Design and validation of high-throughput real-time PCR systems for detection of porcine and bovine respiratory and enteric pathogens using the BioMark HD (Fluidigm) platform

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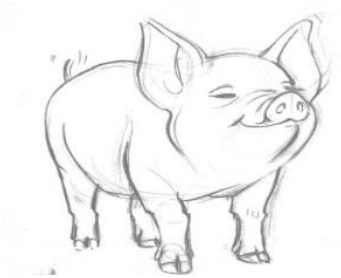
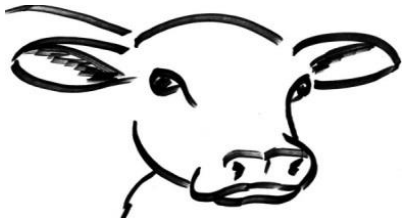
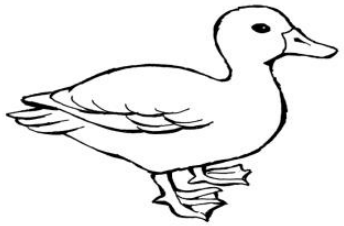
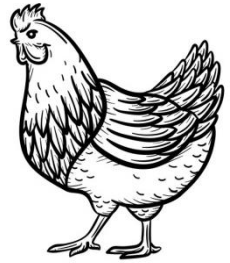
Idea behind using high-throughput real-time PCR

- The Danish National Veterinary Institute
  - Large diagnostic unit
    - Main focus on diseases of domestic animals
    - Identification of cause of disease and pathogens
    - Surveillance of avian and swine influenza A viruses









The diagnostic unit – the flow

Diseased animal

Sample submission

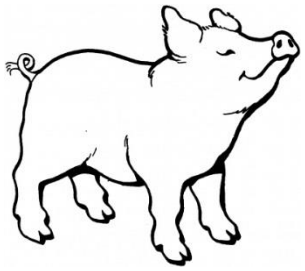
Laboratory

Laboratory analyses

Test results

Veterinarian

Treatment



Healthy animal

Happy farmer

The conventional low-throughput real-time PCR platform

Low-throughput real-time PCR platform

Rotor-Gene Q

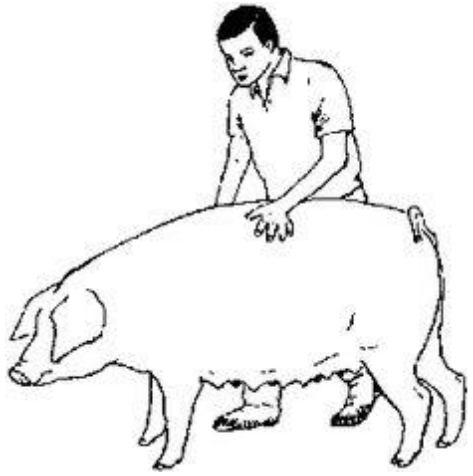
- Sample capacity: 36, 72, 100
- PCR assay per sample:
  - 1 (singleplex) → most test
  - 6 (multiplex) → few test

Expensive analyses
-High analysis cost per sample

Many farmers keep the sample submissions to a minimum

Challenges with the low-throughput real-time PCR platform

- The problem with few sample submissions:
  - Fewer animals get diagnosed
  - Lack of laboratory analysis
    - The disease-causing pathogen remains unknown
  - Medication and vaccination is based on clinical observations
    - Risk of overuse of antibiotics
    - Risk of suboptimal vaccination programs



Changing real-time PCR platform

- Development of high-throughput real-time PCR systems used for detection and typing of pathogens (viruses and bacteria)
  - Pathogens which are important to the health and welfare of Danish pigs and cattle
- Purpose
  - Reducing the analysis costs per sample and working hours

Low-throughput real-time PCR platform

Rotor-Gene Q

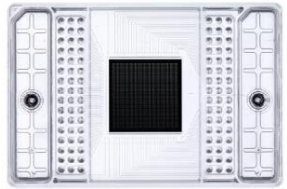
High-throughput real-time PCR platform

BioMark

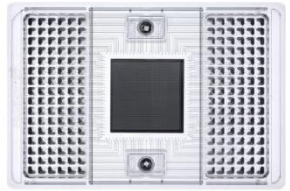
The high-throughput real-time PCR platform

