and DNA sequencing.

The presence and activity of APP during outbreaks, was also confirmed by the presence of antibodies in serum (Paper I). Immunity is an important result of interaction between the agent and the host and is thus discussed further under aspects of the host below. Serology proved to be challenging to use for diagnostic purposes.

5.5.1.3 Classification

Applying WGS was helpful for improved diagnosis and classification of APP. It seems that clinical isolates of APP in Norway have been dominated by serovar 8 since 2004 (Paper I, Paper II). This finding deviates from investigations in the 1990's when APP2 was the most prevalent serovar (Falk *et al.* 1990 , Falk *et al.* 1991b). The APP isolates studied in the 1990's had been collected at slaughter from chronically infected animals (Høie *et al.* 1991), while the isolates in the current studies are from clinical cases. Differences in acute and chronic potential between serovars or strains could exist, thus explaining the different results. Falk *et al*. also detected antibodies to APP6, using serologic methods (Falk *et al.* 1990). No isolates of APP6 were found among the isolates in the current studies, previous APP6 isolates were in fact reclassified as APP8 following genomic analysis in Paper II. Cross-reactions to APP8 antibodies might explain the seeming presence of antibodies to APP6. That implies APP6 was never present in the Norwegian population (Norwegian Veterinary Institute 2021), and APP8 has been underdiagnosed. Similar underestimations and overestimations of serovars have been described in England and Canada (O'Neill *et al.* 2010 , Gottschalk 2015). The advantages of serovar determination using molecular methods have been discussed in the introduction, and due to the increased precision compared to serological methods, differences in the results are to be expected.

Since no known cross-reaction between APP2 and other serovars is common, the finding of APP2 was more reliable. Only one APP2 isolate was observed in the material that was investigated in the current study (Figure 12). In later times, APP2 has been diagnosed from a small number of clinical cases at the NVI, but APP8 remains the most diagnosed serovar (Norwegian Veterinary Institute 2021). Similar shifts in serovar prevalence have been described in Germany. A reduction in serovar diversity was seen in a recent population wide study of historical clinical APP isolates (Schuwerk *et al.* 2021). The prevalence of APP2 has increased in central Europe, while other serovars dominate in more peripheral European countries like Spain (Maldonado *et al.* 2009), the UK (Li *et al.* 2016) and Norway (Paper II).

An upgraded multiplex PCR now differentiates between all 19 described serovars of APP (Stringer *et al.* 2021a), and was recently included in the NVI protocol, replacing serological serovar determination (Falk *et al.* 2020). The reason for not using this PCR in our study was that the APP isolates were already being sequenced and analyzed for the phylogenetic study in Paper II, so applying another molecular based test was redundant.

Although it cannot be concluded that there has been a shift among clinical isolates from APP2 to APP8 in the Norwegian pig population, assessing the dissemination and genetic variability in a large collection of APP8 has clarified some details regarding links to national and regional structure and dynamics of the population. It is not clear what prompted this seeming shift in APP serovar occurrence in Norway. Attempts at eradicating APP in breeding herds by strategic medication in the 1990's (Hofmo *et al.* 1998) could have contributed to a shift in APP serovar carrier status. A test of the current APP carrier state of sows in the breeding and commercial sow herds is needed to clarify this aspect.

5.5.1.4 Other agent traits

Antimicrobial resistance is not likely to complicate the respiratory infection dynamics with APP8 in Norway. A low level of AMR in Norwegian APP isolates has been shown both by the results of Paper II, and the national AMR surveillance program from 2021 (NORM/NORM-VET 2020 2021). Although

the surveillance program did not differentiate between serovars, the isolates are mainly APP8 from the diagnostic archives of NVI. For a comprehensive assessment of phenotypic AMR, clinical breakpoints for more antimicrobial agents need to be established, as many are still missing (Sassu *et al.* 2017). A divergence between the specific results of the MIC testing and the identified AMR genes might exist, as limitations of identifying AMR genes in ResFinder are tied to a lack in knowledge of genetic variants associated with the respective phenotypes. Even so, since the correlation between AMR phenotype and genotype has been found to be high, screening for AMR genes was considered a reliable method to describe AMR profiles of APP isolates.

The results of Paper I confirms APP8s role as a primary respiratory infectious agent. Multimicrobial etiologies thus seems to be less relevant for respiratory disease in Norwegian pigs. With regards to the epidemiological triad (Figure 5) the respiratory disease outbreaks were thus tied to the qualities of APP8, the environment, and the host.

5.5.1 Environment

5.5.1.1 Effect of population structure and health management procedures

International and national biosecurity implementations are likely the most important contributors to a good health status and respiratory disease control in Norway. National import legislation (Ministry of Agriculture and Food 1998) and additional requirements of the production animal sector regarding import of live animals (KOORIMP *et al.* 2021) have contributed to a high national biosecurity in Norway at least for the last 30 years (KOORIMP *et al.* 2021). These systems that regulate the Norwegian pig population have been influencing the dissemination of APP, as shown in Paper II.

5.5.1.1.1 International comparisons

A closed Norwegian pig population has allowed for the evolution of distinct Norwegian clades that differ by comparison to isolates from neighboring nations Denmark and the UK. The estimations of TMRCA between APP8 isolates from Norway, Denmark and the UK were indicative of how long the

three national pig populations have been closed off from each other. Even if the import legislation in Norway have been at play for decades, it seems like the Norwegian population has been isolated from the other two populations for centuries (Paper II). As an alternative to the BEAST model, to estimate a timeline for the evolution of the population, a simplistic linear regression model would calculate the association between SNPs and time. This approach supposes that mutations happen at a constant rate, which they do not (Vandamme 2009).

5.5.1.1.2 Genetic antimicrobial resistance

The geographic separation of the isolates from UK, Denmark and Norway was supported by striking differences in occurrence of AMR genes. While the occurrence of AMR genes in Norwegian APP8 was low, the occurrence was 23-times higher in UK isolates, also somewhat higher in Danish isolates (Paper II). Closed populations, in combination with differences in antimicrobial treatment procedures in the UK, Norway and Denmark have probably led to the widely different AMR patterns (Paper II). Dissemination of AMR genes are driven both by selective pressure towards AMR by exposure to antimicrobial agents, and horizontal transmission from other species in the environment (FAO 2016). Thus, some assumptions can be made about the Norwegian pig population:

- Prudent use of antimicrobial drugs in pigs over time has contributed to a low selective pressure towards AMR in APP8.
- Prudent use of antimicrobial drugs in pigs over time has contributed to a low selective pressure of AMR genes in other livestock associated bacteria, reducing the source of AMR genes for horizontal transmission.
- A closed pig population in Norway, due to national biosecurity considerations, has restricted the possibility of resistant strains from other populations to manifest in Norway.
- Regulation on livestock trade in the future will likely be important to sustain an APP population widely susceptible to antimicrobial drugs, for instance by stopping an introduction of multi-resistant APP strains from other pig populations.

Future detailed genetic analyses could elucidate whether it is likely that the observed AMR genes stem from horizontal transmission. The AMR genes can accumulate in mobilizable genetic elements, facilitating horizontal transmission of multiple genes together, previously demonstrated in APP8 (Bossé *et al.* 2015 , Bossé *et al.* 2016). Whether the genes identified in this study are part of a common mobilizable element requires further investigation.

5.5.1.1.3 The Norwegian pig production structure

The structure of Norwegian pig production is evident in the evolutionary relationship and dissemination of APP8. Regional livestock trading patterns has led to limited variation among APP8 isolates in Norway, with clear geographic clustering (Paper II). A high level of similarity between strains from different herds in Norway limits the traceability of isolates. This restricts the use of a population phylogeny as a background for tracing sources of transmission (Figure 6). This is relevant for instance to assess indirect transmission routes, or persistence of bacteria in the environment after attempted sanitation.

By addressing sampling year, a persistence of genetic lines became evident. The persistence of APP strains in the population could be explained by a slow genetic divergence due to a low selective pressure. Due to the possibility of harboring of bacteria in the tonsils and chronic lesions of adult sows (Fablet et al., 2011), APP could persist in breeding herds in the top tiers of the production pyramid (Figure 2). Random genetic changes have accumulated over time, while the strict management and biosecurity practices that apply for these herds ensure that no horizontal exchange of strains happen. This allows for the dissemination of slightly different strains downward in the pyramid (Figure 2). The findings support direct contact as the main transmission route of APP8. Pigs that descend from different livestock sources will harbor different strains of APP8. Since individual commercial fattening pig herds can purchase livestock from multiple sow herds, this is a likely explanation for the observed within-herd genetic variation in Paper II (Figure 14). A diversity of strains in the tonsils of each pig might also have

contributed to the observed differences, even if they stem from the same source. Capturing the true variation of APP strains from a herd is difficult and might require tonsillar sampling and sampling of pigs from all the supplying sources.

At the same time, a top-down distribution of isolates from breeding herds is a likely explanation for the geographic clustering of isolates in Norway (Figure 14). One nucleus breeding herd can supply gilts directly to several multiplier breeding herds. These can in turn supply pigs to many commercial herds, which are usually located in the same part of the country. This enables transmission of clonal isolates, allowing their persistence within the system and geographic region.

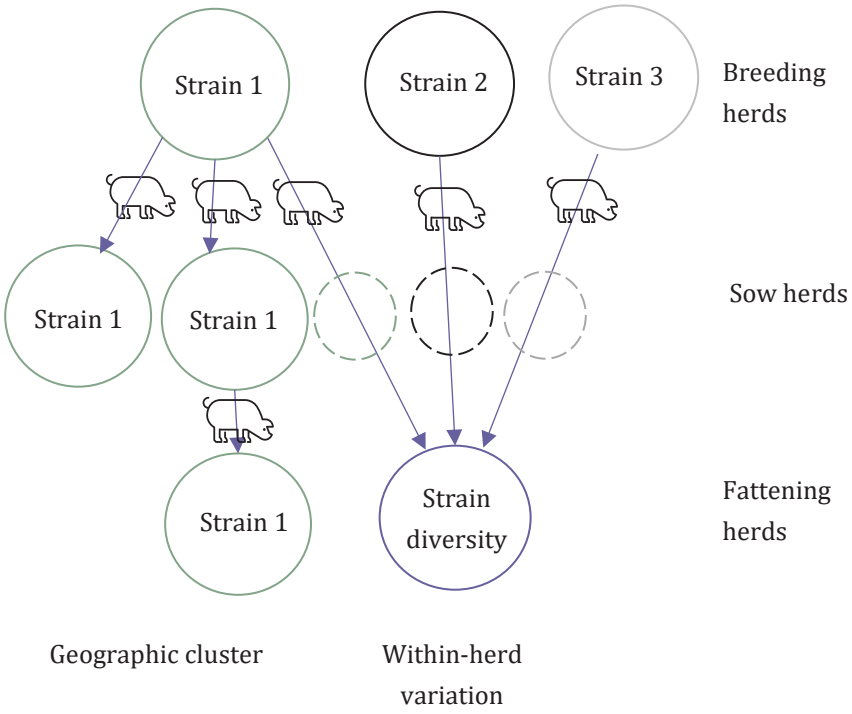

Figure 14 An illustration of Actinobacillus pleuropneumoniae (APP) strain distribution from breeding herds in the Norwegian pig production system.

5.5.1.1 Herd biosecurity

Since national import restrictions hinder the most likely transmission routes of respiratory pathogens that are absent from the Norwegian pig population, the risk of introducing these agents to a herd is generally unlikely. However, APP is assumed to occur endemically in the conventional pig population in Norway (Lium 2002), supported by the results of Paper I. If present in the supplying herd, APP is probably introduced to the fattening herds by tonsillar carriers with every new livestock batch. Only external biosecurity practices related to the livestock source will influence the introduction of APP through this route. Other external biosecurity practices could however be important in limiting potential transmission by other routes. The relevance of transmission of APP by other routes than direct transmission via purchased livestock is not fully understood. Because airborne transmission has not been disconfirmed, there is a general recommendation that herds that are converted to the SPF system in Norway is strategically located away from other pig farms where APP occurs (Animalia 2020b). To establish targeted biosecurity measures, more knowledge regarding transmission of APP is needed.

While rules and regulations form the framework for biosecurity practices, the practical execution on herd level also depends on factors such as tradition, knowledge and understanding of the farmers (Ritter *et al.* 2017). The external biosecurity is probably more influenced by rules and regulations, while internal biosecurity to a larger degree is subject to the farmers own wishes and motivation (Postma *et al.* 2016). Both outbreak and non-outbreak herds scored higher on external than internal biosecurity (Paper III). Better compliance requires a framework tailored to the production type, infrastructure, economic development state and perhaps to preexisting tradition (Ritter *et al.* 2017). Knowledge and understanding of risks and consequences are crucial and will influence the attitudes of the farmers (Laanen *et al.* 2014). As the farmers motivation to implement biosecurity practices was not addressed in this study, we can only make generalized assumptions as to why the biosecurity scores were suboptimal even while regulations promoting biosecurity practices are in place.

Objective scoring of biosecurity in Norwegian fattening pig herds implies there might be a lack of compliance of important biosecurity measures internally at the farm. Suboptimal internal biosecurity practices that increase the risk of transmission of APP between groups of animals in the herd could have contributed towards the outbreaks. Emphasizing this point, all-in-all-out rearing by compartment is one of the most important biosecurity measures to reduce the clinical significance of APP, as discussed by Sjölund in her thesis (Sjölund 2010). However, since the importance of APP transmission in respiratory disease outbreaks is not clear, the effect of herd biosecurity to control this disease is similarly unclear.

5.5.1 Host

Presence of serum antibodies to APP, regardless of disease outbreaks indicated the presence of APP in all but one herd (Paper I). A high proportion of the pigs in both outbreak and non-outbreak herds had positive titers of APP antibodies already at the initial sampling. In Norway, average age at weaning is 33 days (Ingris 2021), which is older than the requirements of the EU (European Parliament and Council 2016a). Due to APP being a late colonizer in piglets (Tobias *et al.* 2014b) the APP carrier rates in Norway are likely to be relatively high. The onset of disease outbreaks occurred at different times during the fattening period (Paper I), at 35 days median after arrival to the fattening unit. The pigs were then around 14 weeks old. Protection by maternally derived antibodies drops prior to this (Cruijsen *et al.* 1995 , Chiers *et al.* 2002a), which indicates that some other criteria must be filled before the disease breaks out.

As the serologic investigations revealed to us, evidence of acquired immunity to APP was present in all but one herd, however, a relatively high proportion were seronegative in the first sample (Paper I). This means they were likely susceptible to a challenge with APP. In our study, antigens were not tested for serovar specificity. As cross-protection between serovars can be lacking (Dubreuil *et al.* 2000), the pigs' immunity towards APP8 was not possible to ascertain in the current study. Although serology is important for the detection of previous and subclinical colonization (Chiers *et al.* 2002a), serologic analyses of APP proved to be challenging for diagnostic purposes

(Paper I). The challenge is due to the unclear mechanisms between subclinical colonization by APP and triggering of the immune system (Gottschalk 2015). The presence of acquired APP antibodies in serum has been described to last for many months (Gottschalk *et al.* 2019). The time of colonization and following seroconversion in these pigs could not be assessed, but it is assumed that APP was present in all the herds prior to the outbreak.

The mechanisms of disease outbreaks were likely tied to additional factors than just the host susceptibility and agent presence in these instances, even if no such risk factors were identified in these studies.

5.5.2 Outbreak mechanisms

The precise events leading up to a lung infection have not been elucidated. Monoclonal infections could result from any of two existing theories to explain the mechanisms behind outbreaks with APP in endemically infected herds (Klinkenberg *et al.* 2014). The theories suggest that outbreaks are caused by a) newly introduced virulent strains of APP and/or by b) descent to the lower respiratory tract of strains already resident in the tonsils. During an outbreak of disease, the infectious pressure of more virulent isolates will increase. Internal biosecurity practices will be important in restricting bacterial transmission within the herd. If descending bacteria from the tonsils are also involved in the infections, a variation in the genomes of bacteria isolated from the lungs of diseased pigs would be expected. Findings of monoclonal infections in the present studies did not support a combination of the two theories. Isolate variation within a herd also indicated that disease occurring in a herd was not solely tied to the spread of a single virulent strain. Several strains could be circulating in a herd during an outbreak. The tonsillar carrying state of the pigs in this study was not assessed but could have contributed to clarify the mechanisms of disease.

5.5.2.1 Triggers for disease outbreaks

Some management and demographic features of the outbreak and non-outbreak herds were described in the materials section of Paper I and III, but there was a lack of power to perform statistical comparisons. Even so, the

outbreak herds were generally larger, had more livestock suppliers and less air space per pig than the non-outbreak herds, all known risk factors for infectious respiratory disease (Rosendal *et al.* 1983). The way pigs are reared in commercial farms today is quite different from 30 years ago, and the need for updated knowledge regarding risk factors has been addressed by Sassu *et al*. (2017).

Ideally, to address the mechanisms behind outbreaks, the above-mentioned demographic features should be accounted for as environmental factors. For APP in particular, theories regarding "trigger" factors, as described by Klinkenberg *et al.* (2014), have been central to understanding mechanisms behind outbreaks, but require further investigation. Targeted risk factor studies to contribute to a trigger factor check list can be utilized at the face of an outbreak, as suggested by Sassu *et al*. (2017).

The interactions between APP, the pigs and the various environmental factors that could trigger disease outbreaks are intricate. Consequently, preventing disease in herds that are colonized with APP is challenging. It has therefore been suggested that efforts to prevent disease should be aimed at hindering bacterial transmission (Velthuis *et al.* 2003 , Tobias *et al.* 2014a). Knowledge of transmission between colonized and healthy animals is thus crucial to prevent the spread of APP and limit disease occurrence and should be prioritized in future research.

5.5.3 Ethical considerations

To be able to perform sampling from live animals, the Norwegian Food Safety Authority approved the study design for 'Grisefine lunger', maintaining compliance of ethical guidelines and the three R's. FOTS Norwegian Food Safety Authority reference ID 13185.

In addition to sampling from live pigs, the field study included sampling of lungs from dead pigs. Serologic investigations did not provide satisfactory diagnostic confidence as seroconversion to APP unrelated to the outbreak was demonstrated both in outbreak and non-outbreak herds. As has been described previously and confirmed by the study in Paper I, diagnosing APP

should be done by post-mortem inspection and bacteriological culture from typical acute lesions. Out of ethical considerations, untreated pigs that had recently succumbed to respiratory disease were selected for sampling in the outbreak herds, if available. However, in the absence of such subjects, a minimum of three pig per herd were euthanized. Collecting pigs' lungs was necessary to perform sound diagnostic procedures and verifying the main etiological agent behind the ongoing outbreaks. Findings from paper I demonstrated that sampling only one typical animal is enough, when the clinical signs are indicative of APP.

5.5.4 Limitations

The aims of this thesis were pursued through observational studies. The field study (Paper I and III) relied on participation by field practitioners and herd owners. Seven case and seven control herds were selected from the same source population, namely conventional commercial farrow-to-finish, and fattening pig herds in Norway. To increase the generalizability of the results, breeding herds and SPF herds were excluded from participation. These herds were excluded because the management of those herd types is likely to differ from commercial herds. Also, both due to stricter health requirements, and due to the general high health status, outbreaks of respiratory disease were expected to happen less frequently in those herds. Herds were subject to convenience sampling rather than random sampling, and no formal assessment of the risk of selection bias was performed.

The initial biologic sampling and clinical examination was performed by the field veterinarians, and prone to observer/operator bias, even if instructions were provided for the sampling. Since Paper I was mainly descriptive, the effect of potential bias is limited, for instance to the report of clinical signs. For body temperature, on the other hand, bias may occur both due to differing techniques and thermometers. Each herd was thus used as its own control, comparing temperatures at visit phases 1 and 3 (Figure 9), measured by the same operators to reduce observer bias.

It is worth noting that the data analyzed in Paper II consists mainly of isolates collected after 2004 and are not equally represented in time. Concentrated

sampling contributed to the reported uncertainty around the estimates of population divergence between countries (Paper II). Since sampling from the whole population was not feasible, an adequate representation of the population over time is necessary for good estimates of divergence. However, it is believed that the isolates in the material have been subject to minimal selection bias due to being passively collected through routine diagnostics of animals sent in during outbreaks. Isolates were collected from all levels of the production system, and from all the major pig producing regions in Norway (Paper II).

5.5.5 Future perspectives

The work described in this thesis has provided new knowledge. To increase our understanding of infectious respiratory disease further, some future perspectives are discussed below.

There are substantial geographical differences in clinical importance of serovars of APP (Dubreuil *et al.* 2000). This is partly due to differences in serovar prevalence and expression of Apx toxins, but also due to differences in expression of other virulence factors between strains within a serovar (Gottschalk *et al.* 2019). So far, the distribution of other virulence factors that could explain this difference has not been clarified. There are currently no available diagnostic tests to differentiate between strains of different virulence, to the author's knowledge. Such a test could be applied to evaluate the potential of a particular strain of APP to cause outbreaks, and to be able to test purchased livestock for their APP carrying status (Gottschalk *et al.* 2019), beyond the classification of serovar. Mapping of virulence-associated traits is a prerequisite for development of a virulence assessment tool. Previous work within this field by Xu *et al*. (2010) was only performed on single isolates of a small range of serovars, however, resulting in an observed conservation of virulence associated genes in typically virulent serovars. The phenotypes and clinical relevance of these virulence-associated genes should be further assessed (Gottschalk *et al.* 2019). In Paper I, severe infections with APP8 were indicative of moderate to high virulence. Virulence factor testing of the isolates would have been interesting. An

annotation of virulence associated genes from a large collection of isolates preferably where all serovars are represented, is needed.

The interest in preventing APP in populations nationwide demands more research regarding risk factors for disease and subclinical manifestation. Paper II revealed that during an outbreak, the strain of APP that causes disease is not necessarily the same among affected pigs in one herd. It also revealed monoclonal infections by APP8. This strengthens the belief that disease outbreaks follow an invasion of the lungs by a single strain of APP that colonized the tonsils prior to the disease. Migration and colonization of the pulmonary tissue by APP is likely to follow a triggering risk factor that affect many pigs in the compartment (Klinkenberg *et al.* 2014). There are studies that have identified risk factors associated with lesions at slaughter. To limit the occurrence of outbreaks, a targeted risk factor analysis for outbreaks of APP is needed.

Only a few Norwegian isolates of APP8 were found to carry AMR genes (Paper II). In depth analysis of the isolates in question including a phenotypic MIC test could elucidate the clinical relevance of the observed genes. In addition, investigation of the genetic segments surrounding the AMR genes could identify whether they seem to be originating from individual acquisition or if they could be part of a common mobilizable genetic element, to assess their spreading potential.

As the results of Paper II showed, there was variation in APP strains isolated from a herd. This means that capturing the range of isolates from a given herd can be difficult. In fattening pig herds, pigs from different sources are expected to carry different strains of APP. Since sows in the supplying herds could be carrying multiple different strains, strain diversity can also occur in pigs from the same source. An assessment of tonsillar strain diversity in sows would increase our knowledge regarding the association between livestock supply and strain diversity in fattening pig herds.

The results of Paper III have indicated that particularly the internal biosecurity was lacking in the fattening herds. A poor internal biosecurity

represents a risk for a spread of endemically occurring infectious agents between animal groups in the pig herds. Although the results from Paper III were not generalizable for the fattening pig herds in Norway, the paper addresses a lack of compliance between the framework of the biosecurity rules and regulations and the farmer compliance. A screening of the biosecurity of Norwegian herds is in place to isolate potential problem areas.

As a direction for future research, the following objectives are suggested:

- Map virulence factors of APP in Norway by phenotypic and genotypic testing

- Targeted risk factor analysis for outbreaks of APP

- Investigation of the acquisition of AMR genes in Norwegian isolates of APP8, and their clinical relevance

- Screening of tonsillar carries state of sows in breeding and commercial sow herds to map diversity of APP in carrier animals

- Screening of biosecurity levels in Norwegian pig herds in different levels of the production pyramid and address compliance to the existing biosecurity framework in Norway

5.6 Conclusion

The work described in this thesis has increased the understanding of infectious respiratory disease in the Norwegian pig population, and new knowledge about APP outbreaks in Norway has been gathered.

The clinical characteristics of respiratory disease outbreaks in Norwegian fattening pig herds were severe and typical for APP. Through comprehensive diagnostic procedures, APP8 was found to be the main etiological agent behind the outbreaks. No other respiratory agents were found to contribute to the disease outbreaks (Paper I). Even with various strains of APP within one herd, pneumonic infections with APP8 were found to be monoclonal within the pig (Paper II).

Historically, APP8 has been underdiagnosed in the Norwegian pig population and has been the dominating clinical serovar in Norway over the last years. Structure of the pig population, such as regional livestock trading patterns has led to limited variation among APP8 isolates in Norway, with clear geographic clustering. The overall genetic variability in isolates of APP8 from Norway is low, and genetically persistent over time.

There was a low occurrence of AMR genes in Norwegian isolates of APP8. International biosecurity considerations allowing for a closed Norwegian pig population has allowed for a distinct separation between Norwegian isolates and those from neighboring nations Denmark and the UK (Paper II). While a high level of national biosecurity in Norway is the most important contribution to the health status of the Norwegian pig population, biosecurity measures at the herd level are important to the prevent the spread of agents that occur in the population.

During outbreaks, objectively scored biosecurity levels were moderate, and showed no difference between herds with and without outbreaks of respiratory disease (Paper III). However, a lower internal biosecurity score in both outbreak and non-outbreak herds was found, implying there might be a lack of compliance with important biosecurity measures internally at

the farm. A low internal biosecurity score implies there is a risk of transmitting infectious agents like APP between pigs within the herd. More information regarding transmission of APP is needed to assess the association between biosecurity measures on occurrence of outbreaks with APP. Genetic investigations were helpful, as studying the genetic variability and evolution of agents increased our understanding of APP and the effect of population structure and production practices, including biosecurity.

Knowledge of the respiratory disease etiology and biosecurity levels in Norwegian pig herds can contribute to prevent future outbreaks. Additionally, the possibilities within WGS to increase our knowledge of APP proved to be particularly useful when studying the effect of pig population structure on genetic variability and AMR status. The work described in this thesis increased our understanding of infectious respiratory disease and may contribute to improved health of pigs.

6 References

Akerley, B.J., Monack, D.M., Falkow, S. and Miller, J.F., 1992. The *bvgAS* locus negatively controls motility and synthesis of flagella in *Bordetella bronchiseptica*. *J Bacteriol* 174(3): 980-990. https://dx.doi.org/10.1128/jb.174.3.980-990.1992

Animalia, 2018. Kjøttets tilstand 2018: Status i norsk kjøtt- og eggproduksjon [The status of meat production]. Available from: https://www.animalia.no/contentassets/ea773e1d55294823802670cac747a264/kjottets-tilstand-2018.pdf

Animalia, 2020a. Helse- og Hygienereglementet for Foredlings- og Formeringsbesetninger [Health and hygiene regulations for nucleus and multiplier herds]. Available from: https://www.animalia.no/contentassets/c91ed15652954a52a631d595cb7b2592/2020-helse--og-hygienereglementet-for-avlsbesetninger.pdf

Animalia, 2020b. Kjøttets tilstand 2020: Status i norsk kjøtt- og eggproduksjon [The status of meat production]. Available from: https://www.animalia.no/contentassets/68810b5267f4423da1065e9591566193/kt20-komplett-origi-web.pdf

Animalia. "Helsegrissystemet" [The healthy pig system]. Animalia; 2021a. Accessed 29. September 2021. Available from: https://www.animalia.no/no/Dyr/svin/helsegrissystemet/.

Animalia. "SPF – systematisk satsing på friskere gris" [SPF - systematic efforts for healthier pigs]. Animalia; 2021b. Accessed 16. August 2021. Available from: https://www.animalia.no/no/gomorning/dyrehelse/ny-spf--systematisk-satsing-pa-friskere-gris/.

Argimón, S., Abudahab, K., Goater, R.J.E., Fedosejev, A., Bhai, J., Glasner, C., Feil, E.J., Holden, M.T.G., Yeats, C.A., Grundmann, H., Spratt, B.G. and Aanensen, D.M., 2016. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. *Microb Genom* 2(11). https://dx.doi.org/10.1099/mgen.0.000093

Assavacheep, P. and Rycroft, A.N., 2013. Survival of *Actinobacillus pleuropneumoniae* outside the pig. *Res Vet Sci* 94(1): 22-26. https://dx.doi.org/10.1016/j.rvsc.2012.07.024

Auger, E., Deslandes, V., Ramjeet, M., Contreras, I., Nash, J.H.E., Harel, J., Gottschalk, M., Olivier, M. and Jacques, M., 2009. Host-pathogen interactions of *Actinobacillus pleuropneumoniae* with porcine lung and tracheal epithelial cells. *Infect Immun* 77(4): 1426-1441. https://dx.doi.org/10.1128/IAI.00297-08

Beskow, P., Norqvist, M. and Wallgren, P., 1998. Relationships between selected climatic factors in fattening units and their influence on the development of respiratory diseases in swine. *Acta Vet Scand* 39(1): 49-60. https://dx.doi.org/10.1186/BF03547806

Bochev, I., 2007. Porcine respiratory disease complex (PRDC): A review. I. Etiology, epidemiology, clinical forms and pathoanatomical features. *Bulg J Vet Med* 10(3): 131–146.

Bossé, J.T., Janson, H., Sheehan, B.J., Beddek, A.J., Rycroft, A.N., Simon Kroll, J. and Langford, P.R., 2002. *Actinobacillus pleuropneumoniae:* pathobiology and pathogenesis of infection. *Microbes Infect* 4(2): 225-235. https://dx.doi.org/10.1016/S1286-4579(01)01534-9

Bossé, J.T., Johnson, R.P., Nemec, M. and Rosendal, S., 1992. Protective local and systemic antibody responses of swine exposed to an aerosol of *Actinobacillus pleuropneumoniae* serotype 1. *Infect Immun* 60: 479 - 484. https://dx.doi.org/10.1128/iai.60.2.479-484.1992

Bossé, J.T., Li, Y., Atherton, T.G., Walker, S., Williamson, S.M., Rogers, J., Chaudhuri, R.R., Weinert, L.A., Holden, M.T.G., Maskell, D.J., Tucker, A.W., Wren, B.W., Rycroft, A.N. and Langford, P.R., 2015. Characterisation of a mobilisable plasmid conferring florfenicol and chloramphenicol resistance in *Actinobacillus pleuropneumoniae*. *Vet Microbiol* 178(3): 279-282. https://dx.doi.org/10.1016/j.vetmic.2015.05.020

Bossé, J.T., Li, Y., Fernandez Crespo, R., Chaudhuri, R.R., Rogers, J., Holden, M.T.G., Maskell, D.J., Tucker, A.W., Wren, B.W., Rycroft, A.N., Langford, P.R. and BRaDP1T Consortium, 2016. ICE*Apl1*, an integrative conjugative element related to ICE*Hin1056*, identified in the pig pathogen *Actinobacillus pleuropneumoniae*. *Front Microbiol* 7(810). https://dx.doi.org/10.3389/fmicb.2016.00810

Bossé, J.T., Li, Y., Fernandez Crespo, R., Lacouture, S., Gottschalk, M., Sarkozi, R., Fodor, L., Casas Amoribieta, M., Angen, O., Nedbalcova, K., Holden, M.T.G., Maskell, D.J., Tucker, A.W., Wren, B.W., Rycroft, A.N., Langford, P.R. and BRaDP1T consortium, 2018a. Comparative sequence analysis of the capsular polysaccharide loci of *Actinobacillus pleuropneumoniae* serovars 1-18, and development of two multiplex PCRs for comprehensive capsule typing. *Vet Microbiol* 220: 83-89. https://dx.doi.org/10.1016/j.vetmic.2018.05.011

Bossé, J.T., Li, Y., Sarkozi, R., Fodor, L., Lacouture, S., Gottschalk, M., Casas Amoribieta, M., Angen, O., Nedbalcova, K., Holden, M.T.G., Maskell, D.J., Tucker, A.W., Wren, B.W., Rycroft, A.N., Langford, P.R. and BRaDP1T consortium, 2018b. Proposal of serovars 17 and 18 of *Actinobacillus pleuropneumoniae* based on serological and genotypic analysis. *Vet Microbiol* 217: 1-6. https://dx.doi.org/10.1016/j.vetmic.2018.02.019

Bossé, J.T., Sinha, S., Li, M.-S., O'Dwyer, C.A., Nash, J.H.E., Rycroft, A.N., Kroll, J.S. and Langford, P.R., 2010. Regulation of *pga* operon expression and biofilm formation in *Actinobacillus pleuropneumoniae* by σE and H-NS. *J Bacteriol* 192(9): 2414-2423. https://dx.doi.org/10.1128/JB.01513-09

Bouckaert, R., Vaughan, T.G., Barido-Sottani, J., Duchene, S., Fourment, M., Gavryushkina, A., Heled, J., Jones, G., Kuhnert, D., De Maio, N., Matschiner, M., Mendes, F.K., Muller, N.F., Ogilvie, H.A., du Plessis, L., Popinga, A., Rambaut, A., Rasmussen, D., Siveroni, I., Suchard, M.A., Wu, C.H., Xie, D., Zhang, C., Stadler, T. and Drummond, A.J., 2019. BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *PLoS Comput Biol* 15(4). https://dx.doi.org/10.1371/journal.pcbi.1006650

Brewer, M.T. and Greve, J.H. 2019. Internal Parasites: Helminths. Diseases of Swine. Zimmermann, J. J., Karriker, L. A., Ramirez, A. et al. Hoboken, NJ, Wiley-Blackwell**:** 1028-1040.

Brockmeier, S.L., Halbur, P.G. and Thacker, E.L. Porcine Respiratory Disease Complex. 2002 [cited 16. August 2021]. In: Polymicrobial Diseases [Internet]. Washington DC: ASM Press. Available from: https://www.ncbi.nlm.nih.gov/books/NBK2481/.

Brooks, J.W., 2016. Postmortem Changes in Animal Carcasses and Estimation of the Postmortem Interval. *Vet Pathol* 53(5): 929-940. https://dx.doi.org/10.1177/0300985816629720

CDC. "Glossary". Centres for Disease Control and Prevention; 2014. Accessed 27. September 2021. Available from: https://www.cdc.gov/csels/dsepd/ss1978/glossary.html.

Chiers, K., De Waele, T., Pasmans, F., Ducatelle, R. and Haesebrouck, F., 2010. Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. *Vet Res* 41(5). https://dx.doi.org/10.1051/vetres/2010037

Chiers, K., Donne, E., Van Overbeke, I., Ducatelle, R. and Haesebrouck, F., 2002a. *Actinobacillus pleuropneumoniae* infections in closed swine herds: infection patterns and serological profiles. *Vet Microbiol* 85(4): 343-352. https://dx.doi.org/10.1016/S0378-1135(01)00518-1

Chiers, K., Donne, E., Van Overbeke, I., Ducatelle, R. and Haesebrouck, F., 2002b. Evaluation of serology, bacteriological isolation and polymerase chain reaction for the detection of pigs carrying *Actinobacillus pleuropneumoniae* in the upper respiratory tract after experimental infection. *Vet Microbiol* 88(4): 385-392. https://dx.doi.org/10.1016/s0378-1135(02)00150-5

Chiers, K., Haesebrouck, F., van Overbeke, I., Charlier, G. and Ducatelle, R., 1999. Early in vivo interactions of *Actinobacillus pleuropneumoniae* with tonsils of pigs. *Vet Microbiol* 68(3): 301-306. https://dx.doi.org/10.1016/S0378-1135(99)00048-6

Christensen, H., Bossé, J., Angen, Ø., Nørskov-Lauritsen, N. and Bisgaard, M., 2020. Immunological and molecular techniques used for determination of serotypes in *Pasteurellaceae*. *Methods Microbiol* 47: 117-149. https://dx.doi.org/10.1016/bs.mim.2020.01.002

Costa, G., Oliveira, S., Torrison, J. and Dee, S., 2011. Evaluation of *Actinobacillus pleuropneumoniae* diagnostic tests using samples derived from experimentally infected pigs. *Vet Microbiol* 148(2-4): 246-251. https://dx.doi.org/10.1016/j.vetmic.2010.08.023

Cruijsen, T., van Leengoed, L.A.M.G., Kamp, E.M., Bartelse, A., Korevaar, A. and Verheijden, J.H.M., 1995. Susceptibility to *Actinobacillus pleuropneumoniae* infection in pigs from an endemically infected herd is related to the presence of toxin-neutralizing antibodies. *Vet Microbiol* 47(3): 219-228. https://dx.doi.org/10.1016/0378-1135(95)00109-3

Davies, P.R., Bahnson, P.B., Grass, J.J., Marsh, W.E. and Dial, G.D., 1995. Comparison of methods for measurement of enzootic pneumonia lesions in pigs. *Am J Vet Res* 56(6): 9-14.

Desrosiers, R. and Moore, C., 1998. Indirect transmission of *Actinobacillus pleuropneumoniae*. *J Swine Health Prod* 6(6): 263-265.

Dreyfus, A., Schaller, A., Nivollet, S., Segers, R.P.A.M., Kobisch, M., Mieli, L., Soerensen, V., Hüssy, D., Miserez, R., Zimmermann, W., Inderbitzin, F. and Frey, J., 2004. Use of recombinant ApxIV in serodiagnosis of *Actinobacillus pleuropneumoniae* infections, development and prevalidation of the ApxIV ELISA. *Vet Microbiol* 99(3-4): 227-238. https://dx.doi.org/10.1016/j.vetmic.2004.01.004

Dubreuil, J.D., Jacques, M., Mittal, K.R. and Gottschalk, M., 2000. *Actinobacillus pleuropneumoniae* surface polysaccharides: their role in diagnosis and immunogenicity. *Anim Health Res Rev* 1(2): 73-93. https://dx.doi.org/10.1017/s1466252300000074

EFSA and ECDC, 2020. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018. Available from: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2020.6007

EFTA Surveillance Authority Decision No 160/10/COL. EFTA Surveillance Authority: Official Journal of the European Union; 2010. Retrieved 18. September 2021, from: https://eur-lex.europa.eu/eli/dec/2010/160(2)/oj

ESVAC, 2015. Sales of veterinary antimicrobial agents in 26 EU/EEA countries in 2013. Available from: https://www.ema.europa.eu/en/documents/report/fifth-esvac-report-sales-veterinary-antimicrobial-agents-26-european-union/european-economic-area-countries-2013_en.pdf

European Comission, 2011. Action plan against the rising threats from antimicrobial resistance. Available from: https://ec.europa.eu/health/sites/default/files/antimicrobial_resistance/docs/communication_amr_2011_748_en.pdf

Council Directive 2008/120/EC of 18 December 2008 laying down minimum standards for the protection of pigs. European Comission; 2016a. Retrieved 08. September 2021, from: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32008L0120

Regulation (EU) 2016/429 of the European Parliament and of the Council of 9 March 2016 on transmissible animal diseases and amending and repealing certain acts in the area of animal health ('Animal Health Law'). European Comission; 2016b. Retrieved 08. September 2021, from: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R0429&from=EN

Eurostat 2009. Pigs: number of farms and heads by size of farm (UAA) and size of breeding sow herd [cited 18. September 2021]. Available from: https://ec.europa.eu/eurostat/databrowser/view/ef_ls_gvsows/default/table?lang=en.

Eurostat 2017. Pigs-sows: number of farms and heads by agricultural size of farm (UAA) and size of breeding sow herd [cited 18. September 2021]. Available from: https://ec.europa.eu/eurostat/databrowser/view/ef_lssowsaa/default/bar?lang=en.

Fablet, C., Marois, C., Kuntz-Simon, G., Rose, N., Dorenlor, V., Eono, F., Eveno, E., Jolly, J.P., Le Devendec, L., Tocqueville, V., Queguiner, S., Gorin, S., Kobisch, M. and Madec, F., 2011. Longitudinal study of respiratory infection patterns of breeding sows in five farrow-to-finish herds. *Vet Microbiol* 147(3-4): 329-339. https://dx.doi.org/10.1016/j.vetmic.2010.07.005

Falk, K., Hoie, S. and Lium, B.M., 1991a. An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. II. Enzootic pneumonia of pigs: microbiological findings and their relationship to pathomorphology. *Acta Vet Scand* 32(1): 67-77. https://dx.doi.org/10.1186/BF03546998

Falk, K. and Lium, B., 1991b. An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. III. Serological findings and their relationship to pathomorphological and microbiological findings. *Acta Vet Scand* 32(1): 79-88. https://dx.doi.org/10.1186%2FBF03546999

Falk, K., Lium, B.M. and Ødegaard, Ø. 1990. Occurrence of lung lesions and antibodies to serotypes 2 and 6 of *Actinobacillus pleuropneumonia*e and *Haemophilus parasuis* in 5176 slaughter weight pigs from 113 elite herds in Norway. International Pig Veterinary Society, Lusanne, CH.

Falk, M. and Hofshagen, M., 2020. Dyrehelserapporten 2019 [Animal health report 2019]. Available from: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2020/dyrehelserapporten-2019

FAO, 2016. Drivers, dynamics and epidemiology of antimicrobial resistance in animal production. Available from: http://www.fao.org/3/a-i6209e.pdf

FAO, 2020. World Food and Agriculture - Statistical Yearbook 2020. Available from: http://www.fao.org/documents/card/en/c/cb1329en

FAO, OIE and World Bank, 2010. Good practices for biosecurity in the pig sector – Issues and options in developing and transition countries. Available from: http://www.fao.org/3/i1435e/i1435e.pdf

Fenwick, B.W. and Osburn, B.I., 1986. Immune responses to the lipopolysaccharides and capsular polysaccharides of *Haemophilus pleuropneumoniae* in convalescent and immunized pigs. *Infect Immun* 54(2): 575-582. https://dx.doi.org/10.1128/iai.54.2.575-582.1986

Filippitzi, M.E., Brinch Kruse, A., Postma, M., Sarrazin, S., Maes, D., Alban, L., Nielsen, L.R. and Dewulf, J., 2017. Review of transmission routes of 24 infectious diseases preventable by biosecurity measures and comparison of the implementation of these measures in pig herds in six European countries. *Transbound Emerg Dis* 2018(65): 381-398. https://dx.doi.org/10.1111/tbed.12758

Foote, S.J., Bossé, J.T., Bouevitch, A.B., Langford, P.R., Young, N.M. and Nash, J.H., 2008. The complete genome sequence of *Actinobacillus pleuropneumoniae* L20 (serotype 5b). *J Bacteriol* 190(4): 1495-1496. https://dx.doi.org/10.1128/JB.01845-07

Frey, J. 2003. Detection, Identification, and Subtyping of *Actinobacillus pleuropneumoniae*. PCR Detection of Microbial Pathogens. Sachse, K. and Frey, J. Totowa, NJ, Humana Press**:** 87-95.

Frey, J., Bossé, J., Chang, Y.-F., Cullen, J., Fenwick, B., Gerlach, G., Gygi, D., Haesebrouck, F., Inzana, T. and Jansen, R., 1993. *Actinobacillus pleuropneumoniae* RTX-toxins: uniform designation of haemolysins, cytolysins, pleurotoxin and their genes. *J Gen Microbiol* 139(8): 1723-1728. https://dx.doi.org/10.1099/00221287-139-8-1723

Fussing, V., Barfod, K., Nielsen, R., Møller, K., Nielsen, J.P., Wegener, H.C. and Bisgaard, M., 1998. Evaluation and application of ribotyping for epidemiological studies of *Actinobacillus pleuropneumoniae* in Denmark. *Vet Microbiol* 62(2): 145-162. https://dx.doi.org/10.1016/S0378-1135(98)00205-3

Gardner, I.A., Bossé, J.T., Sheldrake, R.F., Rosendal, S. and Johnson, R.P., 1991. Serological response to *Actinobacillus pleuropneumoniae* serovar 7 infection in a commercial pig herd. *Aust Vet J* 68(11): 349-352. https://dx.doi.org/10.1111/j.1751-0813.1991.tb00732.x

Genomes OnLine Database. Accessed 07. October 2021. Available from: https://gold.jgi.doe.gov/.

Ghent University. "Biocheck.UGent". Accessed 17. October 2018. Available from: http://www.biocheck.ugent.be/.

Gottschalk, M., 2015. The challenge of detecting herds sub-clinically infected with *Actinobacillus pleuropneumoniae*. *Vet J.* 206(1): 30-38. https://dx.doi.org/10.1016/j.tvjl.2015.06.016

Gottschalk, M. and Broes, A. 2019. Actinobaccilosis. Diseases of Swine. Zimmermann, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. Hoboken, NJ, John Wiley & Sons, Inc.: 749-766.

Gottschalk, M. and Lacouture, S., 2014. *Actinobacillus pleuropneumoniae* serotypes 3, 6, 8 and 15 isolated from diseased pigs in North America. *Vet Rec* 174(18): 452-452. https://dx.doi.org/10.1136/vr.102470

Gottschalk, M. and Lacouture, S., 2015. Canada: Distribution of *Streptococcus suis* (from 2012 to 2014) and *Actinobacillus pleuropneumoniae* (from 2011 to 2014) serotypes isolated from diseased pigs. *Can Vet J* 56(10): 1093-1094.

Gottschalk, M., Lebrun, A., Lacouture, S., Harel, J., Forget, C. and Mittal, K.R., 2000. Atypical *Actinobacillus pleuropneumoniae* isolates that share antigenic determinants with both serotypes 1 and 7. *J Vet Diagn* 12(5): 444-449. https://dx.doi.org/10.1177/104063870001200509

Grøntvedt, C.A., 2017. Veterinærinstituttets faglige aktivitetsrapport 2016 [Annual activity report of the Norwegian Veterinary Institute 2016]. Available from: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2017/veterinaerinstituttets-faglige-aktivitetsrapport

Grøntvedt, C.A., 2018a. Veterinærinstituttets faglige aktivitetsrapport 2017 [Annual activity report of the Norwegian Veterinary Institute 2017]. Available from: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2018/veterinaerinstituttets-faglige-aktivitetsrapport-2017

Grøntvedt, C.A., Er, C., Gjerset, B., Germundsson, A., Framstad, T., Brun, E., Jorgensen, A. and Lium, B., 2011. Clinical impact of infection with Pandemic Influenza (H1N1) 2009 Virus in naive nucleus and multiplier pig herds in Norway. *Influenza Res Treat*. https://dx.doi.org/10.1155/2011/163745

Grøntvedt, C.A., Klevar, S., Nordstoga, A.B., Jonsson, M., Løtvedt, S., Åmdal, S. and Nordström, M. 2019a. First detection of antibodies against Porcine Respiratory Coronavirus in Norway. European Symposium of Porcine Health Management, Utrecht, NL.

Grøntvedt, C.A., Nordstoga, A.B. and Hopp, P., 2020. The surveillance programme for specific viral infections in swine herds in Norway 2019. Available from: https://www.vetinst.no/en/surveillance-programmes/viral-infections-in-swine/_/attachment/download/cd46413d-0741-4747-ae0b-3fa79a88e6d1:9cacbf766827e25338119a188e68c3d394a661ec/2020_OK_Viral%20infections%20in%20swine_Report%202019.pdf

Grøntvedt, C.A., Nordstoga, A.B. and Jonsson, M.E., 2019b. The surveillance programme for specific viral infections in swine herds in Norway 2018. Available from: https://www.vetinst.no/en/surveillance-programmes/viral-infections-in-swine/_/attachment/download/e5793dca-57d2-41eb-bd34-9a99e5d11b98:ef9892272432bafa4b61bc30ae1130952606075b/2019_OK_Viral%20infections%20in%20swine_Report%202018.pdf

Grøntvedt, C.A., Nordstoga, A.B., Er C. , 2018b. The surveillance programme for specific viral infections in swine herds in Norway 2017. Available from: https://www.vetinst.no/en/surveillance-programmes/viral-infections-in-swine/_/attachment/download/7d3452fa-887f-4fd8-95d4-bedc51149c2f:a372b201cddc7a4c4d35fcfcc9010dcdf13fb0a4/2018_OK_Viral%20infections%20in%20swine_Report%202017.pdf

Gulliksen, S., Baustad, B., Framstad, T., Jørgensen, A., Skomsøy, A., Kjelvik, O., Gjestvang, M., Groentvedt, C. and Lium, B., 2021. Successful eradication of *Mycoplasma hyopneumoniae* from the Norwegian pig population – 10 years later. *Porcine Health Manag* 7, 37. https://dx.doi.org/10.1186/s40813-021-00216-z

Gutiérrez-Martín, C.B., Blanco, N.G.d., Blanco, M., Navas, J. and Rodríguez-Ferri, E.F., 2006. Changes in antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolated from pigs in Spain during the last decade. *Vet Microbiol* 115(1): 218-222. https://dx.doi.org/10.1016/j.vetmic.2005.12.014

Hernandez-Garcia, J., Robben, N., Magnee, D., Eley, T., Dennis, I., Kayes, S.M., Thomson, J.R. and Tucker, A.W., 2017. The use of oral fluids to monitor key pathogens in porcine respiratory disease complex. *Porcine Health Manag* 3: 1-13. https://dx.doi.org/10.1186/s40813-017-0055-4

Hill, M.A., Scheidt, A.B., Teclaw, R.F., Clark, L.K., Knox, K.E. and Jordan, M., 1994. Relationship between the indicators of performance and the weight of pneumonic lesions from pigs at slaughter. *Res Vet Sci* 56(2): 240-244. https://dx.doi.org/10.1016/0034-5288(94)90110-4

Hofmo, P.O. and Lium, B. 1998. Attempts to establish elite breeding herds free from *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* by strategic medication. 15th International Pig Veterinary Society's Congress, Nottingham, Nottingham University Press.

Høie, S., Falk, K. and Lium, B.M., 1991. An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. IV. Bacteriological findings in chronic pneumonic lesions. *Acta Vet Scand* 32(3): 395-402. https://dx.doi.org/10.1186/BF03546970

Ingris, 2017. Årsstatistikk 2016 [Annual statistics 2016]. Available from: https://norsvin.no/wp-content/uploads/2018/05/Ingris_%C3%85rsstatistikk_2016.pdf

Ingris, 2018. Årsstatistikk 2017 [Annual Statistics 2017]. Available from: https://www.animalia.no/contentassets/28e0db72674d496186f0570a9e606fca/arsstatistikk_2017.pdf

Ingris, 2021. Årsstatistikk 2020 [Annual statistics 2020]. Available from: https://www.animalia.no/globalassets/ingris---dokumenter/arsstatistikk-2020-007---endelig.pdf

Ito, H., Ogawa, T., Fukamizu, D., Morinaga, Y. and Kusumoto, M., 2016. Nucleotide sequence analysis of a DNA region involved in capsular polysaccharide biosynthesis reveals the molecular basis of the nontypeability of two *Actinobacillus pleuropneumoniae* isolates. *J Vet Diagn* 28(6): 632-637. https://dx.doi.org/10.1177/1040638716656026

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A. and Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods* 14(6): 587-589. https://dx.doi.org/10.1038/nmeth.4285

Kao, R.R., Haydon, D.T., Lycett, S.J. and Murcia, P.R., 2014. Supersize me: how whole-genome sequencing and big data are transforming epidemiology. *Trends Microbiol* 22(5): 282-291. https://dx.doi.org/10.1016/j.tim.2014.02.011

Kaspersen, H., Urdahl, A.M., Groentvedt, C., Gulliksen, S., Tesfamichael, B., Slettemeås, J., Norström, M. and Sekse, C., 2020. *Actinobacillus pleuropneumoniae* eradication with enrofloxacin may lead to dissemination and long-term persistence of quinolone resistant *Escherichia coli* in pig herds. *Antibiotics* 9: 910. https://dx.doi.org/10.3390/antibiotics9120910

Kim, J., Chung, H.K. and Chae, C., 2003. Association of porcine circovirus 2 with porcine respiratory disease complex. *Vet J.* 166(3): 251-256. https://dx.doi.org/10.1016/s1090-0233(02)00257-5

Klinkenberg, D., Tobias, T.J., Bouma, A., van Leengoed, L.A. and Stegeman, J.A., 2014. Simulation study of the mechanisms underlying outbreaks of clinical disease caused by *Actinobacillus pleuropneumoniae* in finishing pigs. *Vet J.* 202(1): 99-105. https://dx.doi.org/10.1016/j.tvjl.2014.06.015

KOORIMP and KIF, 2021. Årsmelding 2020 KOORIMP og KIF [Annual report 2020 KOORIMP and KIF]. Available from: https://www.animalia.no/contentassets/7b27e28ef6bf4e878416cc6664a440e1/koorimp-arsmelding-2020-web.pdf

Kott, B.E., 1983. *Chronological studies of respiratory disease in baby pigs*. Veterinary Microbiology and Preventive Medicine, Iowa State University Master of Science thesis(18164). https://lib.dr.iastate.edu/rtd/18164

Kristensen, C.S., Angen, Ø., Andreasen, M., Takai, H., Nielsen, J.P. and Jorsal, S.E., 2004. Demonstration of airborne transmission of *Actinobacillus pleuropneumoniae* serotype 2 between simulated pig units located at close range. *Vet Microbiol* 98(3): 243-249. https://dx.doi.org/10.1016/j.vetmic.2003.10.026

Lemey, P., Salemi, M. and Vandamme, A. 2009. Glossary. The Phylogenetic Handbook. Lemey, P., Salemi, M. and Vandamme, A. Cambridge, UK, Cambridge University Press. 2**:** 654-671.

Letunic, I. and Bork, P., 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res* 47(W1): W256-W259. https://dx.doi.org/10.1093/nar/gkz239

Li, Y., Bossé, J.T., Williamson, S.M., Maskell, D.J., Tucker, A.W., Wren, B.W., Rycroft, A.N., Langford, P.R. and Bradp1T Consortium, 2016. *Actinobacillus pleuropneumoniae* serovar 8 predominates in England and Wales. *Vet Rec* 179(11): 276. https://dx.doi.org/10.1136/vr.103820

Lium, B., 2002. Luftveislidelser hos gris [Respiratoty diseases in pigs]. *NORSK VET* 114(2): 194-196.

Lium, B. and Falk, K., 1991. An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. I. Prevalence and morphological description of gross lung lesions. *Acta Vet Scand* 32(1): 55-65. https://dx.doi.org/10.1186/BF03546997

Lium, B., Gjerset, B., Er, C., Hopp, P., 2010. The surveillance programme for specific viral infections in swine herdsa in Norway 2009. Available from: https://www.vetinst.no/en/surveillance-programmes/viral-infections-in-swine/_/attachment/download/79858760-beba-41a3-8e93-9b0742ca0921:f8d89dc8d5ead263e326c735c51ac77636628e09/2009_specific%20virus%20infections%20in%20swine%20herds%20in%20Norway.pdf

Loeffen, W.L.A., Kamp, E.M., Stockhofe-Zurwieden, N., van Nieuwstadt, A.P.K.M.I., Bongers, J.H., Hunneman, W.A., Elbers, A.R.W., Baars, J., Nell, T. and van Zuderveld, F.G., 1999. Survey of infectious agents involved in acute respiratory disease in finishing pigs. *Vet Rec* 145(5): 123-129. https://dx.doi.org/10.1136/vr.145.5.123

Loera-Muro, V., Jacques, M., Tremblay, Y., González, F., Loera Muro, A., Ramirez-Lopez, E., Medina, A., Gonzalez-Reynaga, H. and Guerrero-Barrera, A., 2013. Detection of *Actinobacillus pleuropneumoniae* in drinking water from pig farms. *Microbiology.* 159. https://dx.doi.org/10.1099/mic.0.057992-0

Laanen, M., Beek, J., Ribbens, S., Vangroenweghe, F., Maes, D., Dewulf, J. and O'Connor, P., 2010. Bioveiligheid op varkensbedrijven: Ontwikkeling van een online scoresysteem en de resultaten van de eerste 99 deelnemende bedrijven. *Vlaams Diergeneeskd Tijdschr* 79: 302-306.

Laanen, M., Maes, D., Hendriksen, C., Gelaude, P., De Vliegher, S., Rosseel, Y. and Dewulf, J., 2014. Pig, cattle and poultry farmers with a known interest in research have comparable perspectives on disease prevention and on-farm biosecurity. *Prev Vet Med* 115(1-2): 1-9. https://dx.doi.org/10.1016/j.prevetmed.2014.03.015

Maes, D., Deluyker, H., Verdonck, M., Castryck, F., Miry, C., Vrijens, B. and de Kruif, A., 2000. Herd factors associated with the seroprevalences of four major respiratory pathogens in slaughter pigs from farrow-to-finish pig herds. *Vet Res* 31(3): 313-327. https://dx.doi.org/10.1051/vetres:2000122

Maldonado, J., Valls, L., Martínez, E. and Riera, P., 2009. Isolation rates, serovars, and toxin genotypes of Nicotinamide Adenine Dinucleotide-independent *Actinobacillus pleuropneumoniae* among pigs suffering from pleuropneumonia in Spain. *J Vet Diagn* 21(6): 854-857. https://dx.doi.org/10.1177/104063870902100615

Marois, C., Gottschalk, M., Morvan, H., Fablet, C., Madec, F. and Kobisch, M., 2009. Experimental infection of SPF pigs with *Actinobacillus pleuropneumoniae* serotype 9 alone or in association with *Mycoplasma hyopneumoniae*. *Vet Microbiol* 135(3): 283-291. https://dx.doi.org/10.1016/j.vetmic.2008.09.061

Merialdi, G., Dottori, M., Bonilauri, P., Luppi, A., Gozio, S., Pozzi, P., Spaggiari, B. and Martelli, P., 2012. Survey of pleuritis and pulmonary lesions in pigs at abattoir with a focus on the extent of the condition and herd risk factors. *Vet J* 193(1): 234-239. https://dx.doi.org/10.1016/j.tvjl.2011.11.009

Forskrift om tilsyn og kontroll ved import og eksport av levende dyr, annet avlsmateriale og animalsk avfall innen EØS, og ved import av levende dyr fra tredjestater [Regulation on live animal trade]. FOR-1998-12-31-1484. Norway; 1998. Retrieved 23. November 2021, from: https://lovdata.no/dokument/SF/forskrift/1998-12-31-1484

Forskrift om hold av svin [Regulation on keeping pigs]. FOR-2003-02-18-175. Norway; 2003. Retrieved 23. November 2021, from: https://lovdata.no/dokument/SF/forskrift/2003-02-18-175

Forskrift om regulering av svine- og fjørfeproduksjonen [Regulation on pig and poultry production]. FOR-2004-04-01-611. Norway; 2004. Retrieved 23. November 2021, from: https://lovdata.no/dokument/SF/forskrift/2004-04-01-611

Mittal, K.R., Higgins, R., Lariviére, S. and Nadeau, M., 1992. Serological characterization of *Actinobacillus pleuropneumoniae* strains isolated from pigs in Quebec. *Vet Microbiol* 32(2): 135-148. https://dx.doi.org/10.1016/0378-1135(92)90101-X

Nechvatalova, K., Knotigova, P., Krejcí, J., Martin, F., Göpfert, E., Satran, P. and Toman, M., 2005. Significance of different types and levels of antigen- specific immunity to *Actinobacillus pleuropneumoniae* infection in piglets. *Vet Med (Praha)* 107: 47-59. https://dx.doi.org/10.17221/5596-VETMED

Neumann, E.J. and Hall, F. 2019. Disease Control, Prevention and Elimination. Diseases of Swine. Zimmermann, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. Hoboken, NJ, John Wiley & Sons, Inc.: 123-157.

NORM/NORM-VET 2020, 2021. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Available from: https://www.vetinst.no/overvaking/antibiotikaresistens-norm-vet/_/attachment/download/8571ebbe-400c-4126-890b-b704388cc05b:9ad8c83c19b02134461e81ed5951769bdc89a8d2/NORM%20NORM-VET%202020.pdf

NORM/NORM-VET, 2017. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Available from: https://www.vetinst.no/en/surveillance-programmes/norm-norm-vet-report/_/attachment/download/3b568168-1005-4925-8b83-535a384275d7:591d6da99e48213389ab94e986aab3f23707b13f/NORM%20NORM-VET%202017.pdf

Nortura. "Salgsvilkår for omsetning av livgris og smågris til framfôring" [Terms of livestock trade for breeding and nursery pigs]. Nortura SA; 2018. Accessed 11. October 2021. Available from: https://medlem.nortura.no/gris/livdyrformidling/salgsvilkaar_kvalitetsgaranti/.

Norwegian Medicines Agency, 2001. Antiparasittær behandling av produksjonsdyr [Antiparasitic treatment of production animals]. Available from: https://legemiddelverket.no/Documents/Veterin%C3%A6rmedisin/Terapianbefalinger/Antiparasitt%C3%A6rbehandling%20av%20produksjonsdyr.pdf

Norwegian Medicines Agency, 2012. Terapianbefaling: bruk av antibakterielle midler til produksjonsdyr [Therapy recommendations: Use of antimicrobial drugs in production animals]. Available from: https://legemiddelverket.no/Documents/Veterin%C3%A6rmedisin/Terapianbefalinger/Terapianbefaling_bruk%20av%20antibakteriellt%20midler%20til%20produks.pdf

Norwegian Medicines Agency, 2019. Porcilis APP vet. INJEKSJONSVÆSKE, suspensjon. Norwegian Medicines Agency [Porcilis APP vet. INJECTION FLUID, suspention]. https://www.legemiddelsok.no/_layouts/15/Preparatomtaler/Spc/16-11362.pdf

Norwegian Veterinary Institute, 2021. Dyrehelserapporten 2020 [Animal health report 2020]. Available from: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2021/dyrehelserapporten-2020/_/attachment/download/8f53b3fa-4b0e-4861-aa7d-34eff9810631:ce08be5866e2608454b375ac5d41b003a4d4aec3/Dyrehelserapporten%202020.pdf

O'Neill, C., Jones, S.C., Bossé, J.T., Watson, C.M., Williamson, S.M., Rycroft, A.N., Kroll, J.S., Hartley, H.M. and Langford, P.R., 2010. Prevalence of *Actinobacillus pleuropneumoniae* serovars in England and Wales. *Vet Rec* 167(17): 661-662. https://dx.doi.org/10.1136/vr.c5106

Oidtmann, B.C., Thrush, M.A., Denham, K.L. and Peeler, E.J., 2011. International and national biosecurity strategies in aquatic animal health. *Aquaculture* 320(1): 22-33. https://dx.doi.org/10.1016/j.aquaculture.2011.07.032

OIE, 2021a. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2021. https://www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/

OIE. "Terrestrial Animal Health Code " Glossary. World Organization for Animal Health; 2021b. Accessed 23. November 2021. Available from: https://www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-code-online-access/.