- High-throughput real-time PCR platform – BioMark HD (Fluidigm)
  - Requires less sample and reagents (nL vs. µL)
  - Provides up to 9,216 reactions in one chip (96x96)

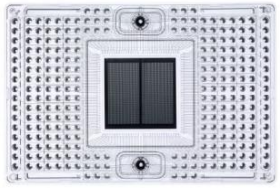
48 samples x 48 assays



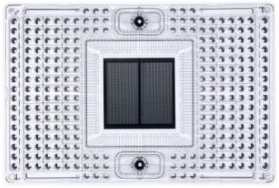
96 samples x 96 assays

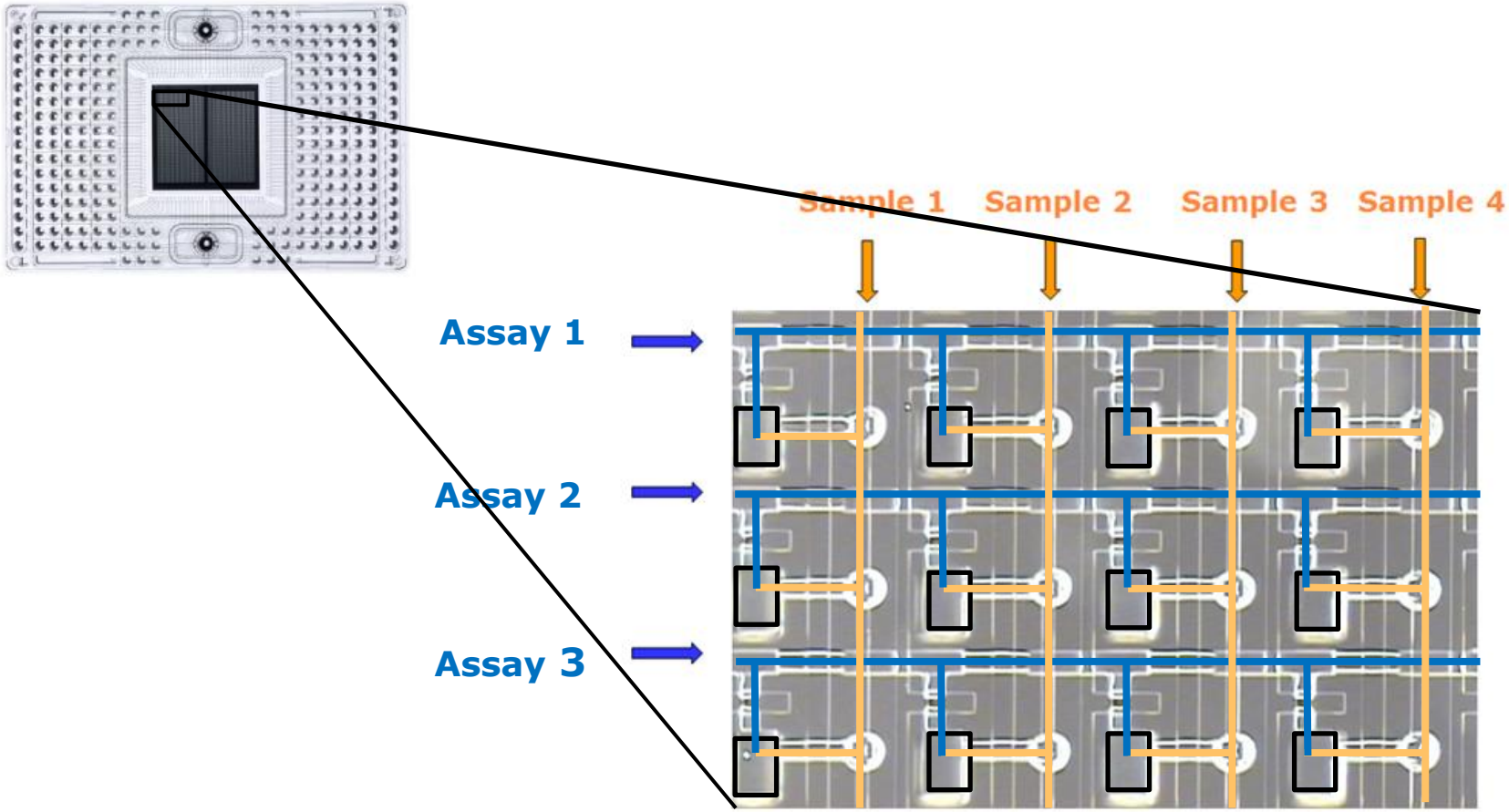


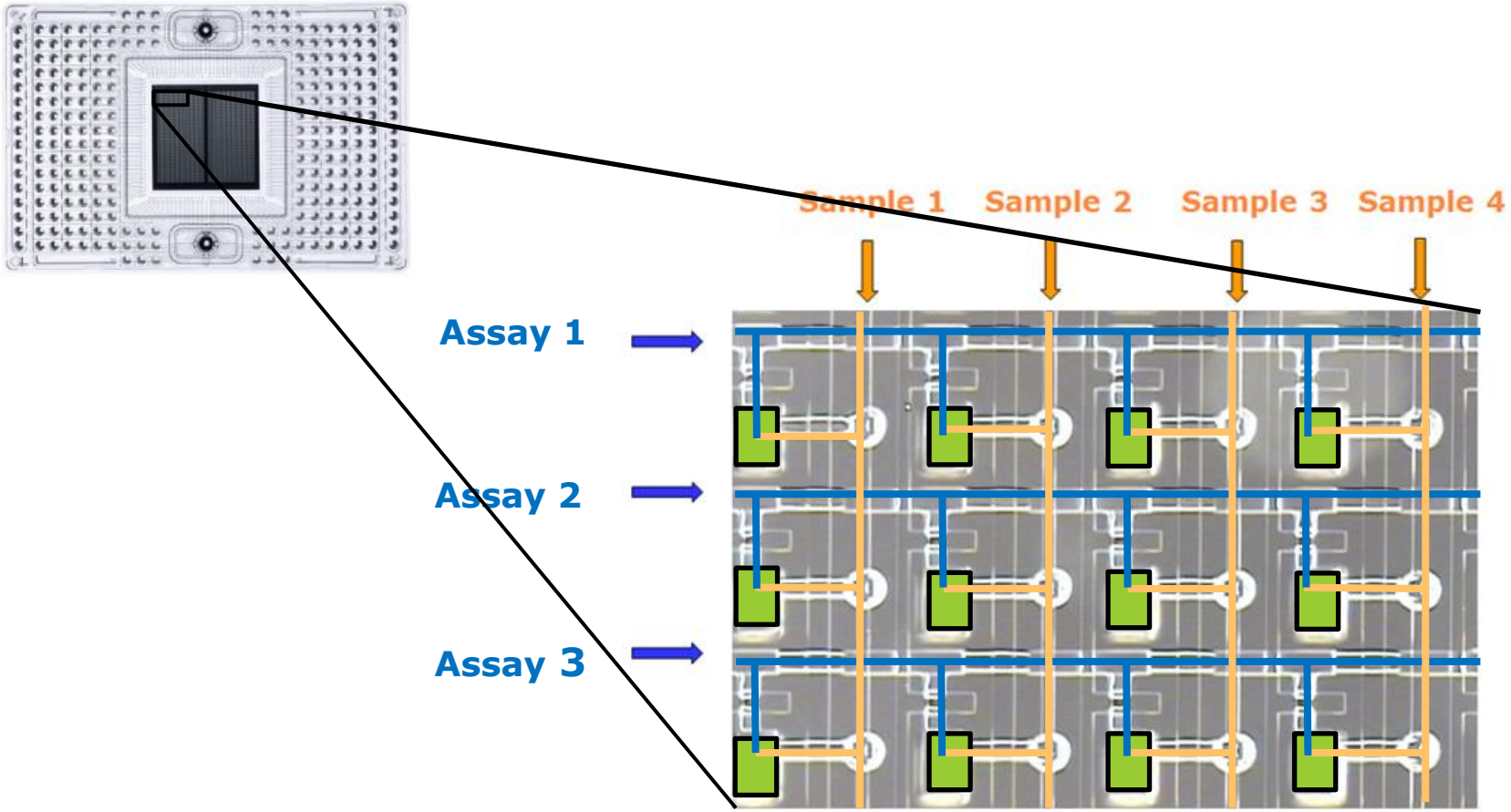
192 samples x 24 assays



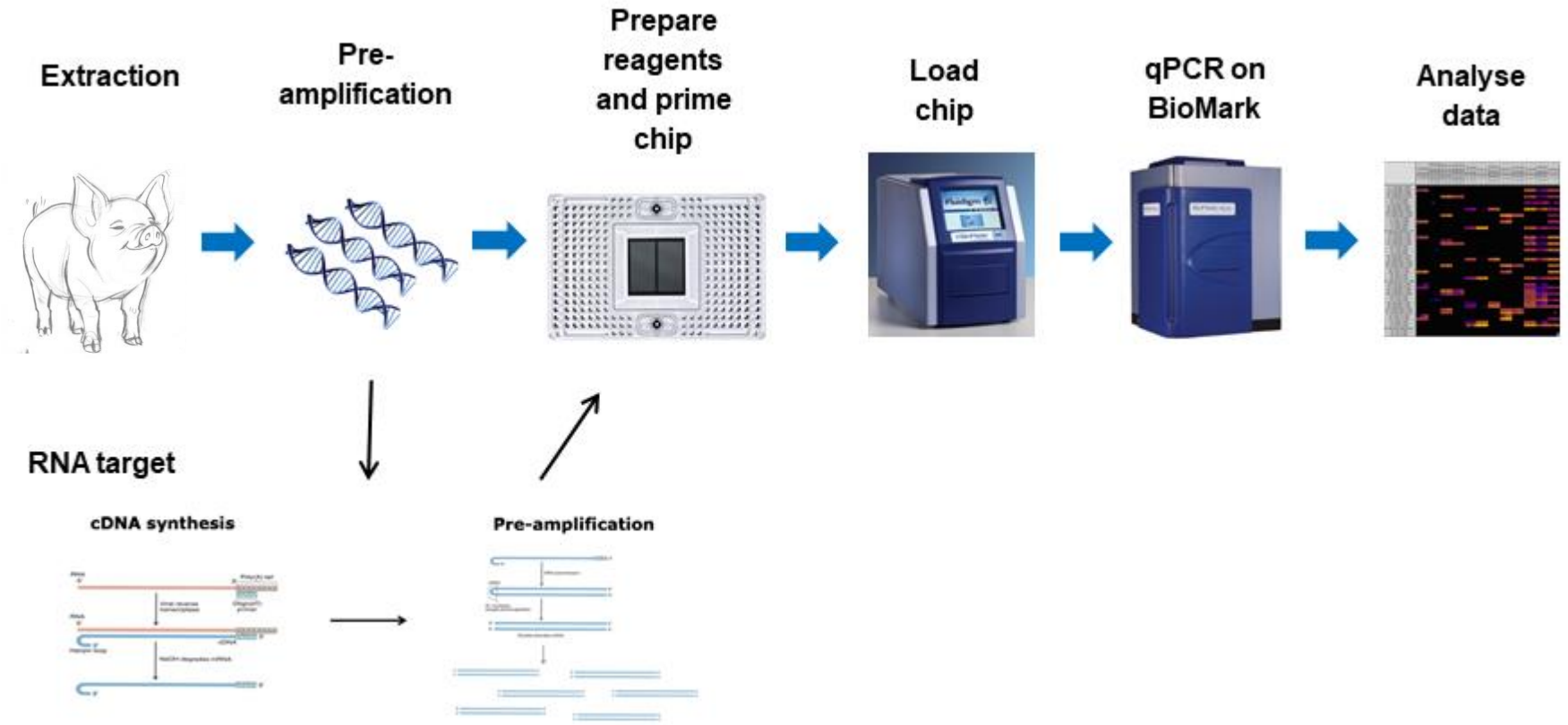
24 samples x 192 assays




Sample 1
Sample 2
Sample 3
Sample 4
Assay 1
Assay 2
Assay 3


Sample 1
Sample 2
Sample 3
Sample 4
Assay 1
Assay 2
Assay 3

The workflow



The first porcine high-throughput real-time PCR system

Swine influenza A virus (swIAV)