Opriessnig, T., Gimenez-Lirola, L.G. and Halbur, P.G., 2011. Polymicrobial respiratory disease in pigs. *Anim Health Res Rev* 12(2): 133-148. https://dx.doi.org/10.1017/S1466252311000120

Overbeke, I.V., Chiers, K., Charlier, G., Vandenberghe, I., Beeumen, J.V., Ducatelle, R. and Haesebrouck, F., 2002. Characterization of the in vitro adhesion of *Actinobacillus pleuropneumoniae* to swine alveolar epithelial cells. *Vet Microbiol* 88(1): 59-74. https://dx.doi.org/10.1016/S0378-1135(02)00080-9

Pattison, I.H., Howell, D.G. and Elliot, J., 1957. A *Haemophilus*-like organism isolated from pig lung and the associated pneumonic lesions. *J comp Pathol* 67: 320-IN337. https://dx.doi.org/10.1016/S0368-1742(57)80031-9

Petersen, H.H., Nielsen, E.O., Hassing, A.G., Ersbøll, A.K. and Nielsen, J.P., 2008. Prevalence of clinical signs of disease in Danish finisher pigs. *Vet Rec* 162(12): 377-382. https://dx.doi.org/https://doi.org/10.1136/vr.162.12.377

Pohl, S.B., H. U.; Frederiksen, W.; Mannheim, W., 1983. Transfer of *Haemophilus pleuropneumoniae* and the *Pasteurella haemolytica*-like organism causing porcine necrotic pleuropneumonia to the genus *Actinobacillus* (*Actinobacillus pleuropneumoniae* comb. nov.) on the basis of phenotypic and deoxyribonucleic acid relatedness. *Int J Syst Evol Microbiol* 33(3): 510-514. https://dx.doi.org/10.1099/00207713-33-3-510

Postma, M., Backhans, A., Collineau, L., Loesken, S., Sjölund, M., Belloc, C., Emanuelson, U., Grosse Beilage, E., Stärk, K.D.C. and Dewulf, J., 2016. The biosecurity status and its associations with production and management characteristics in farrow-to-finish pig herds. *Animal* 10(3): 478-489. https://dx.doi.org/10.1017/S1751731115002487

Ramjeet, M., Deslandes, V., Gouré, J. and Jacques, M., 2008. *Actinobacillus pleuropneumoniae* vaccines: from bacterins to new insights into vaccination strategies. *Anim Health Res Rev* 9(1): 25-45. https://dx.doi.org/10.1017/S1466252307001338

Reiner, G., Fresen, C., Bronnert, S., Haack, I. and Willems, H., 2010. Prevalence of *Actinobacillus pleuropneumoniae* infection in hunted wild boars (*Sus scrofa*) in Germany. *J Wildl Dis* 46(2): 551-555. https://dx.doi.org/10.7589/0090-3558-46.2.551

Ritter, C., Jansen, J., Roche, S., Kelton, D.F., Adams, C.L., Orsel, K., Erskine, R.J., Benedictus, G., Lam, T.J.G.M. and Barkema, H.W., 2017. Invited review: Determinants of farmers' adoption of management-based strategies for infectious disease prevention and control. *Dairy Sci.* 100(5): 3329-3347. https://dx.doi.org/10.3168/jds.2016-11977

Robertson, I.D., 2020. Disease Control, Prevention and On-Farm Biosecurity: The Role of Veterinary Epidemiology. *Engineering* 6(1): 20-25. https://dx.doi.org/10.1016/j.eng.2019.10.004

Rose, N. and Madec, F., 2002. Occurrence of respiratory disease outbreaks in fattening pigs: Relation with the features of a densely and a sparsely populated pig area in France. *Vet Res* 33(2): 179-190. https://dx.doi.org/10.1051/vetres:2002006

Rosendal, S., Boyd, D.A. and Gilbride, K.A., 1985. Comparative virulence of porcine *Haemophilus* bacteria. *Can J Comp Med* 49(1): 68-74.

Rosendal, S. and Mitchell, W.R., 1983. Epidemiology of *Haemophilus pleuropneumoniae* infection in pigs: a survey of Ontario Pork Producers, 1981. *Can J Comp Med* 47(1): 1-5.

Sárközi, R., Makrai, L. and Fodor, L., 2015. Identification of a proposed new serovar of *Actinobacillus pleuropneumoniae*: Serovar 16. *Acta Vet Hung* 63(4): 444-450. https://dx.doi.org/10.1556/004.2015.041

Sassu, E.L., Bossé, J.T., Tobias, T.J., Gottschalk, M., Langford, P.R. and Hennig-Pauka, I., 2017. Update on *Actinobacillus pleuropneumoniae*-knowledge, gaps and challenges. *Transbound Emerg Dis* 65 Suppl 1: 72-90. https://dx.doi.org/10.1111/tbed.12739

Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anderson, T.J., Maclnnes, J.I., Segers, R.P.A.M. and Frey, J., 1999. Characterization of apxlVA, a new RTX determinant of *Actinobacillus pleuropneumoniae*. *Microbiology* 145(8): 2105-2116. https://dx.doi.org/10.1099/13500872-145-8-2105

Schuwerk, L., Hoeltig, D., Waldmann, K.-H., Valentin-Weigand, P. and Rohde, J., 2021. Sero- and apx-typing of German *Actinobacillus pleuropneumoniae* field isolates from 2010 to 2019 reveals a predominance of serovar 2 with regular apx-profile. *Vet Res* 52(1): 10. https://dx.doi.org/10.1186/s13567-020-00890-x

Schwartz, B. and Alicata, J.E., 1932. *Ascaris* larvae as a cause of liver and lung lesions in swine. *J Parasitol* 19(1): 17-24. https://dx.doi.org/10.2307/3271427

Shope, R.E., 1964. Porcine contagious pleuropneumonia. I. Experimental transmission, etiology, and pathology. *J Exp Med* 119(3): 357-368. https://dx.doi.org/10.1084/jem.119.3.357

Sibila, M., Aragón, V., Fraile, L. and Segalés, J., 2014. Comparison of four lung scoring systems for the assessment of the pathological outcomes derived from *Actinobacillus pleuropneumoniae* experimental infections. *BMC Vet Res* 10: 165-165. https://dx.doi.org/10.1186/1746-6148-10-165

Sjölund, M., 2010. *Actinobacillus pleuropneumoniae*. Sveriges lantbruksuniv PhD thesis(ISSN 1652-6880). http://urn.kb.se/resolve?urn=urn:nbn:se:slu:epsilon-3128
Sjölund, M., Martín de la Fuente, A.J., Fossum, C. and Wallgren, P., 2009. Responses of pigs to a re-challenge with *Actinobacillus pleuropneumoniae* after being treated with different antimicrobials following their initial exposure. *Vet Rec* 164(18): 550-555. https://dx.doi.org/10.1136/vr.164.18.550
Statistics Norway 2021a. Jordbruksbedrifter med ymse hudyrslag, etter husdyrslag, statistikkvariabel og år [cited 18. September 2021]. Available from: https://www.ssb.no/statbank/table/05979.
Statistics Norway 2021b. Kjøtproduksjon [cited 18. September 2021]. Available from: https://www.ssb.no/jord-skog-jakt-og-fiskeri/jordbruk/statistikk/kjotproduksjon.
Stringer, O., Bossé, J., Lacouture, S., Gottschalk, M., Fodor, L., Angen, Ø., Velazquez, E., Penny, P., Lei, L., Langford, P. and Li, Y., 2021a. Proposal of *Actinobacillus pleuropneumoniae* serovar 19, and reformulation of previous multiplex PCRs for capsule-specific typing of all known serovars. *Vet Microbiol*: 109021. https://dx.doi.org/10.1016/j.vetmic.2021.109021
Stringer, O., Bossé, J., Lacouture, S., Gottschalk, M., Fodor, L., Angen, Ø., Velazquez, E., Penny, P., Lei, L., Langford, P. and Li, Y., 2021b. Rapid detection and typing of *Actinobacillus pleuropneumoniae* serovars directly from clinical samples: combining FTA® card technology with multiplex PCR. *Front Vet Sci* 8: 728660. https://dx.doi.org/10.3389/fvets.2021.728660
Stärk, K.D.C., 2000. Epidemiological investigation of the influence of environmental risk factors on respiratory diseases in swine - A literature review. *Vet J* 159(1): 37-56. https://dx.doi.org/10.1053/tvjl.1999.0421
Suchard, M.A., Lemey, P., Baele, G., Ayres, D.L., Drummond, A.J. and Rambaut, A., 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol* 4(1). https://dx.doi.org/10.1093/ve/vey016
Svendsby, N., 2013. Biosecure. KOORIMP**:** 16-17. https://www.animalia.no/contentassets/9008c25e37a340c7ba82e834e73d62d7/173211-engelsk-web.pdf
Sørensen, J.T., Edwards, S., Noordhuizen, J. and Gunnarsson, S., 2006. Animal production systems in the industrialised world. *Rev Sci Tech* 25(2): 493-503. https://dx.doi.org/10.20506/rst.25.2.1674
Thacker, E.L., 2001. Immunology of the porcine respiratory disease complex. *Vet Clin North Am Food Anim Pract* 17(3): 551-565. https://dx.doi.org/10.1016/s0749-0720(15)30006-2

The Norwegian Directorate of Health, 2021. UTVIKLINGEN I NORSK KOSTHOLD 2020 Matforsyningsstatistikk [Development in Norwegian Nutrition 2020]. Available from: https://www.helsedirektoratet.no/rapporter/utviklingen-i-norsk-kosthold/Utviklingen%20i%20norsk%20kosthold%202020%20%E2%80%93%20Fullversjon.pdf?download=false

Tobias, T.J.-W., 2014. *Actinobacillus pleuropneumoniae transmission and clinical outbreaks*. Faculty of Veterinary Medicine, Utrech University PhD thesis(ISBN: 978-90-393-6147-4). http://dspace.library.uu.nl/handle/1874/294488

Tobias, T.J., Bouma, A., van den Broek, J., van Nes, A., Daemen, A.J.J.M., Wagenaar, J.A., Stegeman, J.A. and Klinkenberg, D., 2014a. Transmission of *Actinobacillus pleuropneumoniae* among weaned piglets on endemically infected farms. *Prev Vet Med* 117(1): 207-214. https://dx.doi.org/10.1016/j.prevetmed.2014.07.017

Tobias, T.J., Klinkenberg, D., Bouma, A., van den Broek, J., Daemen, A.J., Wagenaar, J.A. and Stegeman, J.A., 2014b. A cohort study on *Actinobacillus pleuropneumoniae* colonisation in suckling piglets. *Prev Vet Med* 114(3-4): 223-230. https://dx.doi.org/10.1016/j.prevetmed.2014.02.008

Urdahl, A.M., Norström, M., Welde, H., Bergsjø, B. and Grøntvedt, C.A., 2021. The surveillance programme for methicillin resistant *Staphylococcus aureus* in pigs in Norway 2020. Available from: https://www.vetinst.no/overvaking/mrsa-svin/_/attachment/download/9e42910f-4a2c-4541-a120-13632e6f6c64:d19e39a87581200204aa9dbe5977abc2520c69d0/2020_OK_MRSA_in_pigs_Report%202019.pdf

USDA, 2017. Swine 2012 Part III: Changes in the U.S. Swine Industry, 1995–2012. Available from: https://www.aphis.usda.gov/animal_health/nahms/swine/downloads/swine2012/Swine2012_dr_Trends.pdf

van Alstine, W.G. 2012. Respiratory system. Diseases of Swine. Zimmermann, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. Oxford, UK, Blackwell Scientific**:** 348-362.

van Seventer, J.M. and Hochberg, N.S., 2017. Principles of Infectious Diseases: Transmission, Diagnosis, Prevention, and Control. *International Encyclopedia of Public Health* 22-39. https://dx.doi.org/10.1016/B978-0-12-803678-5.00516-6

Vandamme, A. 2009. Basic concepts of molecular evolution. The Phylogenetic Handbook. Lemey, P., Salemi, M. and Vandamme, A. Cambridge, UK, Cambridge University Press. 2**:** 3-28.

Vanni, M., Merenda, M., Barigazzi, G., Garbarino, C., Luppi, A., Tognetti, R. and Intorre, L., 2012. Antimicrobial resistance of *Actinobacillus pleuropneumoniae* isolated from swine. *Vet Microbiol* 156(1-2): 172-177. https://dx.doi.org/10.1016/j.vetmic.2011.10.022

Velthuis, A.G., De Jong, M.C., Kamp, E.M., Stockhofe, N. and Verheijden, J.H., 2003. Design and analysis of an *Actinobacillus pleuropneumoniae* transmission experiment. *Prev Vet Med* 60(1): 53-68. https://dx.doi.org/10.1016/S0167-5877(03)00082-5

Vigre, H., Angen, O., Barfod, K., Lavritsen, D.T. and Sorensen, V., 2002. Transmission of *Actinobacillus pleuropneumoniae* in pigs under field-like conditions: emphasis on tonsillar colonisation and passively acquired colostral antibodies. *Vet Microbiol* 89(2-3): 151-159. https://dx.doi.org/10.1016/s0378-1135(02)00149-9

Wallgren, P., Beskow, P., Fellström, C. and Renström, L.H.M., 1994. Porcine lung lesions at slaughter and their correlation to the incidence of infections by *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* during the rearing period. *J Vet Med B Infect Dis Vet Public Health* 41(1-10): 441-452. https://dx.doi.org/10.1111/j.1439-0450.1994.tb00249.x

Ward, C.K. and Inzana, T.J., 1994. Resistance of *Actinobacillus pleuropneumoniae* to bactericidal antibody and complement is mediated by capsular polysaccharide and blocking antibody specific for lipopolysaccharide. *J Immunol* 153(5): 2110.

Wilson, M., Takov, R., Friendship, R., Martin, S., McMillan, I., Hacker, R. and Swaminathan, S., 1986. Prevalence of respiratory diseases and their association with growth rate and space in randomly selected swine herds. *Can Vet J* 50(2): 209-216.

World Healt Organization, 2015. Global Action Plan on Antimicrobial Resistance, World Health Organization. https://apps.who.int/iris/rest/bitstreams/864486/retrieve

WTO, 1995. Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement). Geneva, 1867 U.N.T.S. 493. Available from: https://wto.org

Xu, Z., Zhou, Y., Li, L., Zhou, R., Xiao, S., Wan, Y., Zhang, S., Wang, K., Li, W., Li, L., Jin, H., Kang, M., Dalai, B., Li, T., Liu, L., Cheng, Y., Zhang, L., Xu, T., Zheng, H., Pu, S., Wang, B., Gu, W., Zhang, X.-L., Zhu, G.-F., Wang, S., Zhao, G.-P. and Chen, H., 2008. Genome biology of *Actinobacillus pleuropneumoniae* JL03, an isolate of serotype 3 prevalent in China. *PloS one* 3(1): 1450. https://dx.doi.org/10.1371/journal.pone.0001450

Xu, Z.F., Chen, X.B., Li, L., Li, T.T., Wang, S.Y., Chen, H.C. and Zhou, R., 2010. Comparative genomic characterization of *Actinobacillus pleuropneumoniae*. *J Bacteriol* 192(21): 5625-5636. https://dx.doi.org/10.1128/Jb.00535-10

Yaeger, M.J. and Van Alstine, W.G. 2019. Respiratory System. Diseases of Swine. Zimmermann, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. Hoboken, NJ, John Wiley & Sons, Inc.**:** 393-407.

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F.M. and Larsen, M.V., 2012. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 67(11): 2640-2644. https://dx.doi.org/10.1093/jac/dks261

7 Papers I-III

Cohen *et al. Acta Vet Scand* (2020) 62:35
https://doi.org/10.1186/s13028-020-00529-z

Acta Veterinaria Scandinavica

RESEARCH

Open Access

A descriptive study of acute outbreaks of respiratory disease in Norwegian fattening pig herds

Liza Miriam Cohen[1*], Carl Andreas Grøntvedt[2], Thea B. Klem[2], Stine Margrethe Gulliksen[3], Birgit Ranheim[1], Jens Peter Nielsen[4], Mette Valheim[2] and Camilla Kielland[1]

Abstract

Background: Respiratory diseases are major health concerns in the pig production sector worldwide, contributing adversely to morbidity and mortality. Over the past years there was a rise in reported incidents of respiratory disease in pigs in Norway, despite population wide freedom from Aujeszky´s disease, porcine reproductive and respiratory syndrome, porcine respiratory corona virus and enzootic pneumonia. The main objective of this study was to investigate acute outbreaks of respiratory disease in conventional Norwegian fattening pig herds. The study included 14 herds. In seven herds with reported outbreaks of acute respiratory disease, data on clinical signs was recorded and samples for laboratory examination were collected. Diagnostic protocols were compared by parallel analysis of clinically healthy pigs from seven non-outbreak herds.

Results: The most commonly reported clinical signs were sudden deaths and dyspnea. An average compartment morbidity of 60%, mortality of 4% and case fatality of 9% was recorded in the outbreak herds. Post-mortem examinations revealed acute lesions resembling porcine pleuropneumonia in all 28 pigs investigated from the outbreak herds and in 2 of the 24 (8%) pigs from the non-outbreak herds. Chronic lesions were recorded in another 2 pigs (8%) from the non-outbreak herds. *Actinobacillus pleuropneumoniae* serovar 8 was isolated from lungs and/or pleura from all tested pigs (n = 28) in the outbreak herds, and from 2 out of 24 pigs (8%) in the non-outbreak herds, one pig with an acute and another pig with a chronic infection. No other significant bacterial findings were made. Seroconversion to *A. pleuropneumoniae* antibodies was detectable in all outbreak herds analyzed and in six out of seven non-outbreak herds, but the risk ratio for seroconversion of individual pigs was higher (risk ratio 2.3 [1.50- 3.43 95% CI; $P < 0.001$]) in the outbreak herds. All herds tested positive for porcine circovirus type 2 and negative for influenza A viruses on oral fluid RT-qPCR.

Conclusion: The main etiological pathogen found during acute outbreaks of respiratory disease was *A. pleuropneumoniae* serovar 8. All pigs from outbreak herds had typical lesions of acute porcine pleuropneumonia, and only *A. pleuropneumoniae* serovar 8 was identified. Co-infections were not found to impact disease development.

Keywords: *Actinobacillus pleuropneumoniae*, Acute respiratory disease, Fattening pigs, Outbreak diagnostics

Background

Respiratory diseases give rise to major health concerns in pig populations worldwide. They are believed to contribute adversely to morbidity and mortality, increased use of antimicrobials, poor pig welfare and reduced productivity [1–3]. The direct effect of disease on these parameters

*Correspondence: liza.miriam.cohen@nmbu.no
[1] Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences (NMBU), P.O. Box 8146 Dep, 0033 Oslo, Norway
Full list of author information is available at the end of the article

are however hard to determine in field conditions. Studies show that coinfections with different respiratory agents are common in pigs [4, 5]. Viral infections often predispose for secondary bacterial infections. This has been studied under experimental conditions, i.e. coinfections of porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* [6], PRRSV and *Actinobacillus pleuropneumoniae* [7], swine influenza virus (SIV) and *Bordetella bronchiseptica* [8]. Moderate to marked fever, lethargy, coughing, sneezing and dyspnea are common clinical signs during disease outbreaks [9, 10]. The presence of multiple pathogens often increases the severity of disease and occurrence of lesions in the respiratory tract [8, 11, 12]. There are differences in occurrence and distribution of pathogens between countries, regions and herds [13, 14] that contribute to the complexity of respiratory disease.

Due to strict import regulations in Norway, there is negligible import of live pigs to the commercial pig population [15]. The national yearly yield was approximately 1.6 million slaughtered pigs in 2018, originating mainly from 2580 registered fattener pig herds with a concession limit of maximum 2100 slaughtered pigs per year [16, 17]. The Norwegian pig production is also characterized by stringent regulation of antimicrobial drug use and a tradition of eradicating diseases from animal populations [18, 19]. The commercial pig population in Norway has documented freedom from several important respiratory pathogens including Aujeszky's disease virus, PRRSV, SIV (apart from influenza A [H1N1]pdm09) [20] and *M. hyopneumoniae* [18]. After the pandemic in 2009/2010, antibodies to SIV (H1N1)pdm09 have been detected regularly from 25 to 50% of examined herds in Norway [21], but SIV (H1N1)pdm09 infections in the Norwegian pig population has been considered to have limited clinical impact [22]. In cases of respiratory disease in Norwegian herds, *A. pleuropneumoniae* has regularly been isolated from lungs of carcasses submitted for routine diagnostics [23]. Several studies from other countries conclude that *A. pleuropneumoniae* is normally present in most conventional pig herds, having a main reservoir in the tonsils of carrier pigs [24, 25]. Accordingly, outbreaks in conventional herds are most often triggered by factors related to animal housing, management and environment rather than an introduction of the bacteria in a naïve herd [26]. Preceding infection with a primary viral pathogen is also a possible triggering factor [4]. In the years between 2010 and 2014 there was an increase in reported acute cases of respiratory disease requiring veterinary treatment in Norway [27]. A systematic investigation of porcine respiratory disease outbreaks in Norway has not recently been performed, and updated knowledge is needed for appropriate disease prevention and intervention. The main objective of this study was to investigate clinical outbreaks of acute respiratory disease in Norwegian fattening pig herds, using a group of non-outbreak herds to compare diagnostic procedures.

Methods

Study design

Source population

The source population was the conventional fattening pig herds located in central and southern parts of Norway in the period between September 2017 and October 2018. The conventional herds are not part of the Norwegian Specific Pathogen Free (SPF) sub-population, in which herds are free from e.g. toxin producing *Pasteurella multocida* and all serotypes of *A. pleuropneumoniae*.

Sample population

Seven conventional fattening pig herds with acute outbreaks of respiratory disease (outbreak herds) and seven pig herds without respiratory disease outbreaks (non-outbreak herds) were included in this study.

The inclusion criteria for outbreak herds were; three or more pigs displaying acute signs of respiratory disease including fever and coughing and/or dyspnea, and/or otherwise reduced general condition e.g. lethargy or inappetence. Non-outbreak herds inclusion criteria were; absence of acute clinical signs of respiratory disease at the time of sampling, situated in the same geographical area as the outbreak herds. The non-outbreak herds were not matched to the outbreak herds by means of other parameters. Herds were included only if there were more than three weeks until planned slaughter, due to follow-up sampling per protocol. Two herds were excluded, due to treatment with antimicrobial drugs before sampling could be carried out, and insufficient time from outbreak to planned slaughter, respectively. Descriptive herd data are listed in Table 1.

Recruitment and selection of herds

A network of veterinary practitioners was established to collect samples and herd data. The practitioners were contacted through emails, letters, meetings and announcements in relevant journals and national newspapers. The veterinarians contacted the project group immediately upon being called out to examine pigs with symptoms of acute respiratory disease. Outbreak herds were recruited for participation by the veterinary practitioners after meeting the inclusion criteria. Non-outbreak herds were then recruited by the veterinary practitioners contacting herd owners meeting the matching criteria, asking their participation and arranging a visit. Complete kits containing materials and detailed instructions for sample collection, preservation and transport were

Table 1 Overview of descriptive data in both outbreak and non-outbreak herds (n = 14)

Descriptive herd data	Outbreak herds (n = 7)[a]		Non-outbreak herds (n = 7)[b]	
	Median	Interquartile range	Median	Interquartile range
Production site	Single site production			
No. of suppliers	2	7	1	0
Herd size[c]	650	310	500	350
Yearly yield[d]	2109	1818	1543	1661
Estimations from on-farm registrations:				
Pigs in compartment[e]	155	90	196	255
Compartment volume per pig[f]	3.9 m^3	2 m^3	4.3 m^3	1.5 m^3
Floor space per pig[g]	1.0 m^2	0.2 m^2	1.1 m^2	0.2 m^2

[a] Herd type: 5 finishers, 2 farrow-to-finish. 6 herds: one compartment affected and tested. 1 herd: two compartments affected and tested, compartment average presented

[b] Herd type: 6 finishers, 1 farrow-to-finish. One compartment tested per herd

[c] Number of pigs in the herd/at the production site at the time of the outbreak/sampling

[d] Fattening pigs slaughtered over the last 12 months. Not considering piglets for sale

[e] Number of pigs the compartment with the ongoing outbreak

[f] m^3 in the compartment divided by the number of pigs

[g] total m^2 in the compartment divided by the number of pigs, not considering empty stalls, walkways etc

pre-distributed to designated pick up points at abattoirs and veterinary practice offices and sent to veterinarians across the country upon request.

Herd visits

Each outbreak herd was visited on three occasions (Fig. 1, green boxes); the first visit was conducted as soon as possible during the reported outbreak for initial sampling. The second visit was performed 2 to 5 days later to conduct interviews and register herd demographic data. During the third and final visit two to four weeks after the first, follow-up samples were collected, as described in Fig. 1. Non-outbreak herds were visited on two occasions, once for initial sampling, farmer interviews and herd registrations, and secondly for follow up sampling.

First visit (outbreak sampling)

Details about the diagnostic sampling are shown in Fig. 1. Diagnostic sampling in outbreak herds was performed the day the veterinarian was notified about the disease. The veterinary practitioner reported observed clinical signs on a standardized submission form. In these herds, three to five pigs were selected for organ collection, 28 pigs were sampled in total. The selection was made from pigs with clinical signs of respiratory disease prior to death or euthanasia by captive bolt and exsanguination. Short time from death to sampling was considered, no additional criteria for sampling were applied. In non-outbreak herds three to five pigs were haphazardly selected, 24 pigs were sampled in total. Lungs and mediastinum (including pericardium, excluding the heart) and trachea caudal to the thoracic inlet were collected. Within each herd, care was given not to sample pigs treated with any antimicrobial drugs up to 14 days prior to the sampling.

Blood sampling was performed on a total of 10 pigs per herd by haphazard selection from as many pens in the compartment as possible, up to 10 pens. A total of 141 pigs were sampled. The pigs were selected regardless of clinical presentation and restrained by snaring the upper jaw. During restraint the pigs were ear-tagged for individual identification at follow-up sampling during the final visit. Rectal temperature was measured, and blood samples were collected (details in Fig. 1).

Pooled oral fluid (OF) samples were collected from two haphazardly selected pens (n = 14 pooled OF samples from 28 pens) using chewing rope as described by Prickett et al. [28]. Care was given to keep the stress of the animals during sampling to a minimum.

Second visit (interviews and on-farm demographic data sampling)

Demographic data sampling was obtained by interviewing the farmers using a purpose-built questionnaire, see details in Fig. 1. Relevant information regarding the disease outbreaks including information about the first 5 days after noticing the first clinical signs was registered in

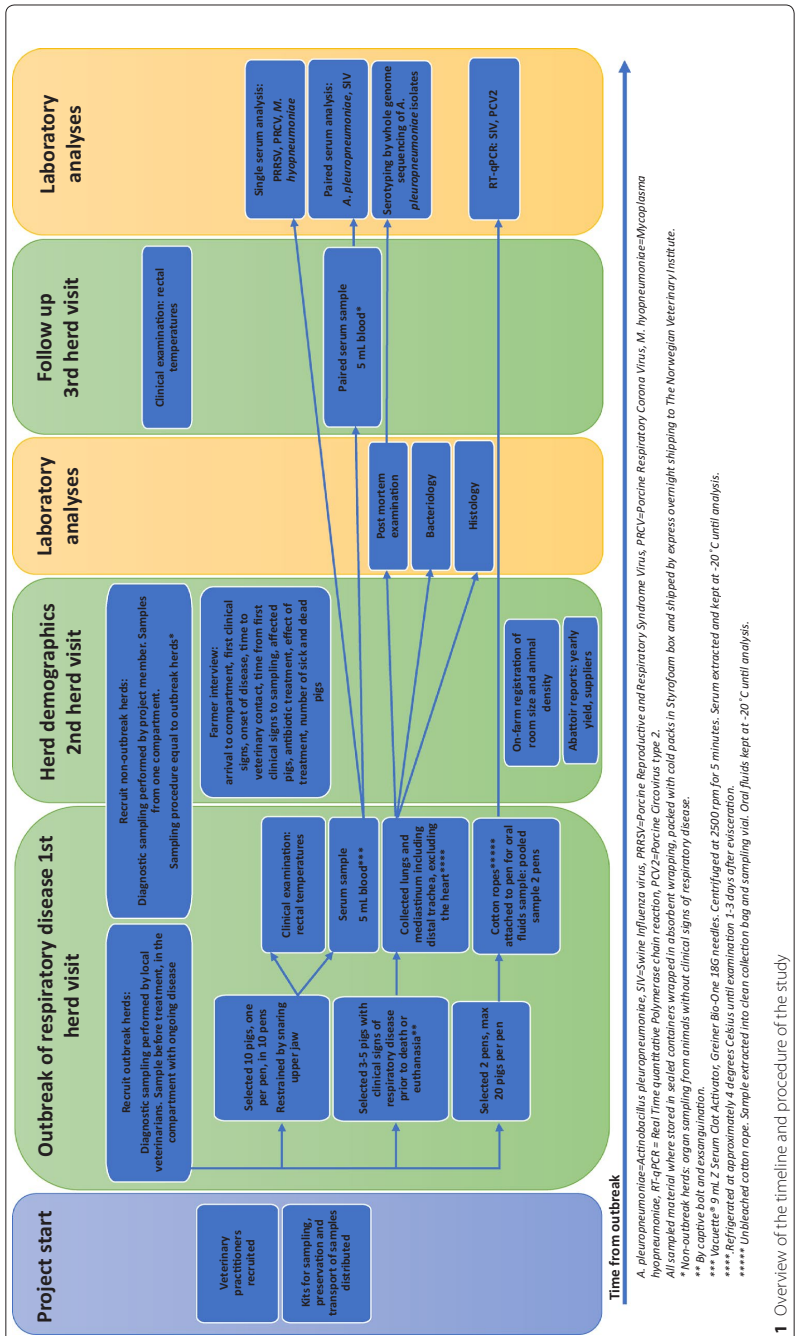

Fig. 1 Overview of the timeline and procedure of the study

outbreak herds. The following data was registered: dates of the pigs' arrival to compartment, a description of earliest observed clinical signs, onset of disease, time to veterinary contact, time from the first clinical signs to the initial sampling, numbers of pigs displaying clinical signs, applied antibiotic treatment, and number of sick and dead pigs from the start of the outbreak until the time of the interview.

Final visit (follow up)

During the final visit, second blood samples were collected from individually ear tagged pigs, and rectal temperature measured in the same pigs.

Sample handling and diagnostics

Procedures for sample handling are presented in Fig. 1.

Pathological examination

Organs from 52 pigs were subject to post-mortem examination. The pericardium, pleura, trachea, bronchi, lung parenchyma and tracheobronchial lymph nodes from 28 to 24 pigs from outbreak herds and non–outbreak herds respectively, were examined at the Norwegian Veterinary Institute (NVI) according to a standardized protocol (Additional file 1). Tissue samples from the lungs, pleura and lymph nodes were fixed, processed, sectioned and stained for histological examination (Additional file 2). In total, 112 histological sections from the outbreak herds and 36 sections from 3 non-outbreak herds were examined following a standardized protocol (Additional file 2).

Bacteriology

Sampling (on charcoal transport swabs) for bacterial cultivation was performed during postmortem examination of 52 lungs and pleurae, see details in Table 3. The lung surface was flamed and aseptically incised before swabbing of lung tissue. The swabs were cultivated as a part of the routine diagnostics at NVI (Additional file 3).

Serotyping by whole genome sequencing

Serovar identification of cultured *A. pleuropneumoniae* (n = 31 isolates) was performed on sequence data, generated through whole genome sequencing of the *A. pleuropneumoniae* isolates at Statens Serum Institut (SSI), Copenhagen, Denmark. The serovar was determined based on the presence of the serovar specific cps operons [29, 30]. Details regarding the method are described in Additional file 3.

Serology

The serum samples (n = 282) were analyzed using commercial diagnostic kits for antibodies to *A. pleuropneumoniae*, influenza A virus, PRRSV, PRCV and *M. hyopneumoniae.* The analyses were performed as described by the manufacturers; details are given in Additional file 3. Interpretation of the test results were categorical, based on the cut-off values recommended by the test manufacturers. Presence of antibodies to PRRSV, porcine respiratory corona virus (PRCV) and *M. hyopneumoniae* were tested in the second serum sample (n = 141). Serum ELISA was conducted on paired serum samples (n = 282) from individual pigs for antibodies to influenza A virus and *A. pleuropneumoniae.*

Virology

The presence of influenza A virus and porcine circovirus type 2 (PCV2) nucleic acids in pooled oral fluids (n = 14) were analyzed with real time polymerase chain reaction (PCR) by in-house procedures (Additional file 3). A cycle threshold (Ct) value for influenza virus below 37 was considered positive. PCV2 quantitative PCR (qPCR) is a quantitative test where results are given as measured nucleotide copies in 200 µL sample, calculated from repeated measures at different Ct values and results are reported as low ($< 10^4$ copies), moderate (10^4–10^7 copies) or high ($> 10^7$ copies).

Statistical analyses

Our sample size of 10 serum samples per herd was chosen based on an estimate of at least one positive animal if the prevalence of our disease in question is around 25% at a 95% confidence level. The same sample size was used for agents not present in the population, that we did not expect to find, due to practical reasons.

Statistical analyses of the data were performed using the software Stata (STATA SE/15 for Windows; Stata Corp., College Station, TX, USA). Descriptive numeric results are presented as average values and the standard deviation (SD) for data with a normal distribution, or median value followed by the interquartile range (iqr) for data that was not normally distributed.

Rectal temperatures from the first visit and from the final visit to the herd were compared. The variable "fever" was defined as a rectal temperature above 39.5 °C. Odds ratios for fever during the outbreak sampling compared to fever during follow-up visits, were calculated using a Stata 15 case–control odds-ratio calculator.

Morbidity was measured as the proportion of pigs with clinical signs of respiratory disease of the total number of pigs in the herd (herd morbidity) and in the compartment (compartment morbidity). Mortality was measured as the proportion of pigs dying during the outbreak, out of the total number of pigs in the herd (herd mortality) and in the compartment (compartment mortality). Case fatality, an indicator of pathogen virulence and disease

lethality, was measured as the proportion of pigs that died during the outbreak and displayed clinical signs of respiratory disease prior to their death, out of the total number of pigs displaying respiratory disease.

A herd was classified as seroconverted if at least one pig shifted from negative to positive status and no pigs shifted from positive to negative status. The proportion of seroconverted pigs in each herd was calculated. Samples from pigs that could not be identified by ear tags (one herd, n = 10) were excluded. When calculating the incidence proportion and risk ratios for seroconversion to *A. pleuropneumoniae*, pigs that were seropositive on the first serum sample were excluded from the population at risk. Incidence proportion was defined as the proportion of the seronegative pigs that seroconvert during the time at risk. Time at risk was defined as time between paired serum samples. The risk ratio (RR) for a pig to seroconvert in outbreak herds, compared to non-outbreak herds, was calculated using a Stata15 Cohort study risk-ratio calculator the 95% confidence interval (CI). The statistical significance of the calculated association, whether it was likely that the RR was different from 1, was indicated by the reported *p* value.

Results

Clinical findings

Median number of days from the farmers noticed clinical signs of respiratory disease until calling the local veterinary practitioner was 1 day. Onset of outbreak was 35 days (median, iqr 43) after the pigs arrived at the compartment. The severity of the clinical signs varied between outbreak herds. Clinical signs reported by the veterinary practitioner were mainly sudden deaths (four herds) and dyspnea (three herds). Signs such as fever, bloody froth from oronasal openings, cough and lethargy were also reported, and it was observed that sick pigs were reluctant to chew on the cotton ropes used for OF sampling.

In all herds, intramuscularly administrated procaine benzylpenicillin was used to treat sick pigs over 3 to 5 days. In one herd, tiamulin was additionally administered in the drinking water for 4 days. Treatments were started by the veterinary practitioner during the first visit after the outbreak of disease. All herd owners reported the treatment to effectively reduce acute clinical signs and stop the further spread of disease.

The average compartment morbidity during the outbreak was 60% (SD 43, range 6–100%), while herd morbidity was 25% (SD 19, range 0.9–51%) in the outbreak herds. Case fatality rate during the disease outbreaks was on average 9% (SD 12, range 0–34%) over 5 days, suggestive of a low virulent agent. During the outbreaks, compartment mortality was 4% on average (SD 3, range 0–10%), while herd mortality was 2% (SD 2, range 0–5%). Proportion of pigs in the outbreak herds measuring a rectal temperature above 39.5 °C was 57.6% (n = 54) and 30% at the first and final visit, respectively. For the non-outbreak herds the proportion of pigs with a rectal temperature above 39.5 °C was 42.4% (n = 50) and 10% at first and final visit, respectively. The odds for a temperature above 39.5 °C were higher (odds ratio = 2.8, 95% CI 1.17–6.70), during outbreak than during follow-up in the outbreak herds. There were no dropouts among the study animals, the number of animals tested at the visits was the same. Median number of days between first and final visit was 22 days (iqr 5) in outbreak herds and 18 days (iqr 4) in non-outbreak herds.

Diagnostics

Pathological examination

Results from the pathological examinations of 52 organs are presented on herd level in Table 2. Gross pathology of the lungs was detected in all pigs (n = 28) from the outbreak herds. Acute pleural lesions were reported in 25 of these pigs (89%) and chronic pleural lesions, were found in one. Typical lesions of acute pneumonia were found in all the pigs. The acute lesions were principally dorsally distributed in all lung lobes, but the caudal lobes were the most affected. Chronic lung lesions were observed in one pig. Moderate to severe enlargement of the tracheobronchial lymph nodes was a prevalent finding (n = 22, 73%) in the pigs with pneumonia. Characteristic gross lung lesions are shown in Fig. 2.

In the non-outbreak herds various gross lung lesions were detected in seven of the 24 pigs (29%). Pleuritis was observed in two of 24 pigs (8%), where one had an acute pleuritis, and the second pig focal chronic pleuritis. Pneumonia was observed in four other pigs. Mild, focal, acute lesions were seen in two of them, while similar acute lesions and abscess formation was seen in another. Multifocal, necrotizing, chronic pneumonia was diagnosed in the fourth pig. A single pig from a non-outbreak herd had gross lung lesions of multifocal bleeding and mottled grayish green areas indicative of larval migration by *Ascaris suum.* Diagnostic results for individual herds, including the gross findings are summarized in Table 2.

Histopathological changes agreed with the acute macroscopic lesions observed. Histological examination revealed fibrin and neutrophil deposits on the pleura. In the lung parenchyma there was alveolar filling with necrotic leukocytes, neutrophils and fibrin. Interstitial edema and hemorrhage, peribronchial and peribronchiolar leukocyte infiltration was observed. Subacute to chronic, necrotic lesions of varying sizes were demarcated by macrophages, lymphocytes and plasma cells

Table 2 Results from gross pathology, bacteriology, serology and virology from seven outbreak herds (from 1 to 7) and seven non-outbreak herds (from 8 to 14)

Herd no.	Number of samples	Pleura gross pathology No. of pigs		Lung gross pathology No. of pigs		Bacteriology[a] No. of pigs (%)		Serology No. of pigs (%)		Virology RT-qPCR ~~on pooled OF sample~~	
	Lungs/serum/OF	Acute lesion[b]	Chronic lesion[c]	Acute lesion[d]	Chronic lesion[e]	APP culture[f]		APP seroconversion[g]	SIV seroconversion	PCV2 detection[h] (category)	SIV detection
						Pleura	Lung				
1	5/10/1	5	–	5	–	4 (80)	5 (100)	3 (30)	–	18,000 (mod)	–
2	3/10/1	3	–	3	1[i]	3 (100)	3 (100)	9 (90)	1(10)[j]	9700 (low)	–
3	5/10/1	4	1	5	–	5 (100)	5 (100)	6 (60)	–	1500 (low)	–
4	3/10/1	2	–	3	–	2 (67)	3 (100)	5 (50)	–	< 10 (low)	–
5	4/10/1	4	–	4	–	4 (100)	4 (100)	10 (100)	–	19,000 (mod)	–
6	5/10/1	5	–	5	–	5 (100)	5 (100)	6 (60)	–	55,000 (mod)	–
7	3/10/1	2	1	3	–	3 (100)	3 (100)	–[k]	–	602 (low)	–
8	3/10/1	1	–	–	–	0	0	4 (40)	–	58,000 (mod)	–
9	3/10/1	–	–	1	1	0	1 (33)	0	–	16,000 (mod)	–
10	4/10/1	–	1	–	–	0	0	2 (20)	–	220,000 (mod)	–
11	4/10/1	–	–	–	–	0	0	1 (10)	–	430,000 (mod)	–
12	3/10/1	–	–	–	–	0	0	8 (80)	–	850,000 (mod)	–
13	4/10/1	–	–	–	–	0	0	4 (40)	–	15,000 (mod)	–
14	3/10/1	–	–	–	1	0	1 (33)	1 (10)	–	5270 (low)	–

OF, oral fluids; APP, *Actinobacillus pleuropneumoniae;* PCV2, Porcine Circovirus type 2; PCR, reverse transcription quantitative polymerase chain reaction; SIV, Swine influenza virus; mod, moderate virus concentration

[a] Swabs from lesions on pleura and lung tissue in right and left, cranial and caudal lobe. If there were no macro-pathological lesions, swabs were collected from pleura, left cranial lobe and right caudal lobe, respectively

[b] Fibrinopurulent pleuritis

[c] Fibrous pleuritis

[d] Demarcated, firm, deep red lung tissue with hemorrhage

[e] Chronic necrotic areas and abscess formation

[f] Growing on lung, pleura and/or pericardium

[g] Proportion of total sample size, in most herds there were seropositive individuals at first sampling, leaving the true population at risk smaller

[h] DNA copies per 200 uL

[i] Acute lesion also present

[j] In this herd, 2 (20%) pigs had a decreasing serum level of SIV antibodies

[k] Unable to identify individual pigs at second sample due to missing unique identifiers

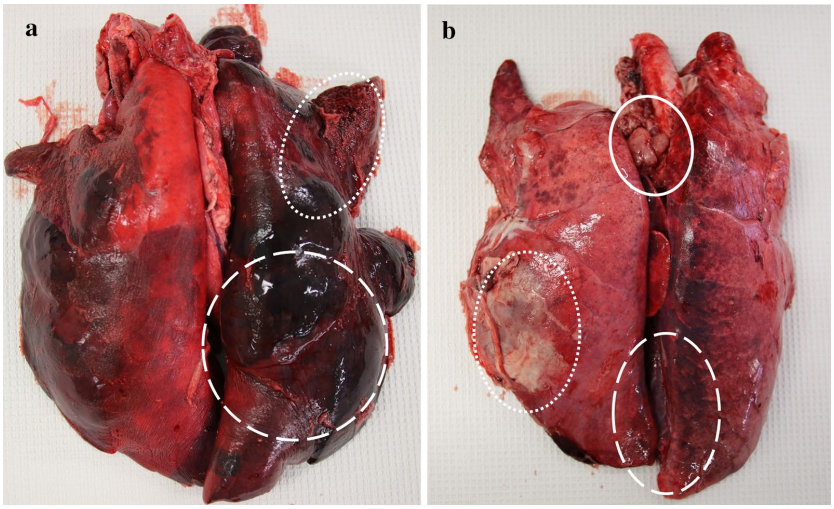

Fig. 2 Lungs from pigs that displayed clinical signs of respiratory disease prior to death. Macroscopic lung lesions. **a.** Acute pleuropneumonia—Macroscopic lesions include fibrinopurulent pleuritis (dotted ring), edematous lungs with multifocal to coalescing hemorrhagic and hyperemic areas (dashed ring). *A. pleuropneumoniae* was cultured in abundance from pleura, left and right caudal lung lobes. **b** Chronic pleuritis, acute pneumonia. Macroscopic lesions include fibrous pleuritis (dotted ring), and focal, dark red areas of condensed lung tissue (dashed ring). The tracheobronchial lymph nodes were moderately enlarged (continuous ring). *A. pleuropneumoniae* was cultured from the right caudal lung lobe; no bacteria were retrieved from the chronic pleuritic lesion

Table 3 Summary of bacteriologic findings on at least one sample from lung and pleura in 28 pigs from outbreak herds and 24 pigs from non-outbreak herds

Microbial species	Outbreak herds[a]		Non-outbreak herds[a]	
	Lung n = 28	Pleura n = 28	Lung n = 24	Pleura n = 24
Actinobacillus pleuropneumoniae	28[b]	26[c]	2[d]	0
Pasteurella multocida	1	3	–	–
Streptococcus spp.	5	10	–	–
Haemophilus parasuis	–	–	–	–
Bordetella bronchiseptica	–	–	1	–
Coliform bacteria	2	2	–	–
Other: *Actinomyces hyovaginalis, Trueperella pyogenes, Proteus mirabilis, Fusobacterium* spp.	3	–	1	–
Unspecific mixed culture	–	1	4	1

[a] In total, 28 pleural swabs and 52 lung swabs were collected in outbreak herds, while 24 pleural and 49 lung swabs were collected in non-outbreak herds. The sum of findings per lung or pleura are presented here

[b] From swabs of acute lung lesions. *A. pleuropneumoniae* was the sole microbial species detected in lung samples from 20 of these pigs

[c] From swabs of pleuritic lesions. *A. pleuropneumoniae* was cultured from 26 pleuritic lesions. *A. pleuropneumoniae* was the sole microbial species detected in pleura samples from 13 of these pigs

[d] From swabs of lung lesions. *A. pleuropneumoniae* was the sole microbial species detected in one lung with chronic lesions, while the other was a mixed culture from an acute lesion

surrounded by connective tissue. Histopathological pathological changes in lymph nodes included neutrophils in subcapsular sinuses in cases of acute pneumonia. When investigating samples from lungs without gross lesions from the non-outbreak herds there was occasional occurrence of mononuclear cell infiltrates and fibrin deposits

on the pleura or in alveolar lumen, and areas of interstitial bleeding.

Bacteriology

Actinobacillus pleuropneumoniae was cultured from all sampled pigs (n = 28) from outbreak herds (n = 7). Abundant growth of *A. pleuropneumoniae* was present in lung tissue in all 28 pigs and on pleura in 26 pigs. In samples from 20 of the lungs and 13 pleurae, *A. pleuropneumoniae* was the sole microbial species detected. In the remaining samples, a range of bacteria were detected in addition to *A. pleuropneumoniae* and the results are shown in Table 3. Swabs from non-outbreak pigs' lungs produced mostly negative bacteriology. From non-outbreak herds, *A. pleuropneumoniae* was isolated from lung parenchyma in two out of 24 pigs. The *A. pleuropneumoniae* isolates originated mainly from areas with acute gross pathology (Table 2). In one non-outbreak pig *A. pleuropneumoniae* was cultured from a chronic lung lesion.

Serotyping of *A. pleuropneumoniae* on genome level revealed that all sampled isolates belonged to serovar 8.

Serology

The 282 serum samples were successfully analyzed in one session. Antibodies to *A. pleuropneumoniae* were detected in samples from six (86%) outbreak herds and four (57%) non outbreak herds. At the first serum sample, 35% (25 of 71) of the pigs in the outbreak herds were seropositive, and 37% (26 of 70) in the non-outbreak herds. At the second serum sample, 89% (63 of 71) and 60% (42 of 70) of the pigs were positive in the outbreak and non-outbreak herds respectively, details are listed in Table 2. Six outbreak herds and six non-outbreak herds were considered seroconverted, indicative of an active infection in the period from the first to the second visit. Seroconversion in the seventh outbreak herd could not be assessed due to missing ear tags. Proportion of seroconverted pigs in each outbreak herd ranged from 30 to 100%, and from 0 to 80% in non-outbreak herds (Table 2). Incidence proportion was 0.96 (SD 0.10) in outbreak herds over the median time at risk of 22 days. Incidence proportion in the non-outbreak herds was 0.44 (SD 0.36) over the median time at risk of 18 days. The risk for seroconversion was more than double compared to pigs from non-outbreak herds (RR 2.3 [1.50–3.43 95% CI; $P < 0.001$]).

Antibodies to influenza A virus were detected in one outbreak herd, where one pig seroconverted during the sampling period, and two pigs were found to have a reduced antibody titer to below cutoff. Influenza A-antibodies were not detected in the remaining six outbreak herds or the non-outbreak herds. The proportion of SIV seropositive herds was 7% out of the herds combined. Antibodies to *M. hyopneumoniae*, PRCV and PRRSV were not detected in samples from any herds.

Virology

The 14 pooled OF samples from 28 pens, median number of pigs per pen was 10 (range 5–19), were all negative for Influenza A Viruses. Quantification of PCV2 by RT-qPCR turned out low or moderate in all samples, results per herd are shown in Table 2.

Discussion

Field outbreaks of acute respiratory disease in Norwegian fattening pigs were investigated and *A. pleuropneumoniae* serovar 8 was the main pathogen detected, with negligible presence of co-infections. Clinical signs reported were in agreement with previous reports of *A. pleuropneumoniae* infections, which are described to have a diverse clinical presentation [31]. Even with the large variation in morbidity and mortality rates, the results from this study were in line with observations from other studies, as research on outbreak characteristics of respiratory disease show that morbidity can range from 10 to 100% [26]. Mortality during outbreaks of acute porcine pleuropneumonia is usually reported to be between 1 and 10% [26]. Case fatality rates are not commonly included in this research literature but is a more precise measure of the lethality of a disease, especially if little information about other illnesses is available. Disease that affects mortality are likely to have common risk factors [32] and the use of case fatality rate is a more robust measurement and less subjected to confounders such as that of other illnesses.

Even as a single infectious primary agent, *A. pleuropneumoniae* can cause severe clinical signs. During acute porcine pleuropneumonia, high fever is common [33, 34]. For pigs in the age range from 3 to 6 months, body temperatures normally span from 38.5 to 39.3 °C [35], and the proportion of pigs displaying a fever can be indicative of an outbreak. In the present study, the pigs were restrained by snaring the upper jaw during clinical examination and blood collection, which is stressful for the animal [36]. The cutoff for fever at 39.3 °C + 0.2 was used in the study to compensate for this stress. Higher odds for displaying fever in the herds during outbreak than at the final visit were found among the pigs in this study. This signified body temperature as a disease characteristic during outbreaks of porcine pleuropneumonia, although technical biases like personnel and thermometers used might have influenced our results. This coincided with results from a recent study from Finland [37].

There are 18 acknowledged *A. pleuropneumoniae* serovars, of which some were recently described [38]. From

the Norwegian pig population, serovars 2, 6, 7, 8 and 10 have previously been reported [39]. Serovar 8 has been most commonly associated with clinical disease in recent years, followed by type 6 [40]. However, these previous findings were all based on antibody agglutination tests which are prone to cross-reactions, for instance between serovars 3, 6, 8 and 15 [41]. All *A. pleuropneumoniae* strains in this study belonged to type 8, raising questions about the importance of serovar 6. Underestimation of serovar 8 has occurred in Canada [42], England and Wales [43]. Serovar 8 is typically viewed as low virulent and is less often associated with clinical disease globally. In a study describing clinical presentation of different serovars in experimentally infected pigs [33], serovars that were less commonly associated with disease were able to produce severe clinical signs, including high fever. This could perhaps be a result of absence of other respiratory agents including more virulent serovars of *A. pleuropneumoniae*.

The macro- and histopathologic findings were typical for acute pleuropneumonia caused by *A. pleuropneumoniae* [44–46], supporting that *A. pleuropneumoniae* was the main etiologic pathogen in these outbreaks. Direct agent detection, primarily by bacteriological culturing in affected lung tissue obtained during necropsy, is considered the most adequate method for diagnosing porcine pleuropneumonia [31]. Direct PCR is a method that would be expected to yield similar results but would not allow for storing of the bacterial isolates for further molecular testing, as was done in this study. We observed a low incidence of pathological lesions in non-outbreak herds, and *A. pleuropneumoniae* was only isolated from lesions resembling porcine pleuropneumonia. Other bacteria, including *P. multocida* and *Streptococcus* spp., were also detected in a few samples in this study. Both are known opportunistic bacteria that colonize the upper respiratory tract of healthy pigs [4]. *Streptococcus suis* is the most important streptococcal swine pathogen found to contribute to bronchopneumonia [47]. It is not unlikely that the bacteria could colonize areas already infected with *A. pleuropneumoniae*. The lesions might then be hard to distinguish from the primary pathogen, particularly if large parts of the lungs are affected. In one outbreak herd all five lungs had growth of other bacteria. They could have been contaminated during collection, transport or sampling. Alternatively, these pigs were all colonized by secondary bacterial pathogens. The number of herds included in this study was too low to investigate whether the presence of these bacteria was linked to any differences in outbreak characteristics or diagnostic results. The low occurrence of common secondary invaders could have been explained by the short time span between registered disease and sampling. It has been questioned whether the actions that led to the eradication of *M. hyopneumoniae* from the Norwegian pig population [18] also significantly reduced the occurrence of other pathogens. This has not yet been investigated.

Treatment with procaine benzylpenicillin was in line with the therapeutic guidelines published by the Norwegian Medicines Agency as the drug of choice for acute porcine pleuropneumonia [48]. Similar recommendations have been published in Finland and Sweden [49, 50]. In Denmark, tilmicosin and tulathromycin have been commonly used against acute pleuropneumonia [51] partly due to the convenience of peroral administration, not due to reduced susceptibility to benzylpenicillin. National surveillance programs for antimicrobial resistance in these countries have recently reported a high proportion of *A. pleuropneumoniae* isolates being susceptible to benzylpenicillin [51–53]. Nevertheless, there are no recently published studies on the efficacy of procaine benzylpenicillin for porcine pleuropneumonia in Norway. Such knowledge of causative pathogens is the fundament for correct and prudent use of antimicrobials. The details to antimicrobial resistance patterns of *A. pleuropneumoniae* in Norway are currently being studied further.

Seroconversion to *A. pleuropneumoniae* had occurred in most of the herds, in many cases in absence of clinical disease. The risk for seroconversion to *A. pleuropneumoniae* for pigs in outbreak herds was more than double compared to pigs from non-outbreak herds, despite small within-herd populations at risk due to many seropositive pigs in the first serum samples. Seroconversion to less virulent strains might have happened without resulting in a cross-protection to the outbreak-causing serovar. In Finland, *Haimi-Hakala* et al. observed no difference in either prevalence of seroconverted herds or proportion of seroconverted pigs per herd in the outbreak case group and non-outbreak control group [37]. They discuss that neither single or paired serum sampling for the diagnosis of acute respiratory disease in field conditions is of much value due to both a lack of details concerning the initiation time of infection and a high prevalence of subclinical infections with *A. pleuropneumoniae*. The risk for seroconversion was not addressed in their paper. A Danish study from 2004 investigated correlations in seroconversion to *A. pleuropneumoniae* and concluded that variation in seroconversion was mainly explained by a common batch level factor, that varies between farms and batches within a farm [54]. Outbreaks of disease might be viewed as a batch level factor in this sense. In cases of all-in-all-out rearing by compartment, which is common, batches of pigs are usually housed separately. As we observed, the outbreaks were often restricted to single compartments. Risk factors can be related to animal

housing, management and environment [26], and infection pressure might be increased during clinical disease and is a likely trigger for seroconversion. Risk factor analyses were beyond the scope of this paper due to a lower number of herds in our study than what was expected. The seeming decrease in outbreak occurrence might have resulted from of a collective effort in the Norwegian pig production system to increase the health status of herds with reoccurring problems with respiratory disease prior to our sampling.

When investigating SIV antibody titers we found that only one outbreak herd was seropositive. Even though one pig seroconverted during the sampling period, two pigs were found to have reduced antibody titer. Since a single false-positive serological reactor could not be excluded, the true status of these animals was uncertain. There being multiple false-positive reactions in one herd, which would have been the case here, was perhaps less likely. The proportion of seropositive herds in this study was less than what is found on a national level, where approximately 25% of the herds are reported positive [21]. The virology results from our study suggested that neither SIV nor PCV2 contributed to the disease outbreaks in the study population. The absence of SIV in all OF samples supported the lack of pathological lesions and serological results indicative of SIV infection. No difference was detected in PCV2 levels between the outbreak- and the non-outbreak herds. Reluctancy of sick pigs to chew on the ropes could have resulted in unrepresentative PCV2 levels. Since PCV2 levels was tested on pooled samples we have no information on the individual pig's contribution to the sample.

The health status of the Norwegian pig population is very good and have many similarities to the one of Finland in the sense that they are free from *M. hyopneumoniae,* PRRSV and until recently PRCV [21]. In Finland, a more diverse outbreak etiology has been observed [37]. In the Finnish study, *A. pleuropneumoniae* was found to be the most likely cause of disease in 14 of the 20 sampled herds. In most of these herds, *A. pleuropneumoniae* was the only etiologic pathogen identified. Similarly, 16 outbreaks of respiratory disease were studied in the Netherlands [10] concluding that five of these were most likely caused by *A. pleuropneumoniae*, while seven were caused by SIV (H1N1) and (H3N2). Like in our study, they did not find any clear evidence of specific dual infections.

Conclusion

The main etiological pathogen of acute outbreaks of respiratory disease in the included Norwegian fattening pigs was *A. pleuropneumoniae*. All pigs from outbreak herds were found to have typical lesions of acute porcine pleuropneumonia, and only *A. pleuropneumoniae* serovar 8 was identified. The clinical presentation and pathology of *A. pleuropneumoniae* was in line with previous reports on field outbreaks internationally. Co-infections did not seem to be of impact on disease development.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13028-020-00529-z.

Additional file 1. Protocol for postmortem sampling. A scheme for a standardized postmortem evaluation and sampling of pigs' lungs. The scheme was compiled at the pathology department at The Norwegian Veterinary Institute to be used in the study of acute respiratory disease outbreaks.

Additional file 2. Histology protocol. A scheme for a standardized histologic evaluation of sections from pigs' lungs, pleura and tracheobronchial lymph nodes, including a description of section preparation. The scheme was compiled by members of the project group Grisefine lunger to be used in the study of acute respiratory disease outbreaks.

Additional file 3. Details of sample handling and diagnostics. A document containing extended details of sample handling and laboratory diagnostic methods performed in the study of acute respiratory disease outbreaks.

Acknowledgements
We would like to thank Professor Mari Heinonen at the University of Helsinki and Tijs Tobias (DVM, Ph.D., MSc) for their advice and contributions to the planning of this study.

At the Norwegian Veterinary Institute several people at different departments have contributed significantly to the diagnostic work that is presented in this study. We would especially like to thank pathologists Øyvor Kolbjørnsen and Helene Wisløff, bacteriologist Marianne Gilhuus, molecular biologist Torfinn Moldal.

In preparation for the field trial, there had been a substantial marketing campaign for the project, reaching out to pig farmers, veterinarians and farm advisors throughout the country. We would like to thank everyone involved in the sampling and data collection for their time and attention.

Prior publication
Results presented in this article have not been previously published.

Authors' contributions
LMC, CAG, TBK, SMG, BR and CK planned this study, LMC, CK and BR performed the outbreak investigations/collected materials. LMC, MV and CK analyzed and interpreted the data. LMC and CK prepared the tables and figures. LMC had the primary responsibility of writing and revising the manuscript. All authors contributed to revising the manuscript. All authors read and approved the final manuscript.

Funding
We are grateful for the funding provided by the Agricultural Agreement Research Fund and the Foundation for Research Levy on Agricultural Products, grant number NFR-267422. Thanks to our collaborators who also funded the research: Animalia, Nortura and KLF. The Norwegian Veterinary institute covered all costs related to administration of sampling equipment and sample analysis for this study.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval
To be able to perform sampling from live animals, the Norwegian Food Safety Authority approved the study design for 'Grisefine lunger' in September 2017, maintaining compliance of ethical guidelines and the three R's. FOTS Norwegian Food Safety Authority reference ID 13185.

Consent for publication
Consent for publication has been given through a signed statement for participation by the recruited herd owners.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences (NMBU), P.O. Box 8146 Dep, 0033 Oslo, Norway. [2] Norwegian Veterinary Institute, P.O. Box 750 Sentrum, 0106 Oslo, Norway. [3] Animalia, P.O. Box 396, Økern, 0513 Oslo, Norway. [4] Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Grønnegårdsvej 2, 1870 Frederiksberg C, Denmark.

Received: 13 December 2019 Accepted: 11 June 2020
Published online: 24 June 2020

References

1. van Alstine WG. Respiratory System. In: Zimmermann JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of Swine. 10th ed. Hoboken NJ: Blackwell Scientific; 2012. p. 348–62.
2. Straw BE, Shin SJ, Yeager AE. Effect of pneumonia on growth rate and feed efficiency of minimal disease pigs exposed to *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae*. Prev Vet Med. 1990;9:287–94.
3. Jager HJ, McKinley TJ, Wood JL, Pearce GP, Williamson SM, Woodger N, et al. A tool to assess the economic impact of pleurisy in slaughter pigs. In: Proceedings of the 21st International Pig Veterinary Society Congress; 2010 July 18-21; Vancouver, Canada. http://www.theipvs.com/wp-content/uploads/2019/07/IPVS-2010-Final-Proceedings.pdf.
4. Brockmeier SL, Halbur PG, Thacker EL. Porcine Respiratory Disease Complex. In: Brogden KA, Guthmiller JM, editors. Polymicrobial Diseases. Washington (DC): ASM Press; 2002.
5. Palzer A, Ritzmann M, Wolf G, Heinritzi K. Associations between pathogens in healthy pigs and pigs with pneumonia. Vet Rec. 2008;162:267–71.
6. Thacker EL, Halbur PG, Ross RF, Thanawongnuwech R, Thacker BJ. *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. J Clin Microbiol. 1999;37:620–7.
7. van Dixhoorn IDE, Reimert I, Middelkoop J, Bolhuis JE, Wisselink HJ, Koerkamp PWGG, et al. Enriched housing reduces disease susceptibility to co-infection with Porcine Reproductive and Respiratory Virus (PRRSV) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) in young pigs. PLoS ONE. 2016;11:9.
8. Loving CL, Brockmeier SL, Vincent AL, Palmer MV, Sacco RE, Nicholson TL. Influenza virus coinfection with *Bordetella bronchiseptica* enhances bacterial colonization and host responses exacerbating pulmonary lesions. Microb Pathog. 2010;49:237–45.
9. Bochev I. Porcine respiratory disease complex (PRDC): a review I Etiology, epidemiology, clinical forms and pathoanatomical features. Bulg J Vet Med. 2007;10:131–46.
10. Loeffen WLA, Kamp EM, Stockhofe-Zurwieden N, van Nieuwstadt APKMI, Bongers JH, Hunneman WA, et al. Survey of infectious agents involved in acute respiratory disease in finishing pigs. Vet Rec. 1999;145:123–9.
11. Pomorska-Mól M, Dors A, Kwit K, Kowalczyk A, Stasiak E, Pejsak Z. Kinetics of single and dual infection of pigs with swine influenza virus and *Actinobacillus pleuropneumoniae*. Vet Microbiol. 2017;201:113–20.
12. Thacker EL, Thacker BJ, Janke BH. Interaction between *Mycoplasma hyopneumoniae* and swine influenza virus. J Clin Microbiol. 2001;39:2525–30.
13. Hansen MS, Pors SE, Jensen HE, Bille-Hansen V, Bisgaard M, Flachs EM, et al. An investigation of the pathology and pathogens associated with porcine respiratory disease complex in Denmark. J Comp Pathol. 2010;143:120–31.
14. Kim J, Chung HK, Chae C. Association of porcine circovirus 2 with porcine respiratory disease complex. Vet J. 2003;166:251–6.
15. KOORIMP, KIF. Annual report 2018 KOORIMP and KIF [In Norwegian]. Animalia. 2019. https://www.animalia.no/contentassets/7b27e28ef6bf4e878416cc6664a440e1/koorimp-arsmelding-2018-web.pdf. Accessed 12 Des 2019.
16. Regulation on swine and poultry production [in Norwegian]. FOR-2004-04-01-611 (2004). Available from: https://lovdata.no/dokument/SF/forskrift/2004-04-01-611.
17. Animalia. Annual statistical report 2018 [in Norwegian]. 2019. https://www.animalia.no/contentassets/28e0db72674d496186f0570a9e606fca/arsstatistikk-2018.pdf. Accessed 01 Nov 2019.
18. Gulliksen SM, Lium B, Framstad T, Jørgensen A, Skomsøy A, Gjestvang M, et al. The successful eradication of *Mycoplasma hyopneumoniae* from Norwegian pig herds – 10 years later. In: Abstract book of the 11th European Symposium of Porcine Health Management; 2019 May 22-24; Utrecth, The Netherlands. https://www.esphm2019.org/download/2569/.
19. Animalia. The Norwegian livestock industry´s joint action plan on antimicrobial resistance. 2017. https://www.animalia.no/contentassets/05c57591f69d4e1da9bb5c44668bd0c1/eng_husdyrnaringas-hplan-amr-endelig-enkeltsider_220617.pdf. Accessed 16 Sept 2019.
20. Grøntvedt CA, Er C, Gjerset B, Hauge AG, Brun E, Jørgensen A, et al. Influenza A(H1N1)pdm09 virus infection in Norwegian swine herds 2009/10: the risk of human to swine transmission. Prev Vet Med. 2013;110:429–34.
21. Grøntvedt CA, Nordstoga AB, Jonsson ME. The surveillance programme for specific viral infections in swine herds in Norway 2018. The Norwegian Veterinary Institute. 2019. https://www.vetinst.no/en/surveillance-programmes/viral-infections-in-swine/_/attachment/download/e5793dca-57d2-41eb-bd34-9a99e5d11b98:ef9892272432bafa4b61bc30ae1130952606075b/2019_OK_Viral%20infections%20in%20swine_Report%202018.pdf. Accessed 07 Nov 2019.
22. Er C, Lium B, Tavornpanich S, Hofmo PO, Forberg H, Germundsson Hauge A, et al. Adverse effects of Influenza A(H1N1)pdm09 virus infection on growth performance of Norwegian pigs—a longitudinal study at a boar testing station. BMC Vet Res. 2014;10:284.
23. Grøntvedt CA. In: The Norwegian Veterinary Institutes activity report 2016 [in Norwegian]. The Norwegian Veterinary Institute. 2017. Available from: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2017/veterinaerinstituttets-faglige-aktivitetsrapport/_/attachment/download/ae016944-69b3-46f1-924d-8df8f82eca8e:94edd0c45b069ed56bfbb15e35c82059556bb988/2017_20_Veterin%C3%A6rinstituttets%20faglige%20aktivitetsrapport%202016%20-%20komplett.pdf. p. 12-13. Accessed 16 Sep 2019.
24. Vigre H, Angen O, Barfod K, Lavritsen DT, Sorensen V. Transmission of *Actinobacillus pleuropneumoniae* in pigs under field-like conditions: emphasis on tonsillar colonisation and passively acquired colostral antibodies. Vet Microbiol. 2002;89:151–9.
25. Chiers K, Donne E, Van Overbeke I, Ducatelle R, Haesebrouck F. *Actinobacillus pleuropneumoniae* infections in closed swine herds: infection patterns and serological profiles. Vet Microbiol. 2002;85:343–52.
26. Klinkenberg D, Tobias TJ, Bouma A, van Leengoed LA, Stegeman JA. Simulation study of the mechanisms underlying outbreaks of clinical disease caused by *Actinobacillus pleuropneumoniae* in finishing pigs. Vet J. 2014;202:99–105.
27. Gulliksen SM. Respiratory disease in pigs—an increasing problem? [in Norwegian]. Go'mørning. 2016;29:8–9.
28. Prickett JR, Kim W, Simer R. Oral-fluid samples for surveillance of commercial growing pigs for porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 infections. J Swine Health Prod. 2008;16:86–91.
29. Bosse JT, Li Y, Fernandez Crespo R, Lacouture S, Gottschalk M, Sarkozi R, et al. Comparative sequence analysis of the capsular polysaccharide loci of *Actinobacillus pleuropneumoniae* serovars 1-18, and development of two multiplex PCRs for comprehensive capsule typing. Vet Microbiol. 2018;220:83–9.
30. Bosse JT, Chaudhuri RR, Li Y, Leanse LG, Fernandez Crespo R, Coupland P, et al. Complete Genome Sequence of MIDG2331, a Genetically Tractable Serovar 8 Clinical Isolate of *Actinobacillus pleuropneumoniae*. Genome Announc. 2016;4:1.
31. Sassu EL, Bosse JT, Tobias TJ, Gottschalk M, Langford PR, Hennig-Pauka I. Update on *Actinobacillus pleuropneumoniae*-knowledge, gaps and challenges. Transbound Emerg Dis. 2017;65(Suppl 1):72–90.
32. Maes DG, Duchateau L, Larriestra A, Deen J, Morrison RB, de Kruif A. Risk factors for mortality in grow-finishing pigs in Belgium. J Vet Med B Infect Dis Vet Public Health. 2004;51:321–6.