- Establishing a high-throughput system for detection and subtyping of swIAVs

frontiers in Cellular and Infection Microbiology
ORIGINAL RESEARCH
published: 22 May 2018
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Subtyping of Swine Influenza Viruses Using a High-Throughput Real-Time PCR Platform

Nicole B. Goecke[1], Jesper S. Krog[1], Charlotte K. Hjulsager[1], Kerstin Skovgaard[1], Timm C. Harder[2], Solvej Ø. Breum[1] and Lars E. Larsen[1]*

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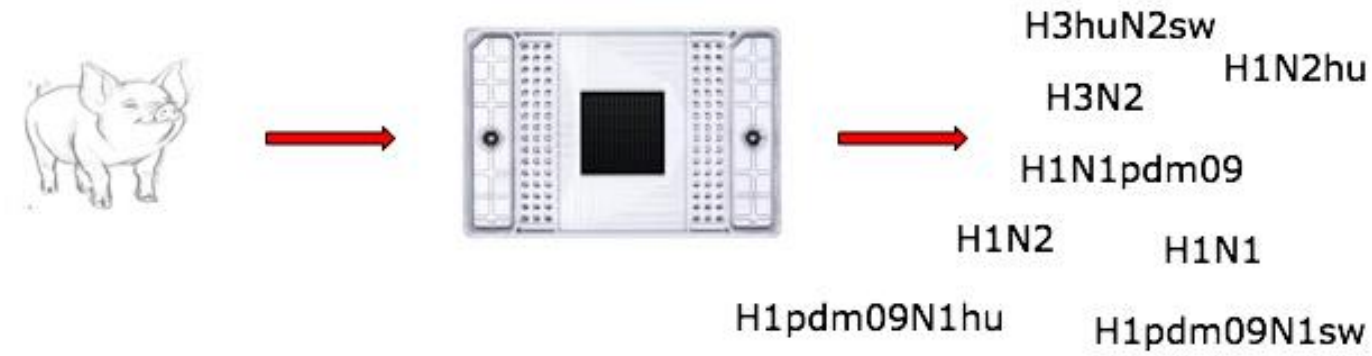
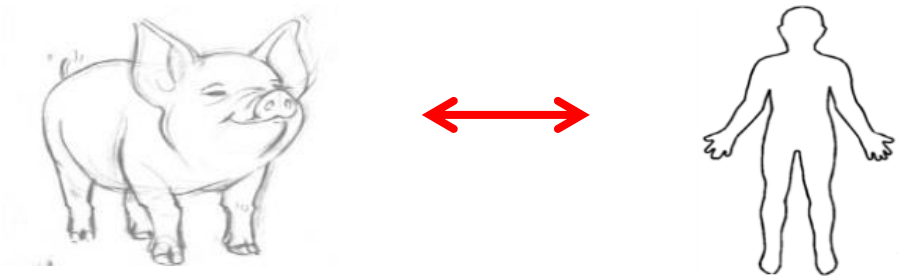


Influenza A virus

Influenza

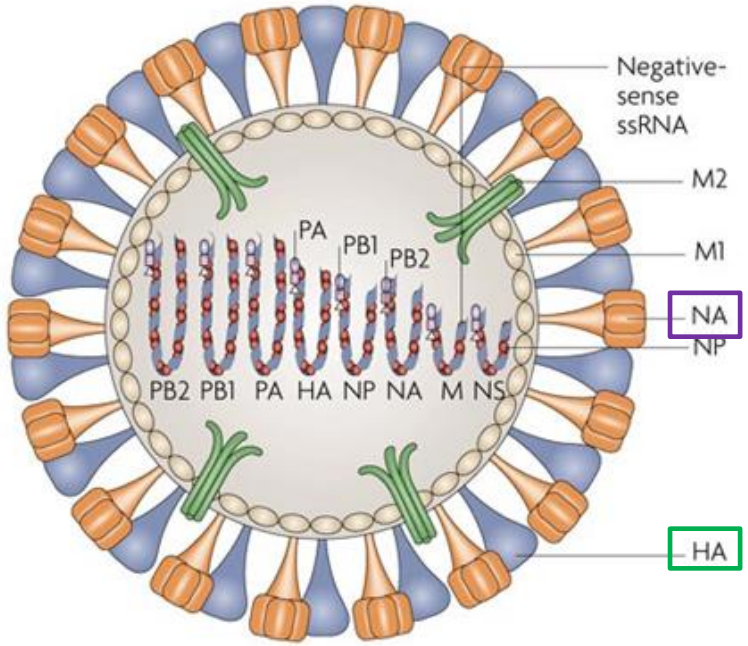
- Respiratory disease caused by multiple subtypes of influenza A virus
- Infection can cause respiratory disease in pigs
- Symptoms: Fever, coughing and sneezing
- Zoonotic potential



Influenza A virus

The structure of the virus

- Single-stranded, negative sense RNA virus
- Segmented genome
  - 8 segments coding for different viral proteins
  - HA: Hemagglutinin
  - NA: Neuraminidase

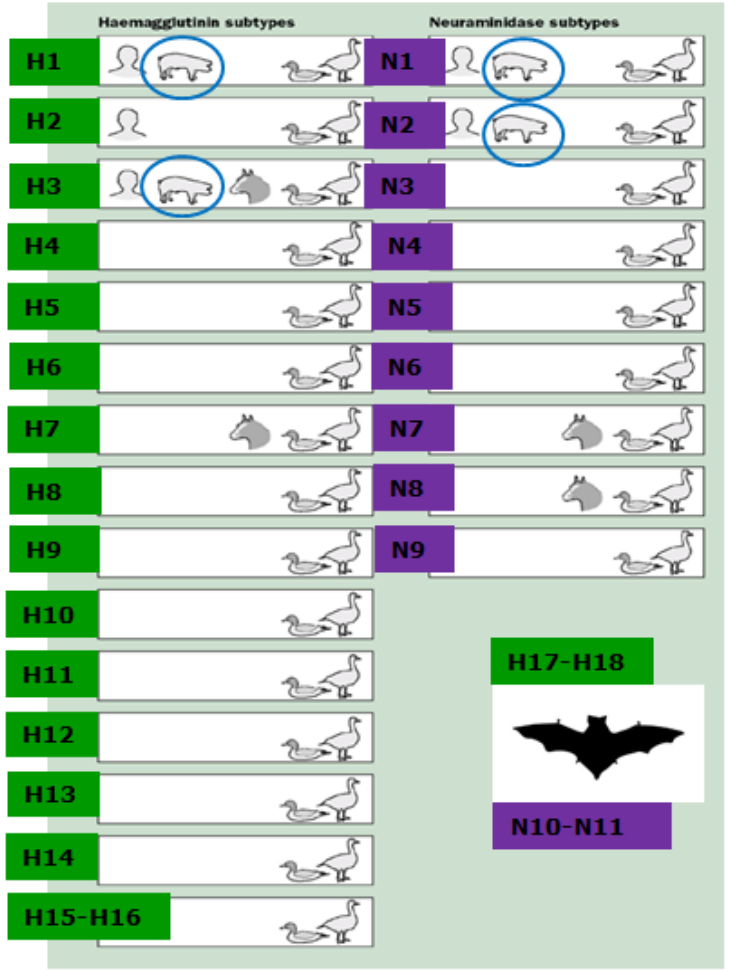


Neuraminidase

Hemagglutinin

Subtyping of influenza A virus

- Currently 18 hemagglutinin (HA) and 11 neuraminidase (NA) genes identified
- Different variants of the HA and NA genes exist
  - H1 lineage
    - H1-human like
    - H1-avian like
    - H1-pandemic 2009
- Subtypes **H1N1**, **H1N2** and **H3N2** are commonly found in pigs