33. Costa G, Oliveira S, Torrison J, Dee S. Evaluation of *Actinobacillus pleuropneumoniae* diagnostic tests using samples derived from experimentally infected pigs. Vet Microbiol. 2011;148:246–51.
34. Tobias TJ, Bouma A, Klinkenberg D, Daemen AJ, Stegeman JA, Wagenaar JA, et al. Detection of *Actinobacillus pleuropneumoniae* in pigs by real-time quantitative PCR for the apxIVA gene. Vet J. 2012;193:557–60.
35. Dewey CE, Straw BE. Herd evaluation. In: Zimmermann JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, Zhang J, editors. Diseases of Swine. 11th ed. Hoboken NJ: John Wiley & Sons, Inc.; 2019. p. 3–16.
36. Parrott RF, Lloyd DM. Restraint, but not frustration, induces prostaglandin-mediated hyperthermia in pigs. Physiol Behav. 1995;57:1051–5.
37. Haimi-Hakala M, Halli O, Laurila T, Raunio-Saarnisto M, Nokireki T, Laine T, et al. Etiology of acute respiratory disease in fattening pigs in Finland. Porcine Health Manag. 2017;3:19.
38. Bosse JT, Li Y, Sarkozi R, Fodor L, Lacouture S, Gottschalk M, et al. Proposal of serovars 17 and 18 of *Actinobacillus pleuropneumoniae* based on serological and genotypic analysis. Vet Microbiol. 2018;217:1–6.
39. Falk K, Lium BM, Ødegaard Ø. Occurrence of lung lesions and antibodies to serotypes 2 and 6 of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* in 5176 slaughter weight pigs from 113 elite herds in Norway. In: Proceedings of the 11th International Pig Veterinary Society Congress; 1990 July 1-5. Lusanne; Switzerland.
40. Norwegian Veterinary Institute. Respiratory diseases [in Norwegian]. 2014. http://multiconsult.eurest.no/nor/Temasider/Svin/Fakta-om-svinesykdommer/LuftveislidelserMycoplasma-hyorhinis.html. Accessed 06 Aug 2019.
41. Mittal KR, Higgins R, Lariviere S. Quantitation of serotype-specific and cross-reacting group-specific antigens by coagglutination and immunodiffusion tests for differentiating *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* strains belonging to cross-reacting serotypes 3, 6, and 8. J Clin Microbiol. 1988;26:985–9.
42. Gottschalk M. The challenge of detecting herds sub-clinically infected with *Actinobacillus pleuropneumoniae*. Vet J. 2015;206:30–8.
43. Li Y, Bosse JT, Williamson SM, Maskell DJ, Tucker AW, Wren BW, et al. *Actinobacillus pleuropneumoniae* serovar 8 predominates in England and Wales. Vet Rec. 2016;179:276.
44. Gottschalk M, Broes A. Actinobaccilosis. In: Zimmermann JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of Swine. 11th ed. Hoboken NJ: John Wiley & Sons, Inc.; 2019. p. 749–66.
45. Lium B, Falk K. An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. I. Prevalence and morphological description of gross lung lesions. Acta Vet Scand. 1991;32:55–65.
46. Falk K, Lium B. An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. III. Serological findings and their relationship to pathomorphological and microbiological findings. Acta Vet Scand. 1991;32:79–88.
47. Gottschalk M, Segura M. Streptococcosis. In: Zimmermann JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of Swine. 11th ed. Hoboken NJ: John Wiley & Sons, Inc.; 2019. p. 934–50.
48. Norwegian Medicines Agency. Therapy recommendations: Use of antimicrobial drugs in production animals [in Norwegian]. 2012. https://legemiddelverket.no/Documents/Veterin%C3%A6rmedisin/Terapianbefalinger/Terapianbefaling_bruk%20av%20antibakteriellt%20midler%20til%20produks.pdf. Accessed 12 Dec 2019.
49. Evira, Finnish Food Safety Authority. Recommendations for the use of antimicrobials in the treatment of the most significant infectious and contagious diseases in animals. 2018. https://www.ruokavirasto.fi/globalassets/viljelijat/elaintenpito/elainten-laakitseminen/hallittu_laakekekaytto/mikrobilaakekaytonperiaatteet/mikrobilaakkeiden_kayttosuositukset_en.pdf. Accessed 12 Dec 2019.
50. Swedish medical products agency. Antimicrobial drug dosage for pigs—new recommendations [in Swedish]. Information från Läkemedelsverket. 2012;23. Available from: https://lakemedelsverket.se/upload/halso-och-sjukvard/behandlingsrek-vet/Rek%20-%20dosering%20av%20antibiotika%20till%20gris%5b1%5d.pdf. Accessed 16 Sept 2019.
51. Statens Serum Institut, National Food Institute. DANMAP 2018 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark 2019. 2019. https://www.danmap.org/-/media/arkiv/projekt-sites/danmap/danmap-reports/danmap-2018/danmap_2018.pdf?la=en. Accessed 20 Sept 2019.
52. Nykäsenoja S, Olkkola S, Pohjanvirta T, Biström M, Kaartinen L, Helin-Soile-vaara H, *et. al.* FINRES-Vet 2018: Finnish Veterinary Antimicrobial Resistance Monitoring and Consumption of Antimicrobial Agents. Finnish Food Authority publications. 2019;6. https://helda.helsinki.fi/bitstream/handle/10138/307519/finres_vet_2018_141119.pdf?sequence=1&isAllowed=y. Accessed 12 Dec 2019.
53. Public Health Agency of Sweden and National Veterinary Institute. Swedres-Svarm 2017: Consumption of antibiotics and occurrence of resistance in Sweden. 2018. https://www.folkhalsomyndigheten.se/contentassets/2ec8ee5ab1674c75beec834ff903ec43/swedres-svarm-2017-18003.pdf. Accessed 12 Dec 2019.
54. Vigre H, Dohoo IR, Stryhn H, Busch ME. Intra-unit correlations in seroconversion to *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae* at different levels in Danish multi-site pig production facilities. Prev Vet Med. 2004;63:9–28.

Publisher's Note

Protocol for post mortem sampling. *A scheme for a standardized post mortem evaluation and sampling of pigs' lungs. The scheme was compiled at the pathology department at The Norwegian Veterinary Institute to be used in the study of acute respiratory disease outbreaks.*

Journal number **Case/control**	**Lung no.1**	**Lung no. 2**	**Lung no. 3**	**Lung no. 4**	**Lung no. 5**
Pericarditis Type + sample for bacteriology (If no pleuritis)	0/ fibrin-pur/ fibrous + bact	0/ fibrin-pur/ fibrous + bact	0/ fibrin-pur/ fibrous + bact	0/ fibrin-pur/ fibrous + bact	0/ fibrin-pur/ fibrous + bact
Pleuritis Type and distribution + sample for bacteriology	0 / fibrin-pur / fibrous R cran-lobe/ caud-lobe L cran-lobe / caud-lobe + bact	0 / fibrin-pur / fibrous R cran-lobe/ caud-lobe L cran-lobe / caud-lobe + bact	0 / fibrin-pur / fibrous R cran-lobe/ caud-lobe L cran-lobe / caud-lobe + bact	0 / fibrin-pur / fibrous R cran-lobe/ caud-lobe L cran-lobe / caud-lobe + bact	0 / fibrin-pur / fibrous R cran-lobe/ caud-lobe L cran-lobe / caud-lobe + bact
Take overview photo	Dorsal and ventral	Dorsal and ventral	Dorsal and ventral	Dorsal and ventral	Dorsal and ventral
Tracheitis/bronchitis Type + sample for bacteriology	0 / fibrin -pur + bact	0 / fibrin-pur + bact	0 / fibrin-pur + bact	0 / fibrin-pur + bact	0 / fibrin-pur + bact
Tracheobronchial lymph nodes	0 / mod enlarged / severely enlarged	0 / mod enlarged / severely enlarged	0 / mod enlarged / severely enlarged	0 / mod enlarged / severely enlarged	0 / mod enlarged / severely enlarged
Pneumonia Consolidation, type	0/acute/subacute/chronic	0/acute/subacute/chronic	0/acute/subacute/chronic	0/acute/subacute/chronic	0 /acute /subacute/chronic
Consolidation, distribution Right side	0 /cran-lobe/caud-lobe Focal/multifo/widespread	0 /cran-lobe/caud-lobe Focal/multifo/widespread	0 /cran-lobe/caud-lobe Focal/multifo/widespread	0 /cran-lobe/caud-lobe Focal/multifo/widespread	0 /cran-lobe/caud-lobe Focal/multifo/widespread
Consolidation, distribution Left side	0 /cran-lobe/caud-lobe Focal/multifo/widespread	0 /cran-lobe/caud-lobe Focal/multifo/widespread	0 /cran-lobe/caud-lobe Focal/multifo/widespread	0 /cran-lobe/caud-lobe Focal/multifo/widespread	0 /cran-lobe/caud-lobe Focal/multifo/widespread
Bact sample, case 2 sites from lesions	R cran-lobe /caud-lobe L cran-lobe/caud-lobe	R cran-lobe /caud-lobe L cran-lobe /caud-lobe	R cran-lobe /caud-lobe L cran-lobe /caud-lobe	R cran-lobe /caud-lobe L cran-lobe/caud-lobe	R cran-lobe /caud-lobe L cran-lobe/caud-lobe
Bact sample, control 2 sites	R caudal lobe L cranial lobe	R caudal lobe L cranial lobe	R caudal lobe L cranial lobe	R caudal lobe L cranial lobe	R caudal lobe L cranial lobe

Hist lung, case 4 samples per animal (preferably from lesions): R and L cran-lobe, R and L caud-lobe (Cran-lobes in same vial, caud-lobes in same vial)	R: cran-lobe / caud-lobe L: cran-lobe / caud-lobe	R: cran-lobe / caud-lobe L: cran-lobe / caud-lobe	R: cran-lobe /caud-lobe L: cran-lobe/caud-lobe	R: cran-lobe / caud-lobe L: cran-lobe / caud-lobe	R: cran-lobe / caud-lobe L: cran-lobe / caud-lobe
Hist lung, control 4 samples per animal	R: cran-lobe / caud-lobe L: cran-lobe / caud-lobe	R: cran-lobe / caud-lobe L: cran-lobe / caud-lobe	R: cran-lobe / caud-lobe L: cran-lobe / caud-lobe	R: cran-lobe / caud-lobe L: cran-lobe / caud-lobe	R: cran-lobe / caud-lobe L: cran-lobe / caud-lobe
Hist ln, case and control	Tracheobronchial ln	Tracheobronchial ln	Tracheobronchial ln	Tracheobronchial ln	Tracheobronchial ln
Hist trachea, case	Trachea	Trachea	Trachea	Trachea	Trachea
Freeze lung tissue for PCR, case: sample from cran-lobe and caud-lobe (from lesions if there are any, in the same tube) Control: L cran-lobe and R caud-lobe (in the same tube) (PCV2)	L cran-lobe/ caud-lobe R cran-lobe/ caud- lobe	L cran-lobe/ caud-lobe R cran-lobe/ caud- lobe	L cran-lobe/ caud-lobe R cran-lobe/ caud- lobe	L cran-lobe/ caud-lobe R cran-lobe/ caud- lobe	L cran-lobe/ caud-lobe R cran-lobe/ caud- lobe
Freeze ln for PCR, case and control (PCV2)	Tracheobronchial ln	Tracheobronchial ln	Tracheobronchial ln	Tracheobronchial ln	Tracheobronchial ln
Freeze lung tissue, case 5 x 5 cm from lesion	lesion	lesion	lesion	lesion	lesion
Photo cut surface (preferably from lesion)	Cut surface lesion	Cut surface lesion	Cut surface lesion	Cut surface lesion	Cut surface lesion
Tentative diagnosis					

Abbreviations: pur= purulent; Cran-lobe= Cranial – and accessory lobe; Caud-lobe= Caudal lobe; R=Right; L=Left; ln=lymph node; Multifo= multifocal; Mod= moderately

Lesion type: acute = hyperemia and increased texture. Subacute = mixture between acute and chronic changes. Chronic = fibrosis, abscessation.

Instructions: circle around the alternative that fits the observation. Tick boxes when a task is done.

Histology protocol. *A scheme for a standardized histologic evaluation of sections from pigs' lungs, pleura and tracheobronchial lymph nodes. The scheme was compiled by members of the project group Grisefine lunger to be used in the study of acute respiratory disease outbreaks. Tissue samples from the lungs, pleura and lymph nodes are fixed and in 10% phosphate-buffered formalin and embedded in paraffin wax. Cut sections (2-3 µm) are stained with hematoxylin and eosin. Samples are, when possible, taken from bordering areas between normal and affected tissue with macroscopic lesions. Sections are examined by microscope (Leica 020-518.500 DM/LS).*

Journal number	**Lung no.1**			**Lung no. 2**			**Lung no. 3**			**Lung no. 4**			**Lung no. 5**		
Case/control*	**Left Cran/caud***	**Right Cran/caud***	**Pleura**	**Left Cran/caud***	**Right Cran/caud***	**Pleura**	**Left Cran/caud***	**Right Cran/caud***	**Pleura**	**Left Cran/caud***	**Right Cran/caud***	**Pleura**	**Left Cran/caud***	**Right Cran/caud***	**Pleura**
Neutrophils															
Fibrin															
Necrotic leukocytes															
Peribronchial/ peribronchiolar infiltration LC and PLC															
Interstitial bleeding															
Lymph node subcapsular granulocytes															
Other remarks															
Other slides**															

*Cross out the alternative that does not apply/was not evaluated

** If other sections have been evaluated, for instance sections of trachea, clarify sample type and findings.

Cran=cranial, caud= caudal, LC = lymphocytes, PLC = plasma cells

For every observation that fits the description in the form, tick the corresponding box.

Additional file 3 - Details of sample handling and diagnostics

Included in this document are extended details of diagnostic procedures that were conducted in *A descriptive study of clinical outbreaks of respiratory disease in Norwegian fattening pig herds.* Some details are repeated in the manuscript to ensure proper context.

Bacteriology

The samples were handled as part of the Norwegian Veterinary Institute (NVI) bacteriology department's routine diagnostic work.

Swabbed material from lungs and pleura was cultured on 5% sheep's blood on agar base including a cross-streak of β-toxic Staphylococcus aureus to help the growth of Nicotinamide adenine dinucleotide (NAD) dependent bacteria like A. pleuropneumoniae and incubated in a humidity chamber in 5% CO2. The swabs were additionally cultivated on blood agar for anaerobic incubation and on a cysteine lactose electrolyte deficient (CLED) agar for aerobic incubation.

Colonies were isolated by secondary culturing. Colony identification was verified by Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MALDI Biotyper®, Bruker Daltonics, Bremen, Germany).

Serotyping by whole genome sequencing

Pure cultures of *A. pleuropneumoniae* isolated from the lungs and pleura were sampled for whole genome sequencing at Statens Serum Institut (SSI), Copenhagen, Denmark.

Extracted bacterial DNA was quantified using the Qubit (Invitrogen, Waltham, MA, USA), followed by library preparation with the Nextera XT DNA Library Prep Kit (Illumina Inc., San Diego, CA, USA) using manufacturers protocol. The libraries were sequenced on the NextSeq 550 platform (Illumina Inc., San Diego, CA, USA) to obtain paired-end 151 bp reads. The serovar was determined based on the presence of the serovar specific cps operons by local BLAST using CLC Genomic Workbench 11.0.1.

Virology

The pooled oral fluid samples were run in real-time PCR according to NVIs molecular biology department's in-house procedures. Nucleic acids were extracted using a Nuclisense easyMAG 2, (Biomerieux, Marcy-l'Étoile, France). PCRs were run in a Stratagene Mx3005P (Agilent Technologies, Santa Clara, California, USA), with respective positive and negative controls on each plate. A cycle threshold (Ct) value for SIV below 37 was considered positive. PCV2 qPCR is a quantitative test where results are given as measures nucleotide copies in 200 µL sample, calculated from repeated measures at different Ct values.

Serology

Details regarding the commercial tests that were used to analyze for agent specific antibodies:

Agent name	**Kit name**	**Manufacturer**	**Sensitivity***	**Specificity***
A. pleuropneumoniae	ID Screen® APP Screening Indirect	IDvet, Montpellier, France	82.9%	99.6%.
Influenza A virus	ID Screen® Influenza A Antibody Competition Multi-species	IDvet, Montpellier, France	69% at the recommended cutoff of 0.5	89% at the recommended cutoff of 0.5
PRRSV	IDEXX PRRS X3	IDEXX, Maine, USA	98.8%	99.9%
PRCV	SVANOVIR® TGEV/PRCV-Ab	Boehringer Ingelheim Svanova, Uppsala, Sweden	93%	97%
M. hyopneumoniae	*Mycoplasma hyopneumoniae* ELISA	Oxoid™, Cheshire, England	100%	98%

PRRSV = Porcine Reproductive and Respiratory Syndrome Virus, PRCV = Porcine Respiratory Coronavirus

*The sensitivity and specificity of the serologic tests have not been evaluated on the Norwegian pig population and might deviate somewhat from the references used by the manufacturers.

The analyses were performed as described by the manufacturers. Plate reading was performed with a Thermo Multiscan EX.

II

frontiers in Microbiology

ORIGINAL RESEARCH
published: 10 September 2021
doi: 10.3389/fmicb.2021.729637

Comparative Genome Sequence Analysis of *Actinobacillus pleuropneumoniae* Serovar 8 Isolates From Norway, Denmark, and the United Kingdom Indicates Distinct Phylogenetic Lineages and Differences in Distribution of Antimicrobial Resistance Genes

Liza Miriam Cohen[1]†, Janine T. Bossé[2†], Marc Stegger[3], Yanwen Li[2], Paul R. Langford[2], Camilla Kielland[1], Thea Blystad Klem[4], Stine Margrethe Gulliksen[5], Birgit Ranheim[1], Carl Andreas Grøntvedt[4] and Øystein Angen[3]*

[1]Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), Oslo, Norway, [2]Section of Paediatric Infectious Disease, Department of Infectious Disease, Imperial College London, London, United Kingdom, [3]Department of Bacteria, Parasites and Fungi, Statens Serum Institut, Copenhagen, Denmark, [4]Norwegian Veterinary Institute, Oslo, Norway, [5]Animalia, Oslo, Norway

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a disease of major impact on pig health, welfare, and productivity globally. Serovar 8 (APP) is the predominant clinical serovar in Norway and the United Kingdom (UK), and has been isolated from clinical cases in Denmark. The primary objective of this study was to characterize the genetic variability of isolates of *A. pleuropneumoniae* APP8 in the Norwegian population. The secondary objectives were to determine the within-host variability of APP8; to compare the APP8 bacterial populations in Norway, Denmark, and the UK, including antimicrobial resistance (AMR) gene profiles and to assess the effect of national differences in antimicrobial drug use and restricted animal movement on the occurrence of resistance. Isolates of APP8 from the UK ($n = 67$), Denmark ($n = 22$), and Norway ($n = 123$) collected between 1983 and 2020 were compared using whole genome sequencing. To investigate genetic variability within individual hosts, an additional 104 APP8 isolates from the lungs of six Norwegian pigs were compared. Very low within-host variation was observed ($\leq$ 2 single nucleotide polymorphisms). The phylogeny of 123 Norwegian APP8 isolates from 76 herds revealed some within-herd genetic variation, but substantial geographical clustering. When inferring the relatedness of the three international APP8 collections, the topology highlighted the existence of two distinct monophyletic branches characterized by the Norwegian and UK isolates, respectively. Three Danish isolates were scattered across the UK branch, whereas the remaining 19 Danish isolates

OPEN ACCESS

Edited by:
Apichai Tuanyok, University of Florida, United States

Reviewed by:
Han Sang Yoo, Seoul National University, South Korea
Adriano de Bernardi Schneider, University of California, San Diego, United States

***Correspondence:**
Liza Miriam Cohen
liza.miriam.cohen@nmbu.no

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Microbiology

Received: *23 June 2021*
Accepted: *13 August 2021*
Published: *10 September 2021*

Citation:
Cohen LM, Bossé JT, Stegger M, Li Y, Langford PR, Kielland C, Klem TB, Gulliksen SM, Ranheim B, Grøntvedt CA and Angen Ø (2021) Comparative Genome Sequence Analysis of Actinobacillus pleuropneumoniae Serovar 8 Isolates From Norway, Denmark, and the United Kingdom Indicates Distinct Phylogenetic Lineages and Differences in Distribution of Antimicrobial Resistance Genes. Front. Microbiol. 12:729637. doi: 10.3389/fmicb.2021.729637

clustered in two monophyletic groups nested in the Norwegian branch. Coalescence analysis, performed to estimate the divergences from a common ancestor, indicated a last common ancestor several centuries ago. The phylogenetic analyses also revealed striking differences in occurrence of AMR genes, as these were 23-times more prevalent among the UK isolates than among the Norwegian isolates. An increased understanding of the effects of population strategies is helpful in surveillance and control of infectious diseases.

Keywords: *Actinobacillus pleuropneumoniae*, phylogeny, whole genome sequencing, antimicrobial resistance genes, evolution, serovar 8

INTRODUCTION

Comparing genome sequence data provides information on molecular and epidemiologic relationships. In microbial infection dynamic studies, population genomics is used to gain insight into species populations, to map diversity and better understand the transmission patterns of pathogens. Genetic variability can be compared at many levels within and between species populations and is influenced both by inherent biologic characteristics that affect the transmission of the pathogen, as well as by host population structures and events (i.e., host population dynamics). Transmission routes, animal population structures and management practices, and patterns of animal movements should all be reflected in the genetic relationships observed between the pathogens.

Systemizing the pig production sector enables efficient surveillance and biosecurity measures which are becoming increasingly important. In order to secure a high level of biosecurity, strict population structures are applied to the pig production in many countries (including Norway, the UK, and Denmark), which include restricted or negligible live animal import from other countries and domestic trade structured through a tiered pyramid. The Norwegian pig production system has a pyramidal structure, with a unidirectional flow of animals from a low number of genetic nucleus breeding herds at the top, to a larger number of commercial producers at the bottom (Norwegian Veterinary Institute, 2021a). *Actinobacillus pleuropneumoniae* is a porcine opportunistic bacterium and the etiological agent of porcine pleuropneumonia. *A. pleuropneumoniae* is transmitted mainly through direct contact between animals, commonly from sows to suckling piglets, or by aerosols over short distances (Chiers et al., 2002; Velthuis et al., 2003; Fablet et al., 2011; Tobias et al., 2014). In most modern pig producing countries, herds are endemically infected with *A. pleuropneumoniae,* with healthy carrier pigs harboring the bacterium in their tonsils (Gottschalk, 2015; Sassu et al., 2017). While more virulent isolates are able to colonize the lower respiratory tract and cause pleuritis and/or pneumonia, other isolates will not (Gottschalk, 2015). To what extent bacteria involved in lung infections are genetically heterogeneous or are solely monoclonal has not been elucidated.

Porcine pleuropneumonia is considered a major health and welfare challenge to pig production worldwide and is a source of considerable use of antimicrobial drugs, both in treatment and prophylaxis (Sassu et al., 2017). As prevalent use of antimicrobial drugs to combat disease leads to emergence of resistant strains (FAO, 2016), national strategies for antimicrobial drug use are based on knowledge of the antimicrobial resistance (AMR) profiles of relevant pathogens. Antimicrobial treatment practices are an important population-wide factor affecting genetic variability through selection of resistance in pathogenic, as well as commensal, bacteria. Population-wide surveillance for AMR genes using whole genome sequencing (WGS) can be useful when forming future national strategies for treatment and control. WGS is a sensitive method for detecting known AMR genes in bacteria (Anjum et al., 2017) including *A. pleuropneumoniae*, where the AMR genotype was shown to correlate nearly 100% with the phenotype for antimicrobial agents other than macrolides (Bossé et al., 2017).

Among the 19 described serovars of *A. pleuropneumoniae* (Stringer et al., 2021), serovar 8 (APP8) is most commonly isolated from cases of acute porcine pleuropneumonia in Norway (Cohen et al., 2020; Norwegian Veterinary Institute, 2021b) and the UK (O'Neill et al., 2010; Li et al., 2016). In Denmark, APP8 has also been isolated from clinical cases, although earlier studies have shown it is not the most prevalent serovar (Møller et al., 1992; Kokotovic and Angen, 2007). Previous analyses of *A. pleuropneumoniae* populations, using multilocus enzyme electrophoresis (Møller et al., 1992; Hampson et al., 1993) or amplified fragment length polymorphism (Kokotovic and Angen, 2007), indicated that the species is divided into clonal groups mainly corresponding to the different serovars. More recent analysis by enterobacterial repetitive intergenic consensus-based PCR, revealed a degree of variation within populations of isolates of serovars 1, 7, and 15 in Australia (Yee et al., 2018). Comparison of whole genome sequences from seven Brazilian APP8 isolates indicated that differences in these were mainly due to prophage and other mobile genetic elements (Prado et al., 2020). To our knowledge, there has previously not been published detailed analysis of large populations of a single serovar of *A. pleuropneumoniae* using genomic data.

The primary objective of this study was to characterize the genetic variability in isolates of APP8 in the Norwegian population. Secondary objectives included determining variability at two further levels, i.e., within-host and between populations in different countries (Norway, Denmark, and the UK), and comparing AMR genes in the different national populations as an indicator of the effect of regional antimicrobial drug

use and closed populations on dissemination of AMR. We used temporal and geographic data to gain knowledge regarding the effect of the pig population structure, animal movement, antimicrobial drug consumption and AMR levels on the evolution of this important pig pathogen.

MATERIALS AND METHODS

Bacterial Isolates

Isolates of APP8 from countries where this serovar has commonly occurred in clinical cases were included in this study. A total of 316 isolates were included, of which 227 isolates originated from Norway, 67 originated from the UK and 22 from Denmark (**Table 1**).

The Norwegian Isolate Repository

A primary isolate repository was established, consisting of 123 APP8 isolates from individual pigs collected through routine diagnostics at the Norwegian Veterinary Institute (NVI) in the period from 2004 to 2019. The isolates originated from a total of 76 herds, of which 23 had given rise to multiple (range 2–6) isolates. These isolates mainly stemmed from cases of clinical pleuropneumonia; however, a minority of isolates ($n = 2$) were cultured by swabbing from pneumonic lungs at slaughter with no prior remarks of clinical signs. Serovar determination was performed by the method as previously described (Cohen et al., 2020). Non-APP8 isolates ($n = 5$), collected at the NVI in the same period, were excluded from the study. In 2019 and 2020, an additional 104 isolates were sampled from pneumonic lungs at the NVI to investigate within-host variation of *A. pleuropneumoniae*. We sampled six pigs from five geographically unrelated herds by swabbing two to five lesions within every set of lungs. Swabs from each lesion were cultured on individual agar plates. From each plate we selected five to eight colonies of *A. pleuropneumoniae*, all serovar 8, resulting in 104 isolates all of which were sequenced.

Swabbed material from lungs and pleura was cultured on 5% sheep's blood on agar base including a cross-streak of β-toxic *Staphylococcus aureus* to support the growth of nicotinamide adenine dinucleotide-dependent *A. pleuropneumoniae* and incubated in a humidity chamber in 5% CO_2. Colonies were purified by secondary culturing, then stored at −80°C. Isolates were revived by the same bacteriological procedures. Colony identification was verified by matrix assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (MALDI Biotyper, Bruker Daltonics, Bremen, Germany). Pure cultures were collected onto sterile swabs and placed in Amies transport medium with charcoal and shipped in bulk by courier to Statens Serum Institut (SSI), Denmark. Prior to DNA extraction, isolates were cultured on chocolate agar and incubated at 35°C over-night.

TABLE 1 | APP8 sample population grouped according to country of origin.

Country of origin	Isolate repository for phylogenetic reconstruction*	Additional isolates sampled for within-host analysis	Total
Norway	123	104	227
UK	67	0	67
Denmark	22	0	22
Total	212	104	316

Isolates stem from routine diagnostics.

The UK Isolate Repository

Genomic sequences from 67 clinical isolates of APP8 have been included from the archives of Imperial College in London. The genome for the APP8 reference strain 405, GenBank accession ID: txid754257, was included here as a UK isolate, although it was isolated in Ireland in 1984 (Nielsen and O'Connor, 1984). Retrieval of the UK isolates was performed from 2003 to 2011 at the Animal and Plant Health Agency (formerly known as the Animal Health and Veterinary Laboratories Agency) diagnostic laboratories in England and Wales. Methods regarding sample collection and handling of the UK isolates have been published previously (Bossé et al., 2015).

The Danish Isolate Repository

Twenty-two Danish clinical isolates of APP8 originate from diagnostic work at Danish Veterinary Laboratory (later National Veterinary Institute – Technical University of Denmark), over the years from 1983 to 2009. Eight of these isolates (sampled between 1983 and 1991) were shipped to Imperial College, London, United Kingdom, in 2007. DNA extraction and WGS of these isolates were performed according to the method previously described (Bossé et al., 2020). The remaining 14 isolates (sampled between 1996 and 2009) were transferred to SSI in 2019 and cultured on chocolate agar, and incubated at 35°C over-night, prior to DNA extraction.

Metadata

The year of sampling was retrieved from the diagnostic records for all included isolates ($n = 316$).