Subtyping of swine influenza A virus

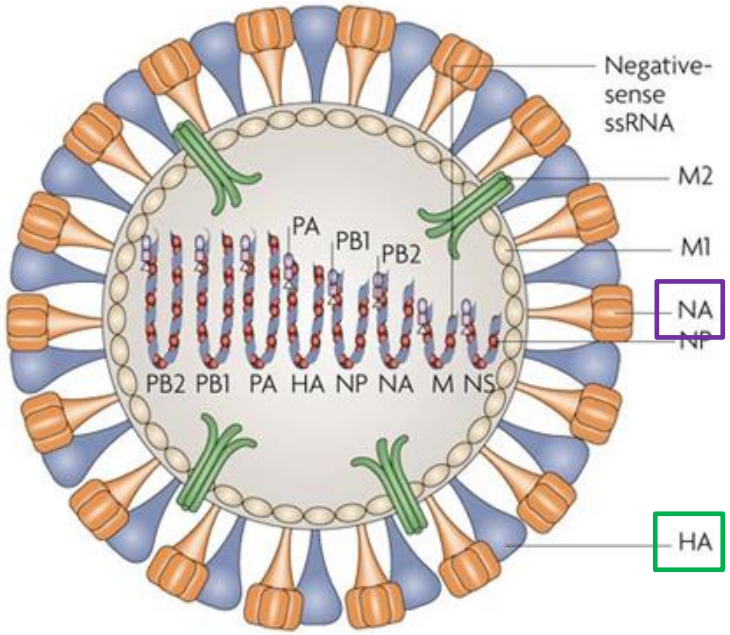
- A passive surveillance program for swIAVs has been conducted in Denmark since 2011
  - The aim is to identify which subtypes circulate in Danish pigs
    - Be able to act fast on new virus variants

- Subtyping of swIAVs
  - Several PCR assays are needed to cover the wide range of circulating subtypes
  - Subtyping is conducted in a multiplex setup
  - High analysis cost → a limited number of samples can be subtyped

Subtyping of swine influenza A virus

- Change of real-time PCR platform

→ A more detailed characterization



Neuraminidase

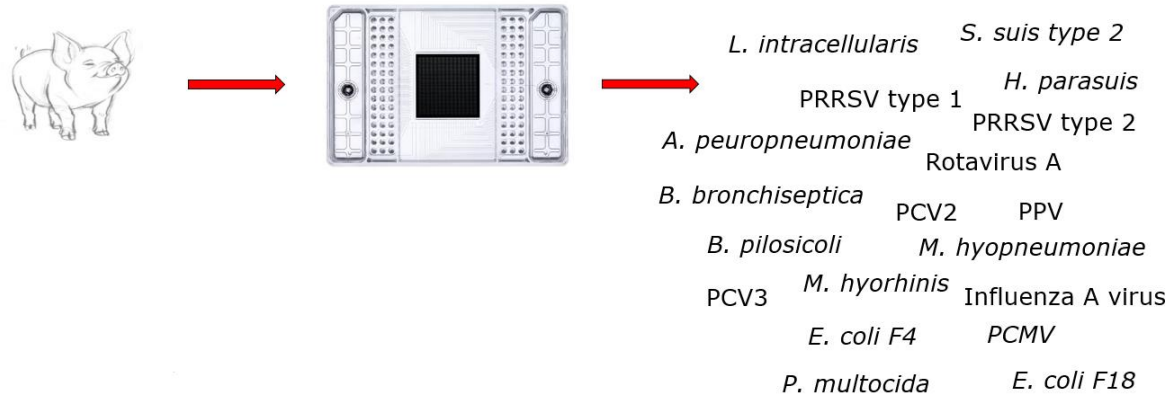
Hemagglutinin

Internal genes:
- PB2
- PB1
- PA
- NP
- M
- NS

The second porcine high-throughput real-time PCR system

Development of a porcine high-throughput system

- Used for detecting 18 different porcine viruses and bacteria
- All the relevant pathogens causing respiratory and intestinal diseases in pigs are included
  - Reduces the risk of not detecting the relevant pathogen(s)



Full Scientific Report

Development of a high-throughput real-time PCR system for detection of enzootic pathogens in pigs

Nicole B. Goecke,[1] Charlotte K. Hjulsager, Jesper S. Krog, Kerstin Skovgaard, Lars E. Larsen