Epidemiology of the Norwegian Isolates

We retrieved the unique farm identification number (ID) for the farm from which the Norwegian APP8 pig isolates ($n = 123$) originated. This ID was in turn used to identify the farm location, production type (herd category) and abattoir affiliation as livestock movement is restricted within the slaughterhouse systems. This information was included to study the effects of geographic origin and livestock trade on genetic variability on population level.

The variable farm location was divided into five geographical categories: North ($n = 6$), Central ($n = 21$), East ($n = 37$), South-West ($n = 46$), and Greater Oslo ($n = 12$). For one isolate, this information was unavailable. The geographic regions defined here are based on the official administrative regions of the Norwegian Food Safety Authorities, as they were in April of 2020 (Norwegian Food Safety Authorities, 2012). For statistical

TABLE 2 | An overview of the distribution of Norwegian APP8 isolates ($n = 123$) divided into two categories; the abattoir and herd of origin.

Abattoir category	No of herds (%)	No of isolates (%)	Number of isolates in herd of origin (% of total)			
			Breeding herds	Commercial herds	Other	Unknown
Private	28 (36.8)	42 (34.1)	2 (1.6)	39 (31.7)	1 (0.8)	0
Cooperative	46 (60.5)	78 (63.4)	6 (4.9)	61 (49.6)	7 (5.7)	4 (3.3)
Unknown	2 (2.6)	3 (2.4)	0	1 (0.8)	0	2 (1.6)
Total	76	123	8 (6.5)	101 (82.1)	8 (6.5)	6 (4.9)

TABLE 3 | Internal SNP differences among 104 APP8 isolates from six pigs from five different herds.

Pig	No isolates	Internal SNP distance
1	25	0
2*	10	0-1
3*	10	0
4	14	0
5	20	0–2
6	25	0–2

Clonal isolates from the same herd, sampled during a disease outbreak.

analyses, these groups were merged to form three geographical regions (**Supplementary Figure 1**) with larger sample sizes: East and Greater Oslo (Region 1), South-West (Region 2), North and Central (Region 3).

Herd of origin was categorized into four types (**Table 2**), based on the structure of the Norwegian pig production pyramid system. The category "Breeding herds" included isolates from genetic nucleus and multiplier breeding herds ($n = 8$), while the "Commercial herds" category included isolates from commercial sow herds and fattening pig herds for consumption ($n = 101$). A 3rd category labeled "Other" ($n = 8$), included isolates from places of origin that differed from the common herd types in the production pyramid, such as isolates from stud quarantine and testing stations, as well as pigs submitted for diagnostics by an abattoir and could not be traced to a herd. Six isolates were grouped as "Unknown."

The variable for abattoir affiliation was divided in three categories: "Cooperative," owned by the members (Nortura, $n = 78$), "Private" (privately owned abattoirs, members of The Meat and Poultry Industry's National Association, $n = 42$), or "Unknown" ($n = 2$; **Table 2**). These categories were chosen because livestock trade in Norway is usually restricted within these abattoir systems.

Summary data for the tables and statistical analyses of the epidemiological data were performed using Stata (STATA SE/15 for Windows; Stata Corp., College Station, TX, United States). The distribution of geographic regions of origin and abattoir affiliation within three clades in the Norwegian APP8 phylogeny was assessed using a cross table and evaluated using the chi squared (χ^2) test. One isolate was excluded from the analysis, as the region of origin was unknown. Herd as a random effect was accounted for.

A dataset of the isolate metadata has been included (**Supplementary Data**).

Analyses of Genetic Variability

Bioinformatic analysis of bacterial genome sequences allow study of genetic variability within and between populations. In this study we assessed genetic variability in whole genome sequences through single nucleotide polymorphisms (SNPs) in the core genome of the included isolates. WGS was also used to characterize the AMR genes carried by the isolates.

Genome Sequencing

Genomic DNA was extracted from the Norwegian ($n = 227$) and 14 of the Danish isolates using the DNeasy Blood and Tissue Kit (QIAGEN, Germantown, MD, United States) and quantified on a Qubit 3.0 Fluorometer (Invitrogen, Waltham, MA, United States). Preparation of the DNA sequence libraries were performed using the Illumina Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, United States) and sequenced on a NextSeq 500 platform (Illumina Inc., San Diego, CA, United States) with paired-end sequencing (2×151 bp) using a 300-cycle NextSeq Mid-Output Kit followed by quality assessment using bifrost.[1] The reads were *de novo* assembled using SPAdes (Bankevich et al., 2012) using default parameters.

Phylogenetic Reconstruction Using SNP Calling

For phylogenetic reconstruction, the sequence reads from all isolates were included. Using the closed chromosomal sequence of APP8 strain MIDG2331 (GenBank accession number LN908249.1) as reference, after removal of duplicated regions using NUCmer (Kurtz et al., 2004), identification of SNPs was performed with NASP v.1.0.0 (Sahl et al., 2016). All positions with less than 10-fold sequencing depth and 90% unambiguous variant calls for any isolate were excluded. Removal of high-density SNP regions, such as those caused by recombination, were identified and removed using Gubbins v.2.3.4 (Croucher et al., 2015) prior to phylogenetic reconstruction using IQ-TREE v.1.5.5 (Nguyen et al., 2015) using ModelFinder as implemented in IQ-TREE, and phylogenetic robustness was assessed with bootstrap analysis using 100 replicates. Visualization and annotation of the phylogenies were performed using iTol v4.314.[2] Geographic visualization was performed in Microreact.[3] In Microreact, each isolate was given the coordinates for the capital of the municipality in which the farm was located to

[1]https://github.com/ssi-dk/bifrost (Accessed April 09, 2021).
[2]https://itol.embl.de (Accessed April 09, 2021).
[3]https://microreact.org (Accessed April 09, 2021).

comply with General Data Protection Regulations. Additionally, a SNP distance between multiple isolates collected from the same pigs was obtained as outlined above and used to investigate within-host variation, using the same-sized core genome. Mean SNP counts for within-pig (internal) variability were calculated (**Table 3**). Isolates were considered clonal if they displayed very limited variation (≤2 SNPs).

Genotyping AMR

The presence of AMR genes was investigated using ABRicate[4] to search the assembled genomes for genes associated with resistance found in the ResFinder database (Zankari et al., 2012). The gene presences were determined based on a combined >80% hit length and >90% sequence identity. Only the primary repository APP8 isolates ($n = 212$) were included in these analyses (**Table 1**).

Coalescence Analyses

To ascertain the temporal relationships of our samples, we utilized coalescent analyses that model how variants sampled from a population may have originated from a common ancestor, by estimating rooted, time-measured phylogenies. For this, BEAST v2.6 was used and run at the CIPRES Science Gateway v3.3[5] public resource. The bModelTest v1.2.1 package was applied using the transitionTransversionSplit model and automatic estimation of mutation rate and log normal nucleotide frequencies. Different coalescent (constant population and Bayesian skyline) and clock models (strict clock and relaxed clock log normal) were applied to allow both constant and variable mutation rates across the branches. For all combinations of models, independent chains ($n = 2$) of 400 million in length were performed with storing of data every 40,000th step, assessing the convergence of key ESS values using Tracer v1.7, after burn-in of 10% by calculation of the $\log_{10}$ Bayes factors for model comparison. Trees were visualized using DensiTree v2.2.7 (Bouckaert, 2010).

RESULTS

Within-Population Variation of Norwegian APP8 Isolates

Phylogeny

Three distinguishable genetic clades were observed among the Norwegian isolates in the phylogeny and named Norway I, Norway II, and Norway III (**Figure 1B**). Median and maximal pairwise SNP distances within the Norwegian population were 112 and 153 SNPs respectively, within a core genome of 1.67 Mbp (71.6% of the reference chromosome), indicating a small within-population variation. Clonal isolates (<3 SNPs) were in some cases isolated from pigs in the same herd. In other instances, isolates from pigs in the same herd sampled at the same point in time displayed a much greater variation (**Figure 2**).

[4]https://github.com/tseemann/abricate (Accessed April 09, 2021).
[5]https://www.phylo.org (Accessed April 23, 2021).

Year of Sampling and Epidemiology of Isolates

The Norwegian isolates were sampled between 2004 and 2020, with a higher sampling frequency in 2013 and between 2017 and 2019 (**Supplementary Figure 1**). Samples from different years were scattered across the phylogeny, with few SNPs between isolates sampled up to 13 years apart, suggesting that APP8 is diversifying at a slow rate in the population.

The clades within the Norwegian phylogeny showed a geographical pattern (**Figure 2**; **Supplementary Figure 1**). The χ^2 of the distribution of isolates between three identified genetic clades and the three combined region categories (**Table 4**) was 72.3 ($p < 0.001$). Accounting for herd as a random effect did not influence the results, hence the associations between phylogenetic clade and region was not random, meaning that isolates from the same geographic regions were more closely related than isolates from different regions. This finding supports geography as a factor of influence to the molecular evolution of *A. pleuropneumoniae*, and that different lineages of the bacterium spread within distinct geographic regions.

The primary repository isolates were mainly (i.e., 101 of 123) sampled from commercial herds. The distribution of herds in the different herd categories is presented in **Table 2**.

There was a visual clustering of isolates within the same abattoir system categories to phylogenetic clade (**Figure 2**), however the statistical analysis did not support this (χ^2 4.1, $p = 0.4$).

Within-Host Variation

A SNP distance ≤2 (median value between 0 and 1) was found between the isolates from the same pig, indicating that they belonged to the same clone. Further statistics on these isolates is shown in **Table 3**.

Comparative Genome Analyses

Phylogenies

The APP8 isolates from UK and Norway were separated by approximately 1,500 SNPs into two distinct phylogenetic branches (**Figure 1A**), where three Danish isolates were found scattered across the UK branch, while the remaining 19 Danish isolates clustered in two monophyletic groups in the Norwegian branch (**Figure 1B**).

Coalescence Analyses

The BEAST analysis performed on the Norwegian branch indicated that the APP8 population separated several centuries ago (**Figure 1C**). The last common ancestor with a Danish isolate, based on strict mutation rates and a coalescent Bayesian skyline tree prior, can be dated back to at least 200 years (95% HDP: 1343–1837). From this analysis, we extrapolated that the Norwegian and UK isolates shared a common ancestor much further back in time, and that no evidence of later introductions can be found in our data. In contrast, our data support recent *A. pleuropneumoniae* transmissions between the UK and Danish pig populations which are reflected in the molecular relationships of APP8 isolated during the last decades.

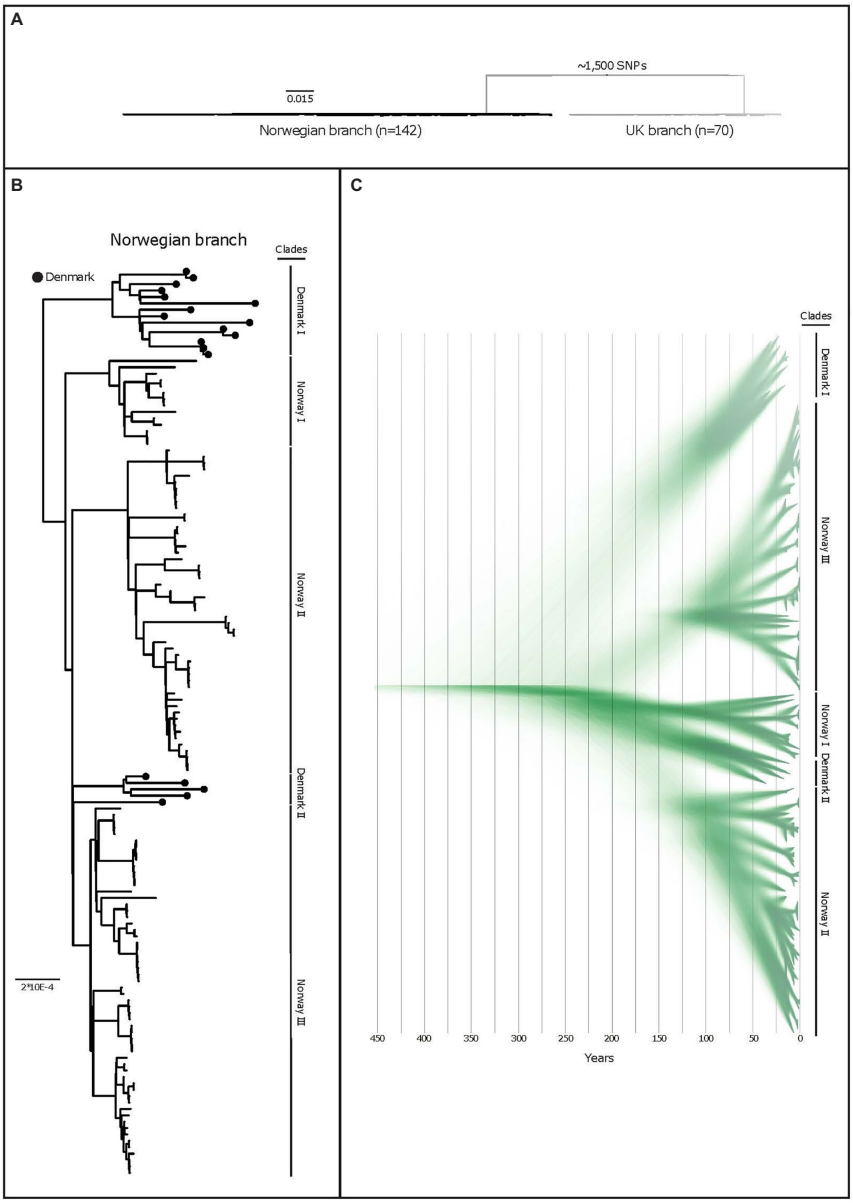

FIGURE 1 | (A) A purged midpoint rooted maximum likelihood phylogeny of isolates of APP8 from Norway, illustrating the two distinct branches of the APP8 collection (the Norwegian branch and the UK branch). The Norwegian branch contains 19 Danish and 123 Norwegian isolates ($n = 142$), whereas the UK branch contains three Danish and 67 UK isolates ($n = 70$) with ~1,500 SNPs separating the branches. **(B)** A rooted maximum-likelihood phylogeny of the Norwegian APP8 branch. Danish isolates are indicated by black dots on the branches. Based on the topology, five distinct phylogenetic clades labelled Norway I-III and Denmark I and II are highlighted. The scale indicates substitutions per site. **(C)** DensiTree representation of 9,000 time-measured phylogenies obtained from BEAST with the best fitting model after removal of 10% burn-in. X-axis is a time scale indicating years since 2019, illustrating the temporal relationships between the 123 Norwegian and 19 Danish isolates. Similar clustering is observed as with the maximum-likelihood approach, however in a different order, highlighted by clade labels.

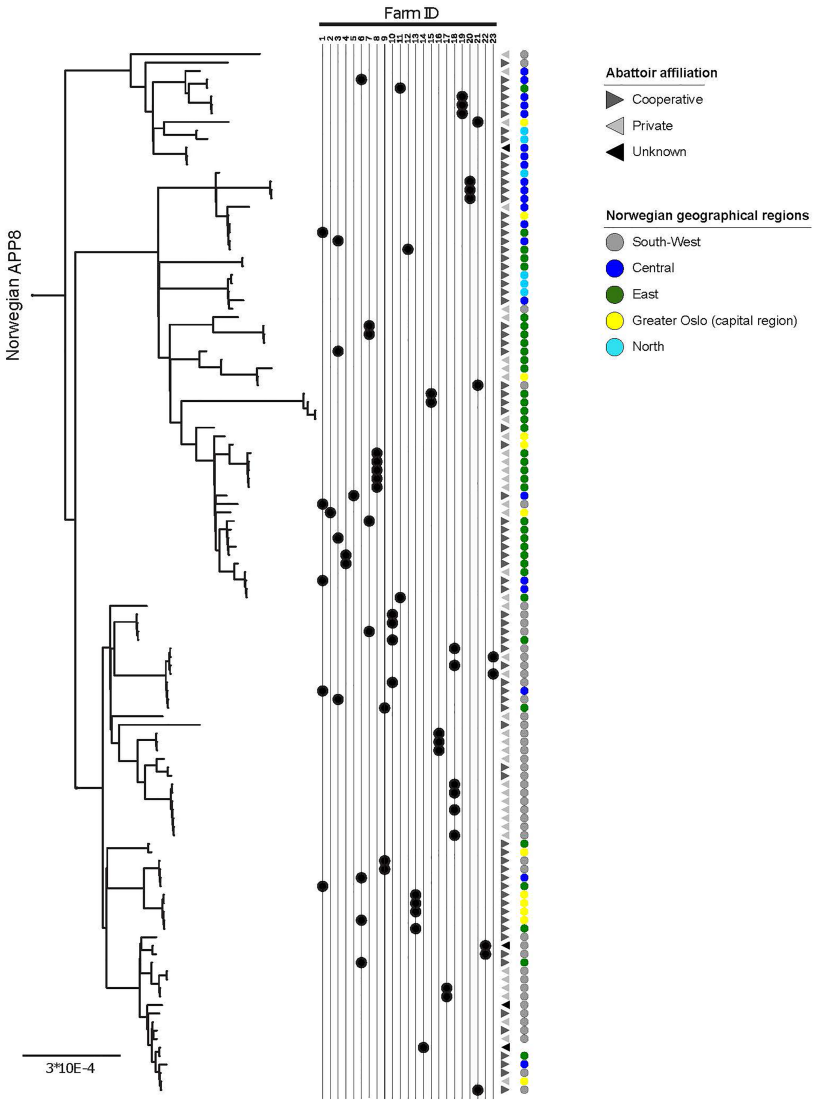

FIGURE 2 | Within-herd variability, geographic origin, and abattoir affiliation of APP8. More than one isolate was sampled from each of 23 herds, indicated by the Farm ID, the black dots point to the location of the isolate in the phylogeny. Geographic origin across five major regions of Norway is indicated by colored circles (legend in figure). Abattoir affiliation to the two main slaughterhouse groups in Norway (Cooperative and Private) and a third Unknown group are indicated with greyscale triangles (legend in figure). The scale indicates substitutions per site.

AMR Genes

The following AMR genes were identified across the entire APP8 collection: *aph(3″)-Ib*, *aph(6)-Id* (streptomycin resistance genes, also called *strA* and *strB*, respectively), *tet*(Y), *tet*(H), *tet*(B; tetracycline resistance), *dfrA14* (trimethoprim resistance), $bla_{\text{ROB-1}}$ (beta-lactam resistance), and *sul2* (sulfonamide resistance). We found statistical differences in occurrence of resistance genes in the three national populations, with the AMR genes most abundant among the UK APP8 isolates (**Figure 3B**; **Table 5**). The sulfonamide resistance gene, *sul2*, was the most common AMR gene occurring in the entire collection, being present in 3.3% of the Norwegian and 66% of the UK isolates. The resistance

TABLE 4 | Distribution of Norwegian isolates of APP8 ($n = 123$) from three phylogenetic clades across three geographical regions in Norway (% of clade total).

Phylogenetic clade	Region 1	Region 2	Region 3	Unknown	Total
Norway I	2 (14.3)	2 (14.3)	10 (71.4)	0	14
Norway II	34 (66.7)	3 (5.9)	14 (27.4)	0	51
Norway III	13 (22.4)	41 (70.7)	3 (5.2)	1 (1.7)	58

Region 1 = East and Greater Oslo, Region 2 = South-West, and Region 3 = North and Central Norway.

profiles of a large collection of UK isolates, including the ones in this study, have been described previously (Bossé et al., 2017). In the UK APP8 branch, the AMR genes were more prevalent in some subclusters, suggestive of a clonal expansion and/or local dissemination by acquisition of mobile genetic elements. The three Danish isolates within the UK branch also resembled the UK isolates in terms of AMR genes. The remaining 19 Danish isolates were nested within the Norwegian branch and had similar low prevalence of AMR genes.

Within the isolates from the Norwegian population, AMR genes were found in only 3.3% ($n = 4$) of the isolates. The four isolates in question did not cluster together, pointing to independent acquisition of the AMR genes. The four isolates originated from the same abattoir system, in three different regions, namely Southwest, East and Greater Oslo, and were isolated over a span of 13 years. A combination of the genes *aph(3″)-Ib*, *aph(6)-Id*, *tet*(Y), and *sul2* was found in three of these. To our knowledge, this is the first time *tet*(Y) has been found within the *Pasteurellaceae* family.

DISCUSSION

In this study we characterized the genetic variability in terms of SNPs, to infer molecular relationships, and distribution of AMR genes in a collection of APP8 isolates. We found that both internationally and within Norway, geographic origin is associated with molecular relationships, as we observe distinct genetic clustering between countries and within major geographical regions in Norway. By applying coalescence analyses, we estimated that the Norwegian isolates separated from UK and Danish isolates several centuries ago. This distinct genetic separation may be due to several historical aspects. Live animal contact between the UK and Norway has not been documented in recent times, reflected by the distinct separation of the APP8 phylogenies of these countries into two branches. Our data show no signs of recent contact. The separation of the UK and Norwegian branches seems to date back to the Middle Ages, during which Vikings from Norway traveled by boat to the British Isles and were likely to bring livestock out or back home. The clustering of Danish isolates within the UK and Norwegian branches are indicative of multiple introductions, also estimated to have occurred at times where live animal exchange was more likely than today. Evidence of contact between the Norwegian and Danish populations is indicated in the phylogeny to have last occurred around 200 years ago. Norway was under Danish rule in the period between 1537 and 1814. Livestock exchange was more likely to have occurred during this period, although no such records have been tracked in the writing of this manuscript. Breeding animals from UK were introduced in Denmark in the 1980s and is probably the reason for the detection of three Danish APP8 isolates among the UK isolates (personal communication, Øystein Angen, senior researcher at SSI, Copenhagen).

By applying geographic and population structure data to our phylogeny, we observed a significant genetic clustering of our isolates. Closely related isolates were identified within geographic regions, supporting geography as a factor of influence to the molecular evolution of *A. pleuropneumoniae*. Surprisingly, there was no clustering of the abattoir categories in the phylogeny, supporting direct contact as the main source of transmission, since livestock trade usually is restricted within these systems. Strains of *A. pleuropneumoniae* are believed to persist within the breeding herds, harbored in the tonsils and chronic lesions of adult sows (Fablet et al., 2011). Since most of these herds are self-supplying, no direct contact with animals from other herds takes place. One nucleus breeding herd can supply pigs (mainly gilts) directly to several multiplier breeding herds, which in turn can supply pigs to many commercial herds, usually located in the same part of the country. This enables transmission of clonal isolates, allowing their persistence within the system and geographic region. Additionally, individual commercial fattening pig herds can purchase livestock from multiple sow herds, in which case we would expect the pigs to be carrying genetically different strains. This is a reasonable explanation for the observed range in genetic within-herd variability but was not investigated further.

At the individual host level, we observed almost no genetic variation. This is likely due to inherent biologic characteristics of the bacterium and the host. It has been shown that pigs can carry a variety of *A. pleuropneumoniae* isolates in their tonsils (Vigre et al., 2002). The isolates can differ in serovar and potential for invasive infection because of virulence factors that enable them to colonize the lower respiratory tract. Diseased and infectious pigs can transmit the clinical isolate through aerosols by coughing, which is a common clinical sign, and in their saliva and nasal secretions by nose-to-nose contact to susceptible pigs (Gottschalk and Broes, 2019). Outbreaks of porcine pleuropneumonia within affected herds are common, though it is not well described in literature if these occur due to newly introduced virulent strains of *A. pleuropneumoniae* and/or by descent to the lower respiratory tract of strains already resident in the tonsils. However, it has been suggested that in endemically infected herds where *A. pleuropneumoniae* is harbored asymptomatically in the tonsils, environmental triggers (such as stress or co-infection with other respiratory pathogens) play a larger role in precipitating disease outbreaks

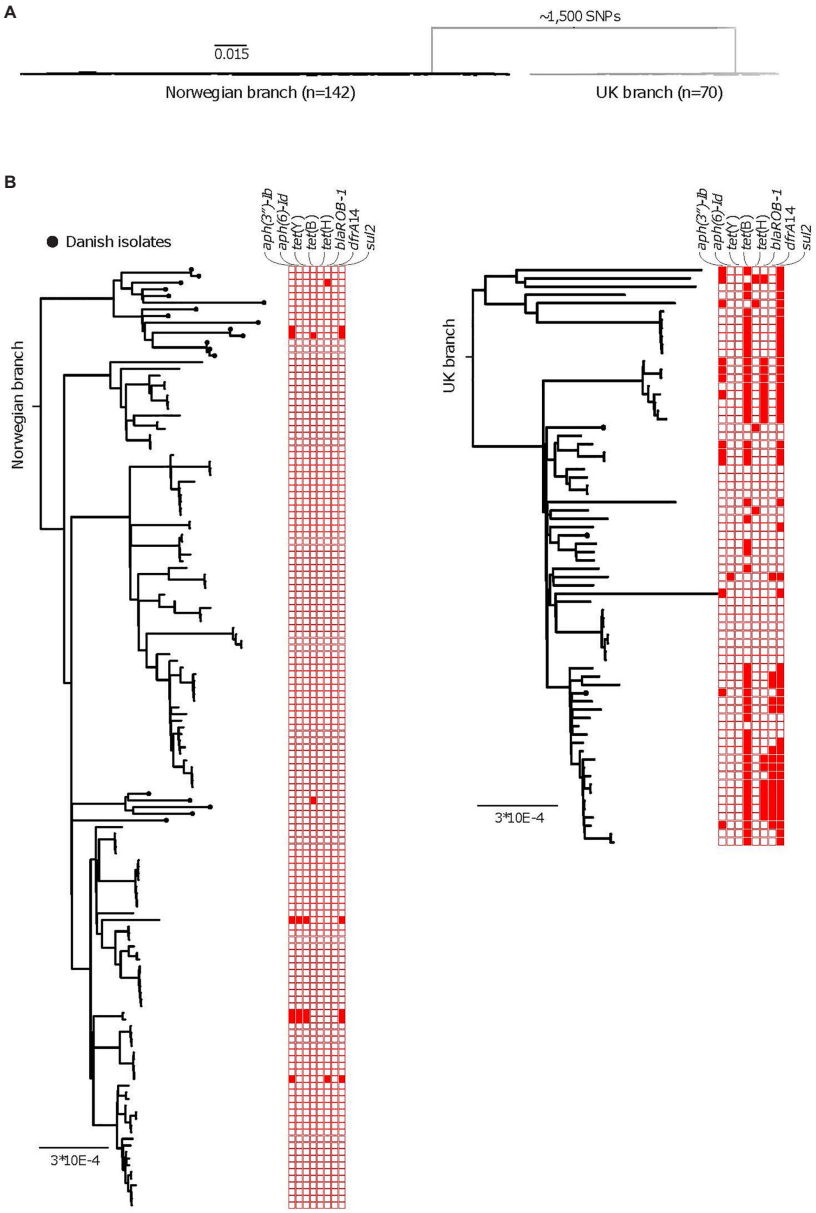

FIGURE 3 | AMR among the APP8 populations collected over 13 years. **(A)** A midpoint rooted phylogeny of isolates of APP8, after removal of recombinant regions, illustrating the two distinct branches of the APP8 collection (the Norwegian branch and the UK branch). The Norwegian branch contains 19 Danish and 123 Norwegian isolates ($n = 142$), whereas the UK branch contains three Danish and 67 UK isolates ($n = 70$) with ~1,500 SNPs separating the branches. **(B)** Rooted phylogenies of the Norwegian branch (left) and the UK branch (right), including AMR gene plot for streptomycin resistance genes *aph(3'')-Ib* and *aph(6)*, tetracycline resistance genes *tet*(Y), *tet*(B), and *tet*(H), beta-lactam resistance gene bla_{ROB-1}, trimethoprim resistance gene *dfrA14* and sulfonamide resistance gene *sul2*. Presence of a resistance gene is indicated by a red colored box, while an empty box indicates absence of resistance gene. The scale indicates substitutions per site.

TABLE 5 | AMR genes identified in isolates of APP8 from Norway ($n = 123$), Denmark ($n = 22$), and the United Kingdom ($n = 67$).

Country of origin	*aph(3'')-Ib/strA*	*aph(6)-Id/strB*	*tet*(Y)	*tet*(H)	*tet*(B)	*dfrA14*	bla_{ROB-1}	*sul2*	Total
Norway	3.3% (4)	2.4% (3)	2.4% (3)	-	-	-	0.8% (1)	3.3% (4)	3.3% (4)
Denmark	9.1% (2)	-	-	4.5% (1)	13.6% (3)	4.5% (1)	4.5% (1)	13.6% (3)	22.7% (5)
United Kingdom	19.4% (13)	1.5% (1)	-	4.5% (3)	67.2% (45)	22.4% (15)	23.9% (16)	67.2% (45)	74.6% (50)

aph(3'')-Ib and aph(6)-Id = streptomycin resistance genes (also called strA and strB, respectively); tet(Y), tet(H), and tet(B) = tetracycline resistance genes; dfrA14 = trimethoprim resistance gene; bla_{ROB-1} *= beta-lactam resistance gene; sul2 = sulfonamide resistance gene. The number of isolates harboring the gene is given in parentheses. Genes that were not present are indicated by "-".*

than transmission of a newly introduced virulent strain (Klinkenberg et al., 2014). During an outbreak of disease, the infectious pressure of more virulent isolates will increase, and if descending bacteria from the tonsils are also involved, a variation in the genomes of bacteria isolated from the lungs of diseased pigs is expected. When diagnosing a case of porcine pleuropneumonia in a herd, due to practical and financial considerations, it is not uncommon that only a single sample per pig or per herd is submitted for in-depth diagnostics including serotyping and AMR testing. Our results support that porcine pleuropneumonia in a pig is caused by a monoclonal infection, indicating that a single sample per pig will be sufficient for a diagnostic purpose. Still, different isolates of APP8 were isolated within a herd, meaning that the mechanisms of disease in a herd was not solely tied to the spread of a single virulent clone. As virulence can vary both between different serovars of *A. pleuropneumoniae* and between isolates of the same serovar (Sassu et al., 2017), there is a possibility that our findings do not apply across all serovars. Our results must still be considered valuable when establishing relevant control strategies against *A. pleuropneumoniae*.

The pangenome of six Brazilian APP8 isolates was recently reported (Prado et al., 2020) and showed that the gene repertoire is well conserved in relation to the available genomes of other serovars, though the presence of serovar-specific patterns of AMR within *A. pleuropneumoniae* is debatable (Asawa et al., 1995; Lee et al., 2015; Kim et al., 2016). Due to a low prevalence of clinical pleuropneumonia caused by APP8 globally, most studies on AMR have been performed on other serovars. AMR genes have many times been found to be linked to, and accumulate in, mobile genetic elements, acting as vehicles for horizontal transmission in many bacterial species. *A. pleuropneumoniae* is no exception (Bossé et al., 2015, 2016b), and many isolates in our study harbor multiple resistance genes that could be tied to such mobile elements. Mobile genetic elements, like plasmids and Integrative and Conjugative Elements (ICE), are not generally tied to specific serovars, though integration of ICE into the chromosome does favor vertical in addition to horizontal transmission, perhaps explaining why some elements have only been found within specific serovars so far. The *tet*(B) gene has previously been shown to be part of a Tn*10* insertion in an integrative conjugative element, ICE*Apl*1 (Bossé et al., 2016b), in the MIDG2331 genome (Bossé et al., 2016a) and in the genomes of a further 21 UK clinical APP8 isolates (Bossé et al., 2017). This gene was also associated with a Tn*7* insertion in the chromosome in 15 (one serovar 7 and the rest APP8), and with possible plasmid sequences in 14 (one each of serovars 2, 6, 7, and 12, and the rest APP8) further isolates (Bossé et al., 2017). The other AMR genes, i.e., *tet*(H), *sul2*, *dfrA14*, bla_{ROB-1}, *aph(3'')-Ib*, and *aph*(*6*; previously referred to as *strA* and *strB*), were all associated with potential plasmids, in a variety of serovars, as indicated by the sequences flanking the AMR genes on the associated contigs (Bossé et al., 2017). The presence of other AMR bacteria in the host population is likely a risk factor for acquisition of these genetic elements. National strategies for handling emergence of multidrug-resistant bacteria in livestock in general could therefore be of relevance to prevent development of AMR in clinically important pathogens like *A. pleuropneumoniae*. It is of high interest to the Norwegian pig production sector to avoid introducing multi-resistant bacteria through contact with other pig populations. Our results conclude that important differences are present also within a serovar, and factors other than the intrinsic properties of serovars contribute to the AMR biodiversity.

The observed differences in AMR gene distribution can also be attributed to the deviating treatment practices in the respective countries. Substantial differences in antimicrobial use exist in pig production in Norway, the UK, and Denmark (Veterinary Medicines Direcorate, 2019; DANMAP 2019, 2020; NORM/NORM-VET 2019, 2020). Sulfonamides were the most used subgroup of antimicrobial drugs for treatment of production animals in Denmark in the 1980s, bypassed by tetracyclines in the 1990s. Resistance to sulfonamides and tetracyclines was found in Danish *A. pleuropneumoniae* isolates already in 1995 (DANMAP 1996, 1997). Around this time, the Danish government implemented measures to significantly reduce the use of antimicrobial drugs in food production. AMR in *A. pleuropneumoniae* has not been systematically investigated in Norway. According to current Norwegian therapeutic guidelines, benzylpenicillin-procaine is the drug of choice for treating porcine pleuropneumonia (Norwegian Medicines Agency, 2012). In a recent field study of acute outbreaks of porcine pleuropneumonia in Norway (Cohen et al., 2020), treatments were found to be in line with these recommendations. In comparison, tilmicosin and tulathromycin have been commonly used in Denmark against acute pleuropneumonia partly due to the convenience of peroral administration (DANMAP 2019,

2020), which is not common practice in Norway (European Medicines Agency, 2018). In the UK, a systematic decrease in general antimicrobial drug use for pigs has been observed since 2015 across most relevant drug classes. However, tetracyclines remain the most used drug class (UK-VARSS, 2020), possibly contributing to the continued selection of tetracycline resistance in *A. pleuropneumoniae*.

We studied genetic variability in three different levels, i.e., within-host, within-population and between populations. The generalizability for the UK and Danish isolates was somewhat hard to ascertain due to limited access to metadata. Isolates were collected from all levels of the production system, and from all the major pig producing regions in Norway. Overall, we observed a low within-population variability of APP8 in Norway and a persistence of genetic lineages over time. Time is only one factor to influence variability, however it is worth noting that our data consists mainly of isolates collected after 2004 and are not equally represented in time. This concentrated sampling contributes to the reported uncertainty around the estimates of population divergence between countries. Since sampling from the whole population is not feasible, an adequate representation of the population over time is necessary for good estimates of divergence. However, we believe that the isolates in our material have been subject to minimal selection bias due to being passively collected through routine diagnostics. A baseline phylogeny of *A. pleuropneumoniae*, as provided by this study, will likely be of great value to future surveillance and control of this pathogen, both because it increases our understanding of the effects of restricted animal movement, and as it enables the discovery of introductions of new genetic lineages. To unveil the clinical relevance of these genetic characteristics, future studies on pathogenicity within genetic lineages are necessary.

CONCLUSION

In this study we utilized genomic data from populations of APP8 to elucidate the population dynamics of the pigs. With modern sequencing techniques and genomic analyses, we were able to study genetic variability, both within and between populations, and to identify evolutionary patterns and relationships. Isolates sampled within-host were nearly identical, and there was little genetic variability between isolates from pigs in a herd during an outbreak, supporting that one sample per animal and only a few samples per herd should suffice for diagnostic sampling. The occurrence of AMR genes in Norwegian isolates is low, and there is a substantial difference in the occurrence of AMR of APP8 in Norway and the UK. By applying relevant metainformation about the source of the strains, we increased our understanding of their correlation to genetic traits such as AMR. Likely a result of the closed pig population strategies, there is no evidence of recent transmission of *A. pleuropneumoniae* into Norway from Denmark and the UK, and the last common ancestor dates more than 200 years back. Our results indicate that the genetic variability found within and among the APP8 populations is influenced not only by inherent biologic characteristics that affect the transmission of the bacteria, but also heavily influenced by social and political strategies and regulations that affect the host population dynamics. A baseline phylogeny of *A. pleuropneumoniae* as provided by this study, will likely be of great value to future surveillance and control of this pathogen, partly because it increases our understanding of the effects of restricted animal movement nationally and internationally and enables the discovery of introductions of new genetic lineages.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena/browser/view/PRJEB47034, ENA, PRJEB47034.

AUTHOR CONTRIBUTIONS

ØA, JB, CG, CK, BR, SG, TK, MS, PL, YL, and LC conceptualized and designed the study. ØA, YL, and LC handled the bacterial samples. MS and ØA performed the bioinformatic analyses. MS, ØA, CK, JB, and LC analyzed the data and interpreted the results. JB and LC wrote the main body of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

We are grateful for the funding provided by the Agricultural Agreement Research Fund and the Foundation for Research Levy on Agricultural Products, grant number NFR-267422, and the UK Biotechnology and Biological Sciences Research Council (BB/S002103/1, BB/S005897/1, and BB/G018553/1). We extend our gratitude to our collaborators who also funded the research: Animalia, Nortura and KLF. The Norwegian Veterinary Institute, Statens Serum Institute and Imperial College covered all costs related to sample handling, storing, and shipping.

ACKNOWLEDGMENTS

The authors would like to thank Hülya Kaya at the Department of Bacteria, Parasites and Fungi at Statens Serum Institut, Bjarne Bergsjø at the NVI bacteriology department and Torfinn Moldal at the NVI molecular biology department for their valuable contributions in sample handling and analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.729637/full#supplementary-material

REFERENCES

Anjum, M. F., Zankari, E., and Hasman, H. (2017). Molecular methods for detection of antimicrobial resistance. *Microbiol. Spectr.* 5, 5–6. doi: 10.1128/microbiolspec.ARBA-0011-2017

Asawa, T., Kobayashi, H., Mitani, K., Ito, N., and Morozumi, T. (1995). Serotypes and antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolated from piglets with pleuropneumonia. *J. Vet. Med. Sci.* 57, 757–759. doi: 10.1292/jvms.57.757

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Bossé, J. T., Chaudhuri, R. R., Li, Y., Leanse, L. G., Fernandez Crespo, R., Coupland, P., et al. (2016a). Complete genome sequence of MIDG2331, a genetically tractable serovar 8 clinical isolate of *Actinobacillus pleuropneumoniae*. *Genome Announc.* 4, e01667–e01615. doi: 10.1128/genomeA.01667-15

Bossé, J. T., Li, Y., Atherton, T. G., Walker, S., Williamson, S. M., Rogers, J., et al. (2015). Characterisation of a mobilisable plasmid conferring florfenicol and chloramphenicol resistance in *Actinobacillus pleuropneumoniae*. *Vet. Microbiol.* 178, 279–282. doi: 10.1016/j.vetmic.2015.05.020

Bossé, J. T., Li, Y., Fernandez Crespo, R., Angen, O., Holden, M. T. G., Weinert, L. A., et al. (2020). Draft genome sequences of the type strains of *Actinobacillus indolicus* (46K2C) and *Actinobacillus porcinus* (NM319), two NAD-dependent bacterial species found in the respiratory tract of pigs. *Microbiol. Resour. Announc.* 9, e00716–e00719. doi: 10.1128/MRA.00716-19

Bossé, J. T., Li, Y., Fernandez Crespo, R., Chaudhuri, R. R., Rogers, J., Holden, M. T. G., et al. (2016b). ICE*Apl1*, an integrative conjugative element related to ICE*Hin1056*, identified in the pig pathogen *Actinobacillus pleuropneumoniae*. *Front. Microbiol.* 7:810. doi: 10.3389/fmicb.2016.00810

Bossé, J. T., Li, Y., Rogers, J., Fernandez Crespo, R., Li, Y., Chaudhuri, R. R., et al. (2017). Whole genome sequencing for surveillance of antimicrobial resistance in *Actinobacillus pleuropneumoniae*. *Front. Microbiol.* 8:311. doi: 10.3389/fmicb.2017.00311

Bouckaert, R. (2010). DensiTree: making sense of sets of phylogenetic trees. *Bioinformatics* 26, 1372–1373. doi: 10.1093/bioinformatics/btq110

Chiers, K., Donne, E., Van Overbeke, I., Ducatelle, R., and Haesebrouck, F. (2002). *Actinobacillus pleuropneumoniae* infections in closed swine herds: infection patterns and serological profiles. *Vet. Microbiol.* 85, 343–352. doi: 10.1016/S0378-1135(01)00518-1

Cohen, L. M., Grøntvedt, C. A., Klem, T. B., Gulliksen, S. M., Ranheim, B., Nielsen, J. P., et al. (2020). A descriptive study of acute outbreaks of respiratory disease in Norwegian fattening pig herds. *Acta Vet. Scand.* 62:35. doi: 10.1186/s13028-020-00529-z

Croucher, N. J., Page, A. J., Connor, T. R., Delaney, A. J., Keane, J. A., Bentley, S. D., et al. (2015). Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res.* 43:e15. doi: 10.1093/nar/gku1196

DANMAP 1996 (1997). Consumption of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria From Food Animals, Food and Humans in Denmark. Available at: https://www.danmap.org/-/media/Sites/danmap/Downloads/Reports/1996-2010/Danmap_1996_UK.ashx?la=da&hash=4629AF44E3CCAD9961DF026498A04E17B52002C8 (Accessed April 13, 2021).

DANMAP 2019 (2020). Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria From Food Animals, Food and Humans in Denmark. Available at: https://www.danmap.org/-/media/Sites/danmap/Downloads/Reports/2019/DANMAP_2019.ashx?la=da&hash=AA1939EB449203EF0684440AC1477FFCE2156BA5 (Accessed April 13, 2021).

European Medicines Agency (2018). Sales of Veterinary Antimicrobial Agents in 30 EU/EEA Countries in 2016. Available at: https://www.ema.europa.eu/en/veterinary-regulatory/overview/antimicrobial-resistance/european-surveillance-veterinary-antimicrobial-consumption-esvac#annual-report-on-sales-of-veterinary-antibiotics-section (Accessed April 13, 2021).

Fablet, C., Marois, C., Kuntz-Simon, G., Rose, N., Dorenlor, V., Eono, F., et al. (2011). Longitudinal study of respiratory infection patterns of breeding sows in five farrow-to-finish herds. *Vet. Microbiol.* 147, 329–339. doi: 10.1016/j.vetmic.2010.07.005

FAO (2016). Drivers, Dynamics and Epidemiology of Antimicrobial Resistance in Animal Production. Available at: http://www.fao.org/3/a-i6209e.pdf (Accessed April 13, 2021).

Gottschalk, M. (2015). The challenge of detecting herds sub-clinically infected with *Actinobacillus pleuropneumoniae*. *Vet. J.* 206, 30–38. doi: 10.1016/j.tvjl.2015.06.016

Gottschalk, M., and Broes, A. (2019). "Actinobaccilosis," in *Diseases of Swine. 11th Edn*. eds. J. J. Zimmermann, L. A. Karriker, A. Ramirez, K. J. Schwartz and G. W. Stevenson (Hoboken NJ, USA: John Wiley & Sons, Inc.), 749–766.

Hampson, D. J., Blackall, P. J., Woodward, J. M., and Lymbery, A. J. (1993). Genetic analysis of *Actinobacillus pleuropneumoniae*, and comparison with *Haemophilus* spp. taxon "minor group" and taxon C. *Zentralbl. Bakteriol.* 279, 83–91. doi: 10.1016/S0934-8840(11)80494-9

Kim, B., Hur, J., Lee, J. Y., Choi, Y., and Lee, J. H. (2016). Molecular serotyping and antimicrobial resistance profiles of *Actinobacillus pleuropneumoniae* isolated from pigs in South Korea. *Vet. Quart.* 36, 137–144. doi: 10.1080/01652176.2016.1155241

Klinkenberg, D., Tobias, T. J., Bouma, A., van Leengoed, L. A., and Stegeman, J. A. (2014). Simulation study of the mechanisms underlying outbreaks of clinical disease caused by *Actinobacillus pleuropneumoniae* in finishing pigs. *Vet. J.* 202, 99–105. doi: 10.1016/j.tvjl.2014.06.015

Kokotovic, B., and Angen, O. (2007). Genetic diversity of *Actinobacillus pleuropneumoniae* assessed by amplified fragment length polymorphism analysis. *J. Clin. Microbiol.* 45, 3921–3929. doi: 10.1128/JCM.00906-07

Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C., et al. (2004). Versatile and open software for comparing large genomes. *Genome Biol.* 5:R12. doi: 10.1186/gb-2004-5-2-r12

Lee, K.-E., Choi, H.-W., Kim, H.-H., Song, J.-Y., and Yang, D.-K. (2015). Prevalence and characterization of *Actinobacillus pleuropneumoniae* isolated from Korean pigs. *J. Bacteriol. Virol.* 45, 19–25. doi: 10.4167/jbv.2015.45.1.19

Li, Y., Bossé, J. T., Williamson, S. M., Maskell, D. J., Tucker, A. W., Wren, B. W., et al. (2016). *Actinobacillus pleuropneumoniae* serovar 8 predominates in England and Wales. *Vet. Rec.* 179:276. doi: 10.1136/vr.103820

Møller, K., Nielsen, R., Andersen, L. V., and Kilian, M. (1992). Clonal analysis of the *Actinobacillus pleuropneumoniae* population in a geographically restricted area by multilocus enzyme electrophoresis. *J. Clin. Microbiol.* 30, 623–627. doi: 10.1128/jcm.30.3.623-627.1992

Nielsen, R., and O'Connor, P. J. (1984). Serological characterization of 8 *Haemophilus pleuropneumoniae* strains and proposal of a new serotype: serotype 8. *Acta Vet. Scand.* 25, 96–106. doi: 10.1186/BF03547283

Nguyen, L.-T., Schmidt, H. A., von Haeseler, A., and Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32, 268–274. doi: 10.1093/molbev/msu300

NORM/NORM-VET 2019 (2020). Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Available at: https://www.vetinst.no/en/surveillance-programmes/norm-norm-vet-report/_/attachment/download/df4ceec7-f93c-435d-85ba-4376819bc1db:b912eb920994ba60f90889595a43d4e5e63c9ea1/NORM%20NORM-VET%202019.pdf (Accessed April 13, 2021).

Norwegian Food Safety Authorities (2012). Organisation. Available at: https://www.mattilsynet.no/language/english/about_us/organisation.2315 (Accessed January 19, 2021).

Norwegian Medicines Agency (2012). Terapianbefaling: bruk av antibakterielle midler til produksjonsdyr [Therapy recommendations: Use of antimicrobial drugs in production animals]. Available at: https://legemiddelverket.no/Documents/Veterin%C3%A6rmedisin/Terapianbefalinger/Terapianbefaling_bruk%20av%20antibakteriellt%20midler%20til%20produks.pdf (Accessed April 13, 2021).

Norwegian Veterinary Institute (2021b). "Smittsom lunge- og brysthinnebetennelse (APP)," [Porcine pleuropneumonia (APP)]. Available at: https://www.vetinst.no/sykdom-og-agens/smittsom-lunge-og-brysthinnebetennelse (Accessed 23.08.2021).

Norwegian Veterinary Institute (2021a). Dyrehelserapporten 2020 [Animal health report]. Available at: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2021/dyrehelserapporten-2020/_/attachment/download/8f53b3fa-4b0e-4861-aa7d-34eff9810631:ce08be5866e2608454b375ac5d41b003a4d4aec3/Dyrehelserapporten%202020.pdf (Accessed August 03, 2021).

O'Neill, C., Jones, S. C., Bossé, J. T., Watson, C. M., Williamson, S. M., Rycroft, A. N., et al. (2010). Prevalence of Actinobacillus pleuropneumoniae serovars in England and Wales. *Vet. Rec.* 167, 661–662. doi: 10.1136/vr.c5106

Prado, I. G. O., da Silva, G. C., Crispim, J. S., Vidigal, P. M. P., Nascimento, M., Santana, M. F., et al. (2020). Comparative genomics of *Actinobacillus pleuropneumoniae* serotype 8 reveals the importance of prophages in the genetic variability of the species. *Int. J. Genomics* 2020:9354204. doi: 10.1155/2020/9354204

Sahl, J. W., Lemmer, D., Travis, J., Schupp, J. M., Gillece, J. D., Aziz, M., et al. (2016). NASP: an accurate, rapid method for the identification of SNPs in WGS datasets that supports flexible input and output formats. *Microb. Genom.* 2:e000074. doi: 10.1099/mgen.0.000074

Sassu, E. L., Bossé, J. T., Tobias, T. J., Gottschalk, M., Langford, P. R., and Hennig-Pauka, I. (2017). Update on *Actinobacillus pleuropneumoniae*-knowledge, gaps and challenges. *Transbound. Emerg. Dis.* 65(Suppl. 1), 72–90. doi: 10.1111/tbed.12739

Stringer, O., Bossé, J., Lacouture, S., Gottschalk, M., Fodor, L., Angen, Ø., et al. (2021). Proposal of *Actinobacillus pleuropneumoniae* serovar 19, and reformulation of previous multiplex PCRs for capsule-specific typing of all known serovars. *Vet. Microbiol.* 255:109021. doi: 10.1016/j.vetmic.2021.109021

Tobias, T. J., Klinkenberg, D., Bouma, A., van den Broek, J., Daemen, A. J., Wagenaar, J. A., et al. (2014). A cohort study on *Actinobacillus pleuropneumoniae* colonisation in suckling piglets. *Prev. Vet. Med.* 114, 223–230. doi: 10.1016/j.prevetmed.2014.02.008

UK-VARSS (2020). Veterinary Antibiotic Resistance and Sales Surveillance Report (UK-VARSS 2019). Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/950126/UK-VARSS_2019_Report__2020-TPaccessible.pdf (Accessed April 13, 2021).

Velthuis, A. G., De Jong, M. C., Kamp, E. M., Stockhofe, N., and Verheijden, J. H. (2003). Design and analysis of an *Actinobacillus pleuropneumoniae* transmission experiment. *Prev. Vet. Med.* 60, 53–68. doi: 10.1016/S0167-5877(03)00082-5

Veterinary Medicines Direcorate (2019). UK Veterinary Antibiotic Resistance and Sales Surveillance Report (UK-VARSS 2018). Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/909987/_1587146-v23-VARSS_2018_Report__2019_-accessible.pdf (Accessed April 13, 2021).

Vigre, H., Angen, O., Barfod, K., Lavritsen, D. T., and Sorensen, V. (2002). Transmission of *Actinobacillus pleuropneumoniae* in pigs under field-like conditions: emphasis on tonsillar colonisation and passively acquired colostral antibodies. *Vet. Microbiol.* 89, 151–159. doi: 10.1016/S0378-1135(02)00149-9

Yee, S., Blackall, P. J., and Turni, C. (2018). Genetic diversity and toxin gene distribution among serovars of *Actinobacillus pleuropneumoniae* from Australian pigs. *Aust. Vet. J.* 96, 17–23. doi: 10.1111/avj.12660

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

III

A descriptive study of the biosecurity levels in Norwegian fattening pig herds with and without outbreaks of respiratory disease

Liza Miriam Cohen[1]*, Carl Andreas Grøntvedt[2], Stine Margrethe Gulliksen[3] and Camilla Kielland[1]

[1] Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU) P.O. Box 5003, NO-1432 Ås, Norway

[2] Norwegian Veterinary Institute, P.O. Box 64, Ås, Norway

[3] Animalia, P.O. Box 396, Økern, Norway

Abstract

Background

Biosecurity is a set of measures designed to reduce the risk of introduction, establishment and spread of animal diseases, from and within an animal population. In Norway there is a high level of national biosecurity protecting the favorable health status of Norwegian pigs. However, there are no studies describing the current external and internal biosecurity levels of Norwegian fattening pig herds. This study used Biocheck.UGent™ which is an acknowledged tool for objective soring of biosecurity at the herd level. Biocheck.UGent™ uses a questionnaire to assign a score of the total, external and internal biosecurity for each herd. The maximum score is 100.

The objective of this study was to describe the biosecurity levels in Norwegian fattening pig herds with outbreaks of respiratory disease (case) caused by *Actinobacillus pleuropneumoniae* and in non-outbreak (control) herds.

Results

The total, subtotal external and subtotal internal Biocheck.UGent™ scores for the outbreak herds (n=7) were 61, 64 and 57, respectively. The total, subtotal external and subtotal internal Biocheck.UGent™ scores for the non-outbreak herds (n=7) were 60, 69 and 61, respectively. Both herd groups scored higher on external than internal biosecurity. Both herd groups had implemented measures to reduce the risk of introduction and spread of infectious disease through purchase of livestock, and management implementation in the finishing unit, while measures between compartments were poorly implemented.

Conclusion

Objective scoring of biosecurity in Norwegian fattening pig herds showed no difference between herds with outbreaks of respiratory disease and in non-outbreak herds.

Keywords

Biosecurity, Biocheck.UGent™, fattening pigs, pig production, respiratory disease

Background

What is biosecurity

Biosecurity is of utmost importance to prevent infectious diseases both in human and veterinary medicine. The term biosecurity is defined as *a set of management and physical measures designed to reduce the risk of introduction, establishment and spread of animal diseases, infections, or infestations to, from and within an animal population* (1). An animal population is defined as *a group of units sharing a common defined characteristic* (1), e.g., nation or herd. Measures to mitigate risk of introduction and spread of disease build on knowledge of general infection biology and specific disease epidemiology (2). Hence, we can address biosecurity through generalized principles or more tailored measures for specific production types and diseases. Biosecurity is often divided into external biosecurity (EXT) and internal biosecurity (INT) (2). The EXT prevents introduction of infectious agents to susceptible populations, whereas the INT prevents the spread of infectious agents in susceptible populations when the agent is already present (2).

How can we quantify biosecurity?

While quantifying biosecurity in a herd can be challenging, it can provide better grounds for objective assessment and comparison. The overall biosecurity is impacted by many factors representing different risks. A weighted scoring system is therefore useful. Biocheck.UGent™ is a tool established by Ghent University, Belgium (3), for objective biosecurity scoring at herd level in poultry, cattle and pig herds (4). A detailed questionnaire is the basis for a biosecurity scoring within 12 defined areas of the production, differentiating between EXT and INT biosecurity. The questionnaire is paired with a knowledge database that provides advice targeted at the areas of improvement (4).

The Biocheck.UGent™ is risk based, accounting for risks associated with known transmission routes of infectious agents (5). In a review of transmission routes for 24 porcine pathogens of global importance, direct contact between animals is the only route that applies for all pathogens (6). This is in agreement with the international standards of the World Organization for Animal Health (OIE), stating that segregation of animals is the most important element of herd biosecurity, followed by cleaning and disinfection (2). Other, indirect, transmission routes include via semen, manure, animal vectors, insect vectors, aerosols, water and feed and fomites (transport vehicles, people, equipment, and clothing).

Respiratory infections and biosecurity

Targeted biosecurity measures against porcine respiratory disease requires knowledge of relevant infectious agents. Infectious respiratory diseases contribute to reduced animal welfare, decreased production efficiency, and increased use of antimicrobial drugs. Respiratory diseases is one of the most common health concerns to fattening pigs (7). In the current paper, "fattening" is referring to both the fattening and finishing stages. Infectious agents that cause respiratory disease in pigs can be divided into primary and secondary invaders (8). Primary invaders can cause disease on their own. Secondary invaders are often resident in the respiratory tract and will only contribute to disease development following a primary infection and/or a reduction in the pig's respiratory defenses (8). Important and widespread primary respiratory viruses include Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Swine Influenza Virus (SIV) and Porcine Circovirus type 2 (PCV2)(7). Common primary respiratory bacteria are *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* (7). Parasitic infestations that affect the respiratory tract also occur, particularly larval migration of *Ascaris suum* (8). Generally, respiratory viruses can be considered more infective than bacteria and parasites, reflected by known transmission routes (6). Direct transmission applies to all the above-mentioned agents, while aerosol transmission is common for viruses. Potential for indirect transmission depend on the agents' ability to survive outside the host (6, 9). Due to differences between agents, there are no specific biosecurity measures for control of all respiratory diseases. For instance, possible transmission routes for PRRSV includes direct

contact, and via people, semen, manure, animal and insect vectors, aerosols, animal feed, water, and fomites. Relevant biosecurity measures therefore encompass most areas of the production. On the other hand, *A. pleuropneumoniae* transmits by direct contact, short distance aerosols, and to some extent water and fomites (9-11). Relevant EXT measures include routines for purchase of animals from sources with known health status, and distance from neighboring farms. Relevant INT measures include all-in-all-out practice per compartment and pen, and routines for cleaning and disinfection between batches (9). Biosecurity measures may be less effective in reducing the risk of disease by commensal secondary invaders (12). Biocheck.UGent™ is not targeted towards specific agents. The results must be interpreted with attention to transmission routes of agents that one wishes to prevent.

Norwegian conditions

There are substantial differences in the occurrence of infectious respiratory agents between countries, subpopulations and farms within a country (8). Differences in population health status is closely linked to structure and regulation of the pig production. The commercial pig production system in Norway is structured in a tiered pyramid. The livestock flow is unidirectional from a limited number of self-recruiting genetic nucleus breeding herds at the top, through multiplier herds, to commercial farrow-to-feed, farrow-to-finish or specialized fattening herds in the lower tiers (13). Commercial herds purchase livestock from one or multiple supplier herds. Most livestock trade is coordinated regionally by representatives of the pig production sector, usually the slaughterhouse. There has been a shift towards larger farms during the last 30 years in many countries globally including Norway (14). Production volume in Norwegian pig farms is regulated by national legislation (15). Fattening pig herds are limited to 2100 fatteners yearly, which is smaller than farm sizes in many other modern pig producing countries (16, 17). National measures to maintain a favorable health status for Norwegian pigs include, but are not limited to, strict import regulation (18-20), and legal biosecurity requirements in the *Regulation on keeping of pigs* aimed at good hygienic practices and prevention of disease transmission (21).

Occurrence of respiratory agents

Comprehensive active serosurveillance document the population-wide absence of several important porcine pathogens including PRRSV (22) and *M. hyopneumoniae* (23). Today, SIV H1N1pdm09 and *A. pleuropneumoniae* are perhaps the most widespread clinically relevant primary infectious respiratory agents in the Norwegian pig population (24, 25). *Actinobacillus pleuropneumoniae* is detected regularly during routine diagnostics of clinical respiratory disease (13), and was recently found as the etiologic agent in a study of outbreaks of respiratory disease in seven Norwegian fattening herds (25). Although PCV2 is endemically occurring in the population, its impact to the respiratory health in Norwegian pigs is not clear. Common respiratory secondary invaders, including *Pasteurella multocida* (7) and *Streptococcus suis* (8) and to some extent *Glässerella (Haemophilus) parasuis*, are also present in the population.

Practices to improve respiratory health

Due to endemicity of primary respiratory invaders in Norway (26) reducing the impact of especially *A. pleuropneumoniae* in fattening pigs has been prioritized. The Norwegian pig production sector have established high health breeding herds in the production pyramid (27), nationally eradicated *M. hyopneumoniae* (23) from the pig population, and the sector encourages herds to convert to Specific Pathogen Free (SPF) production (28). SPF herds are free from both porcine pleuropneumonia (*A. pleuropneumoniae*) and atrophic rhinitis (toxigenic *P. multocida)* in addition to swine dysentery (*Brachyspira hyodysenteriae*) and sarcoptic mange (*Sarcoptes scabiei var suis*). Furthermore, the sector has organized efforts to improve the respiratory health in herds with repeated problems of respiratory disease, although no records of the following activities have been published. Herd owners are advised to perform initial diagnostic procedures and initiate appropriate preventive measures, before establishing a vaccination protocol in combination with assignment of regular livestock suppliers to reduce mixing of animals from different sources (Gulliksen SM, swine veterinarian at Animalia, personal communication by email, 2021 Oct. 31.). After the eradication of *M. hyopneumoniae*, the pig production sector claims the biggest challenge has been related to the occurrence of *A. pleuropneumoniae* (28).

Health and biosecurity monitoring in fattening pig herds

Previous recordings of health parameters in Norwegian pig herds have varied with production type. There are specific requirements to health documentation in herds that supply livestock, i.e. breeding herds (27) and commercial sow herds (29). While health and welfare scorings are performed at slaughter, fattening pig herds have been less closely monitored through production. A system for documenting health, welfare, and hygiene data from all herds in the production system was established in 2019 (30). Systematic investigations of the current biosecurity levels in Norwegian fattening pig herds are lacking, and the available literature regarding scoring the biosecurity in fattening pig herds internationally is somewhat scarce (31-33).

In 2017 and 2018 a case/control study of respiratory disease in Norwegian fattening pig herds was performed, enrolling seven case herds with outbreaks of respiratory disease caused by *A. pleuropneumoniae*, and seven control herds (34). The biosecurity levels in these herds were assessed using the Biocheck.UGent™ tool.

Aim

The objective of this study was to describe the biosecurity levels in Norwegian fattening pig herds with outbreaks of respiratory disease (case) and in non-outbreak (control) herds.

Material and methods

Sampling

In 2017 and 2018, 14 fattening pig herds were investigated in a field study of respiratory disease in the Norwegian pig population to map the etiology of acute outbreaks of respiratory disease. Seven herds with outbreaks of respiratory disease (outbreak herds) where included. Seven control herds (non-outbreak herds) with no clinical signs of an ongoing outbreak of respiratory disease, were matched from the same practice area. The non-outbreak herds were suggested by veterinary field practitioners that contributed to the sampling. All herds were commercial fattening pig producers, either specialized fattening herds (n= 5 outbreak herds and 3 non-outbreak) or farrow-to-finish herds (n= 2 outbreak herds and 4 non-outbreak).

A more detailed description of the selection of herds has been described previously (25). The data collection happened according to the timeline in Figure 1.

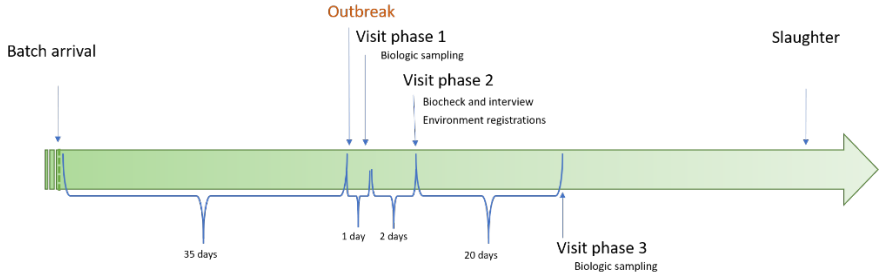

Figure 1 Timeline of data collection for this study. Median number of days between events are given.

Herd characteristics

Herd characteristics of the outbreak and non-outbreak herds, including number of livestock suppliers, pigs produced in a year, the herd size, the animal capacity of the compartment, the number of pigs in the compartment, the compartment volume per pig (m^3), floor space per pig (m^2), number of years of experience of the owner, and the number of people working on the herd (personnel) are presented in Table 1. Data were collected per compartment due to the nature of the sampling, which was performed during an ongoing outbreak of respiratory disease in the compartment. A description of the collection of herd characteristics data and a presentation of these descriptive statistics have been published previously (25).

220

221 ***Table 1*** Descriptive herd characteristics of the outbreak and non-outbreak herds.

	Outbreak (n=7)		**Non-outbreak (n=7)**	
Herd characteristics	M	IQR	M	IQR
Number of suppliers	2	7	1	0
Pigs produced (Yearly yield)	2109	1818	1543	1660
Herd size	650	310	500	350
Animal capacity in compartment	240	125	197	145
Pigs in compartment	155	90	199	249
Compartment volume per pig, m^3	3.9	2	4.3	1.5
Floor space per pig, m^2	1	0.2	1.1	0.2
Biocheck farm characteristics*				
Owner's years of experience	24	16	13	10
Personnel	2	1	3	2

222 M = median, IQR = Interquartile range

223 *Questions regarding herd characteristics are asked in the Biocheck.UGent™

224 questionnaire, but not factored into the biosecurity scoring.

225

Biosecurity quantification

The biosecurity levels in each herd were quantified using Biocheck.UGent™ for pigs. A Norwegian translation of the questionnaire was made for this study, in collaboration with the developers, and has been made available to the public on the Biocheck.UGent™ website (4). Biocheck.UGent™ for pigs is comprised of a detailed questionnaire that forms the basis for a quantitative scoring of biosecurity within 12 defined areas of the pig production (A-L). The first part of questionnaire is divided into six areas (A-F) which are used to quantify the EXT of a herd. The second part of the questionnaire covers another six areas (G-L), which quantifies the INT of a herd. The questionnaire was performed as an interview in Norwegian, by a single interviewer. The results are given as scores of the total biosecurity (TOT), EXT, and INT respectively. The score ranges from 0-100, where a high score indicates better implementation of the biosecurity measures in question. Both INT and EXT are comprised of several subcategories (Table 2). EXT includes purchase of livestock and semen, transport of animals, cadaver and manure handling, supply of equipment, feed and water, personnel and visitors, vermin and bird control and the location of the farm. INT includes the different compartments at the farm, and person and equipment traffic between compartments, handling of sick animals, cleaning, and disinfection during production and between batches. The subtotal EXT and INT score calculation is based on a weighting (Table 2) which reflects the relative associated risk of introducing or spreading infectious disease (5). Here we have reported the scores in the two groups within each subcategory, as well as the subtotal INT, EXT, and TOT (Table 3). For the specialized fattening herds (n=2 non-outbreak and 4 outbreak herds), questions related to farrowing and piglet management were not evaluated. Therefore, in the subcategories “H. Farrowing and suckling period” and “I. Nursery unit” only the farrow-to-finish herds are represented.

255 ***Table 2*** Worldwide average scores and weighting factors of Biocheck.UGent™, as per

256 September 9. 2021.

Biocheck.UGent™ category	*Worldwide average*	*Weigh (%)*
External biosecurity		
A. Purchase of breeding pigs, piglets, and semen	89	25
B. Transport of animals, removal of carcasses and manure	77	23
C. Feed, water, and equipment supply	50	15
D. Visitors and farmworkers	72	17
E. Vermin and bird control	73	10
F. Location of the farm	67	10
Subtotal external biosecurity	**74**	
Internal biosecurity		
G. Disease management	73	10
H. Farrowing and suckling period	58	*14*
I. Nursery unit	68	14
J. Finishing unit	75	14
K. Measures between compartments, working lines and use of equipment	53	28
L. Cleaning and disinfection	69	20
Subtotal internal biosecurity	**64**	
Total	**69**	

257 Number of completed surveys: 4494. Adapted from the Biocheck.UGent™ web page

258 (35).

Questions regarding herd characteristics that are asked in an introductory section of the Biocheck.UGent™ questionnaire, but not factored into the biosecurity scoring (Table 1).

Statistical analysis

Stata (STATA SE/15 for Windows; Stata Corp., College Station, Texas, USA), was utilized for descriptive analysis of data from the biosecurity scoring. Due to the small sample sizes, median values were used both when reporting biosecurity scores, and herd characteristics, where IQR was used to show variability about the mean.

Two-sample Wilcoxon rank-sum (Mann-Whitney) test was used to compare the scores of the outbreak and with the non-outbreak group, a non-parametric test for small sample sizes. It tests the hypothesis that there is no difference between the distribution of scores in the groups, indicated by a p-value of 0.05.

Results

The median TOT score was 61 in outbreak herds and 60 in non-outbreak herds. In both groups the score of EXT was higher than the INT. The median TOT and subtotal INT and EXT Biocheck.UGent™ scores, including all 12 subcategories A to L, are listed per group (outbreak/non-outbreak) in Table 3 and visualized in Figure 2.

279 ***Table 3*** Biocheck.UGent™ scores of the outbreak (n=7) and non-outbreak herds (n=7).

	Outbreak		Non-outbreak		Ranked sum test*
Biosecurity category	M	IQR	M	IQR	P-value
Total Biosecurity score	61	21	60	10	0.8
Subtotal external biosecurity score	64	17	69	16	0.18
A. Purchase of breeding pigs, piglets, and semen	80	32	74	28	0.75
B. Transport of animals, removal of carcasses and manure	67	18	67	13	0.75
C. Feed, water, and equipment supply	47	24	57	10	0.26
D. Visitors and farmworkers	24	35	65	64	0.19
E. Vermin and bird control	80	40	80	10	0.9
F. Location of the farm	60	30	70	40	0.22
Subtotal internal biosecurity score	57	36	61	25	1
G. Disease management	60	40	60	20	0.94
H. Farrowing and suckling period	33	7	40	26	0.62
I. Nursery unit	46	50	71	7	0.41
J. Finishing unit	86	50	100	14	0.15
K. Measures between compartments, working lines and the use of equipment	43	32	32	54	0.75
L. Cleaning and disinfection	75	35	50	25	0.48

280 M = median, IQR = Inter quartile range

281 *Wilcoxon ranked sum test

282 Farrowing and Nursing: n = 2 and 4 outbreak and non-outbreak herds, respectively.

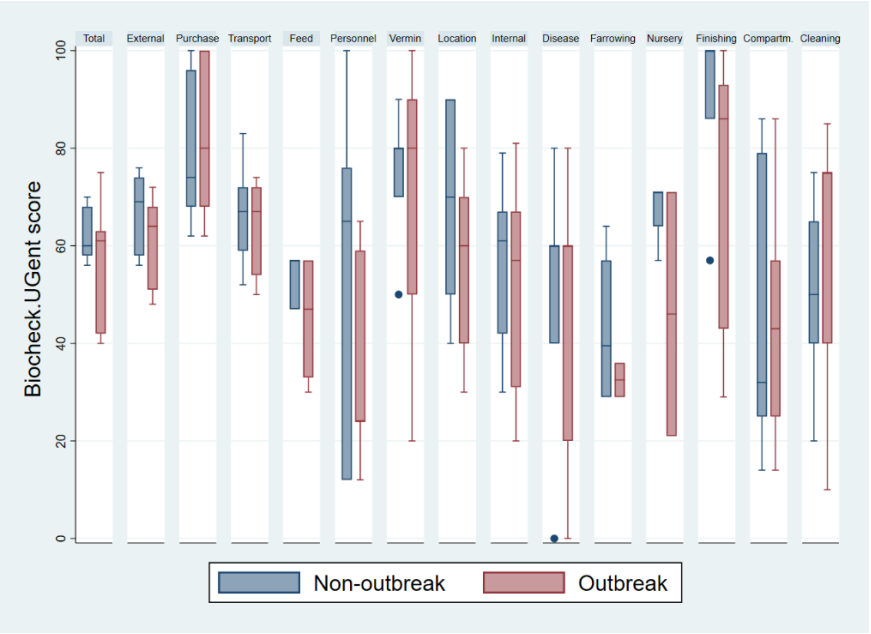

Figure 2 Box and whisker plot of Biocheck.UGent™ scores of the outbreak (n=7) and non-outbreak (n=7) herds.

Farrowing and Nursing: n = 2 and 4 outbreak and non-outbreak herds, respectively. Each graph representing the group scores for total, subtotal external, subtotal internal and 12 subcategories respectively. Horizontal line indicates median score. Blue dots outside the boxes represent outliers, single observations that are inconsistent with the remainder of the data.
Total = Total biosecurity score, External = Subtotal external biosecurity score, Purchase = Purchase of breeding pigs, piglets and semen, Transport = Transport of animals, removal of carcasses and manure, Feed = Feed, water, and equipment supply, Personnel = Visitors and farmworkers, Vermin = Vermin and bird control, Internal = Subtotal internal biosecurity score, Location. = Location of the farm, Disease = Disease management, Farrowing = Farrowing and suckling period, Nursery = Nursery unit, Finishing = Finishing unit, Compartm. = Measures between compartments, working lines and the use of equipment, Cleaning = Cleaning and disinfection.

In the outbreak herds, the subcategory with the lowest score was "D. Visitors and farmworkers" and in the control group, "K. Measures between compartments, working lines and the use of equipment" scored the lowest. In both herd groups, the highest subcategory scores were in the categories "J. Finishing unit", "A. Purchase of breeding pigs, piglets and semen" and "E. Vermin and bird control".

Non-outbreak herds were smaller and had fewer livestock suppliers than the outbreak herds (Table 1).

Discussion

The results in a global perspective

A quantification of the biosecurity levels of two groups of Norwegian fattening pig herds has been described in this study. There was no significant difference between the level of biosecurity in the outbreak herds and non-outbreak herds. The median EXT scores were higher than the INT scores in both groups.

The results from this study agree with results from comparable herd types from other European countries. The worldwide, and per country average scores (Table 2) are available on the Biocheck.UGent™ web page(4). According to Biocheck.UGent™, the average TOT score was 71, ranging from 54 to 77 per country, with 100 indicating a maximum level of biosecurity. These scores, and much of the literature regarding the use of Biocheck.UGent™ for pigs, discriminate poorly between herd types. Comparisons should be made between comparable herd types. The use of Biocheck.UGent™ to quantify biosecurity levels in fattening pigs has only been reported from a few other European countries (31, 32). Both groups in this present study scored relatively low compared to fattening pig herds in the UK, that had a median TOT score of 68.3, EXT score of 74.8 and INT score of 59.6 (31). Fattening pig herds from Belgium, Finland, Poland, and the UK, where anonymously scored in a study from 2020 (32) arguing that there is a variation in biosecurity scores in European countries. The groups in the present study scored within the range of these four countries, in which there was a median EXT score of 66 [58.5-75.3] and median INT score of 71 [45.5-71.2] (32). In three of the four

countries the EXT score was higher than the INT score (32), as observed for both groups in the present study.

Biosecurity in Norwegian pig herds

According to the scoring of Biocheck.UGent™ there was room for improved biosecurity in the herds in this study, similar to indications of biosecurity scorings in the UK, Finland, Poland, and Belgium (5, 31, 32). We can differentiate between the installations and procedures that constitute the hardware, and the actual implementation, understanding and compliance with measures and protocols as the software of biosecurity. For countries in the European Union (EU), the Animal Health law (36) encourages a more unified approach to biosecurity that is aligned with international standards like the OIE. While Norway is not a member of the EU, it is required to implement EU legislation through the European Economic Area (EEA) agreement. There are specific biosecurity requirements in Norwegian national legislation regarding pig production. The biosecurity requirements include but are not limited to the presence of a hygiene lock at the farm entrance and suitable areas for loading and unloading livestock for transport. There are also specific requirements regarding facilities for cleaning animal compartments and equipment, and facilities for changing clothes and personal hygiene (21). International and national legislation or regulations to implement biosecurity measures on farm level will lay down formal requirements to the farmers but will not automatically ensure compliance. The biosecurity framework of rules and regulations is probably more influential to the EXT, while INT to a larger degree is subject to the farmers own wishes and motivation (37). Better compliance requires a framework tailored to the production type, infrastructure, economic development state and perhaps to preexisting tradition (38). Knowledge and understanding of risks and consequences are crucial and will influence the attitudes of the farmers (39).

High national biosecurity level

Import restrictions hinder the most probable transmission routes for all agents, and thus reduces the likelihood of introducing infectious respiratory agents that are absent from the Norwegian pig population to a herd. Import legislation (19) and additional requirements of the production animal sector regarding import of live animals (18) have contributed to a high national

biosecurity in Norway at least for the last 30 years (18). Still, other transmission pathways could be relevant to the introduction of infectious agents to Norway, in which case the biosecurity measures implemented at the herd will be significant to the national health situation. Although more attention to biosecurity is likely in densely populated farms (5), small production units and a long distance between units contributes to segregation of pigs. Thus, a less concentrated production strengthens the EXT level of Norwegian farms. In the event of an introduction of new infectious respiratory agents, sound biosecurity practices to reduce contact points between herds could reduce the potential for spreading.

The first introduction of Porcine Respiratory Coronavirus (PRCV) to Norway was discovered through the national serosurveillance program (40). Although the source of this introduction remains unidentified this is a recent example of a breach in the national and herd EXT. PRCV has not been found to cause clinical disease in Norwegian pigs. The introduction of pandemic swine influenza virus (SIV H1N1 pdm09) in 2009 (41) is another example of an EXT breach, where virus most likely transmitted to pig herds from infectious personnel. No introduction of other SIV serovars to the Norwegian pig population prior to or in the years after the SIV pandemic has been detected (22), as these are not commonly transmitted via humans, and the biosecurity measures in place likely provide sufficient protection against their introduction.

Characteristics of the outbreak and non-outbreak herds

Some herd characteristics such as farm size and age of farm buildings might be relevant to disease prevention. Non-outbreak herds were smaller and had fewer livestock suppliers than the outbreak herds. Previous studies have found a link between farm size and level of biosecurity, but disagree whether the association is positive (33) or negative (42). It has been discussed that bigger farms pay more attention to biosecurity due to relatively bigger economic consequences of introducing infectious agents to the herd (33). Since production volume per holding in Norway is restricted by law, the batch sizes (fattening pigs) are limited by these regulations. Many producers are contracted to regular supply from the same supplying herd. Mixing of pigs from different sources still occurs in herds that have a higher yield than a single

supplier can deliver or when producers buy fatteners on the open market. One of the two biggest slaughterhouse groups in Norway, reported that 70-80% of fattening pigs were sold through regular contracts in 2017 (43). However, if supplying herds ensure a homogenous health status to relevant infectious agents, the biosecurity is not compromised simply by having a higher number of suppliers. Targeted testing and surveillance of relevant infectious agents is already implemented and documented in the Norwegian breeding herds (27), and within the SPF system (44). Alternatively, proper segregation of pigs from sources of different health status, and all-in-all-out rearing per compartment, could prevent transmission of infectious agents between them. A negative association between age of the buildings and EXT has been described (5). Older buildings that were not built with biosecurity practices in mind is an example of how facilities unsuitable to ensure proper segregation through compartmentalization can influence the biosecurity. Questions regarding livestock supply and health status (EXT) as well as compartmentalization (INT) are included in the Biocheck.UGent™ assessment.

Subcategories

Purchase of animals and management implementation in the finishing unit restrict the direct animal contact and are thus likely the areas of the production that are most relevant to the control of respiratory disease agents. High EXT scores in subcategory A suggest that measures to reduce the risk of introducing infectious agents through purchased livestock and genetic material (semen) had been implemented. Herds that are self-recruiting will achieve a high score in this category (5). High scores were also achieved because of the health requirements that apply for supplier herds in Norway (27, 30, 45). The difference in number of suppliers was thus not reflected in a different EXT score between the two herd groups in this study. High INT scores in subcategory J implied that the herds practiced measures in the finishing pig unit to limit direct animal contact points and animal density, including all-in-all-out systems per pen and per unit.

Even if direct contact is thought to be the primary transmission route, measures between compartments could be relevant, for instance for the spread of *A. pleuropneumoniae* (11). Low INT scores in the subcategory K

indicated that change of clothing or shoes, handwashing or use of separate equipment between compartments was lacking.

A high EXT score in the subcategory E implied that routines to keep vermin, birds, and companion animals out of contact with the pigs were in place, including a rodent-control program. A low EXT score in category D indicated that either the structure or implementation of the hygiene lock at the farm entrance was suboptimal. Norwegian pig farmers are required to have a hygiene lock (21), which are regulated by national guides. Measures related to the hygiene lock and vermin and bird control may however not be so relevant to the introduction of respiratory disease agents, particularly *A. pleuropneumoniae* which is the most relevant agent to the herds in this study.

The link between biosecurity and respiratory disease

Biocheck.UGent™ is a general and all-encompassing biosecurity tool to quantify the general level of biosecurity. The tool is not fit for mapping risk factors for specific infectious agents. Associations between biosecurity and other herd parameters such as antimicrobial drug use, animal welfare, pig health and production efficiency have been made, however causal relationships were not determined (12, 31, 46). Although biosecurity influences the introduction, establishment and spread of infectious respiratory agents, respiratory diseases are multifactorial and a causal relationship between biosecurity implementation and occurrence of respiratory outbreaks cannot be made in this study.

Bias and generalizability

Due to the study design, the inclusion criteria for the case and control group, and the low number of included herds, generalizability to the general pig population of Norway was low. The small sample sizes also made the results uncertain. Even so, results such as a high EXT and a lower INT, regardless of the herd group, was similar to findings from other studies. This indicates that even with few herds, this descriptive study may be indicative of biosecurity levels in Norwegian fattening pig herds. A strength of this study is that the biosecurity assessment was performed by one interviewer, to minimize the observer bias. There is some doubt regarding the fit of Biocheck.UGent™ for a comparison of Norwegian herds due to the absence of many infectious agents

that the biosecurity measures are targeted at, and farm sizes are small and farm density and animal density is so low that nuances will not be registered. The tool was however deemed fit for a general scoring of biosecurity in Norwegian fattening pig herds, and context was gained by viewing the scores against other European countries, and what is known about the national biosecurity in Norway.

Conclusion

Quantifying the biosecurity using Biocheck.UGent™, where the maximum scores is 100, the median total score was 61 in outbreak herds and 60 in non-outbreak herds. Both herd groups scored higher on external than internal biosecurity. Both herd groups had implemented measures to reduce the risk of introduction and spread of infectious disease through purchase of livestock, and management implementation in the finishing unit, while measures between compartments were poorly implemented. A high level of national biosecurity in Norway which reduces the risk of introduction, establishment and spread of porcine respiratory diseases is arguably the most important contribution to the health status of the Norwegian pig population. The objective scoring of biosecurity in Norwegian fattening pig herds showed no difference between herds with outbreaks of respiratory disease and in non-outbreak herds.

List of abbreviations

EXT: external biosecurity

INT: internal biosecurity

IQR: Inter quartile range

PRCV = Porcine Respiratory Corona Virus

PCV2 = Porcine Circovirus type 2

PRRSV: Porcine Reproductive and Respiratory Syndrome Virus

SIV: Swine Influenza Virus

TOT: total Biocheck.UGent™ score

Declarations

Ethics approval and consent to participate

An ethics approval was not applicable for this study since no personal identifiable data or data from animals was included. Consent to participate has been given through a signed statement by the recruited herd owners.

Consent for publication

Consent for publication has been given through a signed statement for participation by the recruited herd owners.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests.

Funding

We are grateful for the funding provided by the Agricultural Agreement Research Fund and the Foundation for Research Levy on Agricultural Products, grant number NFR-267422. We extend our gratitude to our collaborators who also funded the research: Animalia, Nortura and KLF.

Authors' contributions

LMC, CAG, SMG and CK conceptualized and designed the study. LMC translated the biosecurity test. LMC and CK collected the data and performed the data analysis. LMC, CAG, SMG and CK interpreted the results. LMC wrote the main body of the manuscript. All authors contributed to manuscript revisions and manuscript approval.

Acknowledgements

The authors would like to thank all members of the project Grisefine lunger (NFR-267422) for their support and contributions to discussions during this work.

References

1. OIE. "Terrestrial Animal Health Code ". World Organization for Animal Health; 2021. https://www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-code-online-access/. Accessed 23. November, 2021.

2. FAO, OIE, World Bank. Good practices for biosecurity in the pig sector – Issues and options in developing and transition countries: Food and Agriculture Organization of the United Nations, World Organisation for Animal Health, World Bank; 2010. Available from: http://www.fao.org/3/i1435e/i1435e.pdf.

3. Laanen M, Beek J, Ribbens S, Vangroenweghe F, Maes D, Dewulf J, et al. Bioveiligheid op varkensbedrijven: Ontwikkeling van een online scoresysteem en de resultaten van de eerste 99 deelnemende bedrijven. Vlaams Diergeneeskd Tijdschr. 2010;79:302-6.

4. Ghent University. "Biocheck.UGent". http://www.biocheck.ugent.be/. Accessed 17. October, 2018.

5. Laanen M, Persoons D, Ribbens S, de Jong E, Callens B, Strubbe M, et al. Relationship between biosecurity and production/antimicrobial treatment characteristics in pig herds. Vet J 2013;198(2):508-12. https://dx.doi.org/10.1016/j.tvjl.2013.08.029.

6. Filippitzi ME, Brinch Kruse A, Postma M, Sarrazin S, Maes D, Alban L, et al. Review of transmission routes of 24 infectious diseases preventable by biosecurity measures and comparison of the implementation of these measures in pig herds in six European countries. Transbound Emerg Dis. 2017;2018(65):381-98. https://dx.doi.org/10.1111/tbed.12758

7. Brockmeier SL, Halbur PG, Thacker EL. Porcine Respiratory Disease Complex. 2002 [cited 16. August 2021]. In: Polymicrobial Diseases [Internet].

549 Washington DC: ASM Press. Available from:
550 https://www.ncbi.nlm.nih.gov/books/NBK2481/.

551 8. Yaeger MJ, Van Alstine WG. Respiratory System. In: Zimmermann JJ,
552 Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of
553 Swine. 11 ed. Hoboken, NJ: John Wiley & Sons, Inc.; 2019. 21 p. 393-407.

554 9. Amass SF. Biosecurity: Stopping the bugs from getting in (part 1 of
555 2). Pig Journal. 2005; 55:104-14. Available from:
556 https://www.thepigsite.com/articles/biosecurity-stopping-the-bugs-from-
557 getting-in-part-1-of-2

558 10. Tobias TJ, Bouma A, van den Broek J, van Nes A, Daemen AJJM,
559 Wagenaar JA, et al. Transmission of *Actinobacillus pleuropneumoniae* among
560 weaned piglets on endemically infected farms. Prev Vet Med.
561 2014;117(1):207-14. https://dx.doi.org/10.1016/j.prevetmed.2014.07.017

562 11. Assavacheep P, Rycroft AN. Survival of *Actinobacillus*
563 *pleuropneumoniae* outside the pig. Res Vet Sci. 2013;94(1):22-6.
564 https://dx.doi.org/10.1016/j.rvsc.2012.07.024

565 12. Stokstad M, Klem TB, Myrmel M, Oma VS, Toftaker I, Østeras O, et al.
566 Using biosecurity measures to combat respiratory disease in cattle: the
567 Norwegian control program for Bovine Respiratory Syncytial Virus and
568 Bovine Coronavirus. Front Vet Sci. 2020;7:167.
569 https://dx.doi.org/10.3389/fvets.2020.00167

570 13. Norwegian Veterinary Institute. Dyrehelserapporten 2020 [Animal
571 health report 2020]. 2021. Available from:
572 https://www.vetinst.no/rapporter-og-
573 publikasjoner/rapporter/2021/dyrehelserapporten-
574 2020/_/attachment/download/8f53b3fa-4b0e-4861-aa7d-
575 34eff9810631:ce08be5866e2608454b375ac5d41b003a4d4aec3/Dyrehelser
576 apporten%202020.pdf.

577 14. Jordbruksbedrifter med ymse hudyrslag, etter husdyrslag,
578 statistikkvariabel og år. Statistics Norway. 2021 [cited 18. September 2021].
579 Available from: https://www.ssb.no/statbank/table/05979.

15. Forskrift om regulering av svine- og fjørfeproduksjonen [Regulation on pig and poultry production]. FOR-2004-04-01-611. [Retrieved 23. November 2021] Available from: https://lovdata.no/dokument/SF/forskrift/2004-04-01-611

16. USDA. Swine 2012 Part III: Changes in the U.S. Swine Industry, 1995–2012: United States Department of Agriculture; 2017. Available from: https://www.aphis.usda.gov/animal_health/nahms/swine/downloads/swine2012/Swine2012_dr_Trends.pdf.

17. Pigs-sows: number of farms and heads by agricultural size of farm (UAA) and size of breeding sow herd. Eurostat. 2017 [cited 18. September 2021]. Available from: https://ec.europa.eu/eurostat/databrowser/view/ef_lssowsaa/default/bar?lang=en.

18. KOORIMP, KIF. Årsmelding 2020 KOORIMP og KIF [Annual report 2020 KOORIMP and KIF]. 2021. Available from: https://www.animalia.no/contentassets/7b27e28ef6bf4e878416cc6664a440e1/koorimp-arsmelding-2020-web.pdf.

19. Forskrift om tilsyn og kontroll ved import og eksport av levende dyr, annet avlsmateriale og animalsk avfall innen EØS, og ved import av levende dyr fra tredjestater [Regulation on live animal trade]. FOR-1998-12-31-1484. [Retrieved 23. November 2021] Available from: https://lovdata.no/dokument/SF/forskrift/1998-12-31-1484

20. Forskrift om dyrehelsemessige betingelser for innførsel og utførsel av svin [Regulation on live animal trade]. FOR-2002-03-25-304. [Retrieved 23. November 2021] Available from: https://lovdata.no/dokument/SF/forskrift/1998-12-31-1484

21. Forskrift om hold av svin [Regulation on keeping pigs]. FOR-2003-02-18-175. [Retrieved 23. November 2021] Available from: https://lovdata.no/dokument/SF/forskrift/2003-02-18-175

22. Grøntvedt CA, Nordstoga AB, Hopp P. The surveillance programme for specific viral infections in swine herds in Norway 2019: Norwegian Veterinary Institute; 2020. Available from:

https://www.vetinst.no/en/surveillance-programmes/viral-infections-in-swine/_/attachment/download/cd46413d-0741-4747-ae0b-3fa79a88e6d1:9cacbf766827e25338119a188e68c3d394a661ec/2020_OK_Viral%20infections%20in%20swine_Report%202019.pdf.

23. Gulliksen S, Baustad B, Framstad T, Jørgensen A, Skomsøy A, Kjelvik O, et al. Successful eradication of *Mycoplasma hyopneumoniae* from the Norwegian pig population – 10 years later. Porcine Health Manag. 2021; 7. https://dx.doi.org/10.1186/s40813-021-00216-z.

24. Grøntvedt CA, Er C, Gjerset B, Germundsson A, Framstad T, Brun E, et al. Clinical impact of infection with Pandemic Influenza (H1N1) 2009 Virus in naive nucleus and multiplier pig herds in Norway. Influenza Res Treat. 2011. https://dx.doi.org/10.1155/2011/163745.

25. Cohen LM, Grøntvedt CA, Klem TB, Gulliksen SM, Ranheim B, Nielsen JP, et al. A descriptive study of acute outbreaks of respiratory disease in Norwegian fattening pig herds. Acta Vet Scand. 2020; 62. https://dx.doi.org/10.1186/s13028-020-00529-z.

26. Ingris. Årsstatistikk 2016 [Annual statistics 2016]. Animalia, Norsvin; 2017. Available from: https://norsvin.no/wp-content/uploads/2018/05/Ingris_%C3%85rsstatistikk_2016.pdf.

27. Animalia. Helse- og Hygienereglementet for Foredlings- og Formeringsbesetninger [Health and hygiene regulations for nucleus and multiplier herds]. 2020. Available from: https://www.animalia.no/contentassets/c91ed15652954a52a631d595cb7b2592/2020-helse--og-hygienereglementet-for-avlsbesetninger.pdf.

28. Animalia. "SPF – systematisk satsing på friskere gris" [SPF - systematic efforts for healthier pigs]. Animalia; 2021 [updated 09.02.2021]. https://www.animalia.no/no/gomorning/dyrehelse/ny-spf--systematisk-satsing-pa-friskere-gris/. Accessed 16. August, 2021.

29. Ingris. Årsstatistikk 2020 [Annual statistics 2020]. Animalia, Norsvin; 2021. Available from: https://www.animalia.no/globalassets/ingris---dokumenter/arsstatistikk-2020-007---endelig.pdf.

30. Animalia. "Helsegrissystemet" [The healthy pig system]. Animalia; 2021 [updated 14. September 2021]. https://www.animalia.no/no/Dyr/svin/helsegrissystemet/. Accessed 29. September, 2021.

31. Pandolfi F, Edwards SA, Maes D, Kyriazakis I. Connecting Different Data Sources to Assess the Interconnections between Biosecurity, Health, Welfare, and Performance in Commercial Pig Farms in Great Britain. Front Vet Sci. 2018;5(41). https://dx.doi.org/10.3389/fvets.2018.00041

32. Chantziaras I, Dewulf J, Van Limbergen T, Stadejek T, Niemi J, Kyriazakis I, et al. Biosecurity levels of pig fattening farms from four EU countries and links with the farm characteristics. Livest Sci. 2020; 237. https://dx.doi.org/10.1016/j.livsci.2020.104037.

33. Boklund A, Alban L, Mortensen S, Houe H. Biosecurity in 116 Danish fattening swineherds: descriptive results and factor analysis. Prev Vet Med. 2004;66(1):49-62. https://dx.doi.org/10.1016/j.prevetmed.2004.08.004

34. Cohen LM, Bossé JT, Stegger M, Li Y, Langford PR, Kielland C, et al. Comparative Genome Sequence Analysis of *Actinobacillus pleuropneumoniae* Serovar 8 Isolates From Norway, Denmark, and the United Kingdom Indicates Distinct Phylogenetic Lineages and Differences in Distribution of Antimicrobial Resistance Genes. Front Microbiol. 2021; 12:2558. https://dx.doi.org/10.3389/fmicb.2021.729637.

35. Ghent University. Statistics for Pigs - Worldwide. Biocheck.UGent™, Available from: https://biocheck.ugent.be/en/worldwide?species=1&questionnaire=57&country=world

36. Regulation (EU) 2016/429 of the European Parliament and of the Council of 9 March 2016 on transmissible animal diseases and amending and repealing certain acts in the area of animal health ('Animal Health Law'). [Retrieved 08. September 2021] Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R0429&from=EN

37. Postma M, Backhans A, Collineau L, Loesken S, Sjölund M, Belloc C, et al. The biosecurity status and its associations with production and management characteristics in farrow-to-finish pig herds. Animal. 2016;10(3):478-89. https://dx.doi.org/10.1017/S1751731115002487

38. Ritter C, Jansen J, Roche S, Kelton DF, Adams CL, Orsel K, et al. Invited review: Determinants of farmers' adoption of management-based strategies for infectious disease prevention and control. Dairy Sci. 2017;100(5):3329-47. https://dx.doi.org/10.3168/jds.2016-11977

39. Laanen M, Maes D, Hendriksen C, Gelaude P, De Vliegher S, Rosseel Y, et al. Pig, cattle and poultry farmers with a known interest in research have comparable perspectives on disease prevention and on-farm biosecurity. Prev Vet Med. 2014;115(1-2):1-9. https://dx.doi.org/10.1016/j.prevetmed.2014.03.015

40. Grøntvedt CA, Nordstoga AB, Jonsson ME. The surveillance programme for specific viral infections in swine herds in Norway 2018: Norwegian Veterinary Institute; 2019. Available from: https://www.vetinst.no/en/surveillance-programmes/viral-infections-in-swine/_/attachment/download/e5793dca-57d2-41eb-bd34-9a99e5d11b98:ef9892272432bafa4b61bc30ae1130952606075b/2019_OK_Viral%20infections%20in%20swine_Report%202018.pdf.

41. Grøntvedt CA, Er C, Gjerset B, Hauge AG, Brun E, Jørgensen A, et al. Influenza A(H1N1)pdm09 virus infection in Norwegian swine herds 2009/10: the risk of human to swine transmission. Prev Vet Med. 2013;110(3-4):429-34. https://dx.doi.org/10.1016/j.prevetmed.2013.02.016

42. Scali F, Santucci G, Dewulf J, Alban L, Maisano AM, Giudici F, et al. Biosecurity in Italian pig farms - monitoring as a basis for targeted improvements. In: 13th SafePork; 26-29 August; Berlin 2019: p. 184-5.

43. Borgen B. "Salgsskjema for omsetting av smågris" [Sales form for fattening pigs]. Nortura; 2017. https://medlem.nortura.no/gris/livdyrformidling/salgsskjema/. Accessed 01. October, 2021.

44. Animalia. "SPF-besetninger" [SPF-herds]. 2020 [updated June 23. 2020]. https://www.animalia.no/no/Dyr/svin/spf--besetninger/. Accessed June 30., 2020.

45. Animalia. "Smittebeskyttelse og helseovervåkning i den norske svinepopulasjonen" [Biosecurity and health surveillance in the Norwegian pig population]. 2021 [updated 04. June 2021]. https://www.animalia.no/no/Dyr/svin/smittebeskyttelse-og-helseovervakning-i-norske-svinebesetninger/. Accessed 01. October, 2021.

46. Calderon Diaz JA, da Costa MR, Manzanilla EG. Biosecurity practices associated with negative farm status for *Mycoplasma hyopneumoniae*, porcine reproductive and respiratory syndrome virus, and swine influenza virus in farrow-to-finish pig farms. J Anim Sci. 2020;98(Supplement 3):6. https://dx.doi.org/10.1093/jas/skaa054.009

ISBN: 978-82-575-1879-0
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no