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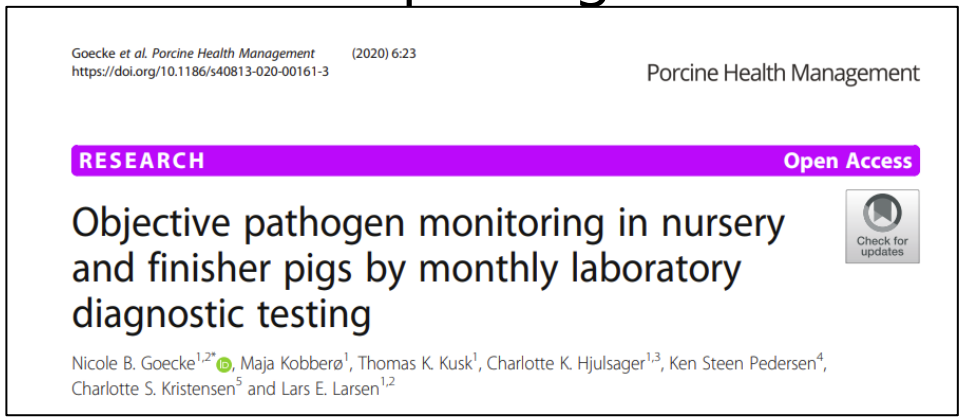
jvdi.sagepub.com

The use of the porcine high-throughput real-time PCR system

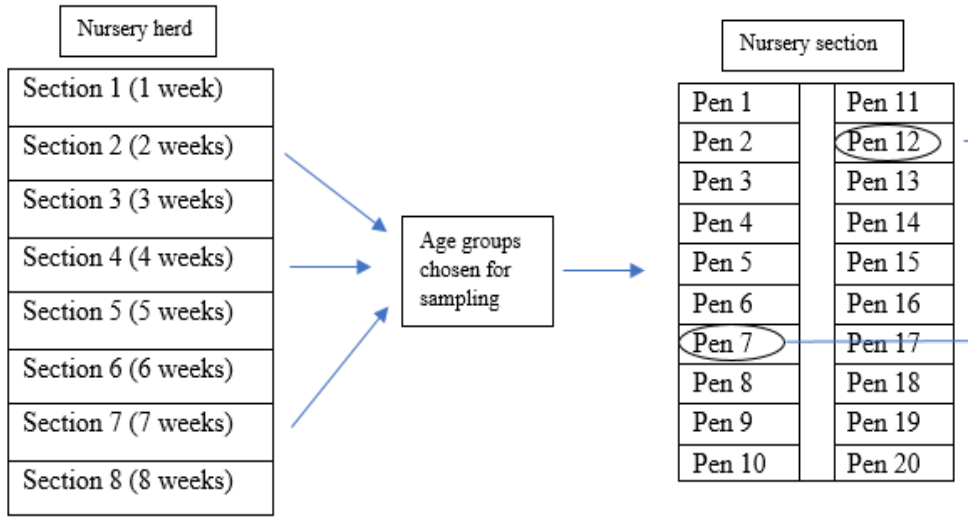
- Test the performance of the high-throughput diagnostic system on field samples
  - Samples collected from ten Danish pig production units (six herds)
- Test if monthly monitoring of pathogens on herd level could be a supportive tool for the veterinarians
  - Create a more objective basis for intervention
- Investigate the relationship between prevalence of different pathogens and clinical signs observed



The study design

- The study was carried out in ten Danish pig production units
  - Six nursery units
  - Four finisher units

- Sampling and clinical observations were carried in each unit in September, October and November 2017



The study design

Sample types

- Faecal sock samples and oral fluid samples

Clinical observations

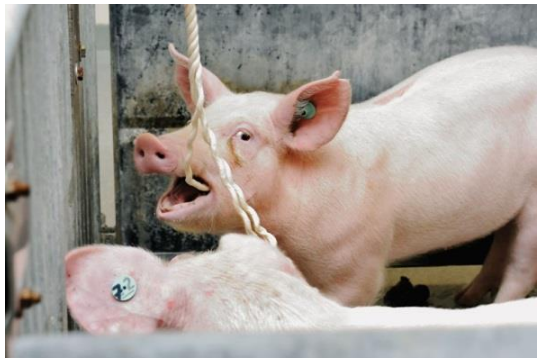
- Coughing and diarrhoea

In total
- 174 faecal sock samples
- 172 oral fluid samples

} 18 pathogens

Faecal sock sample

Oral fluid sample



Example of the results obtained from a nursery unit

C) 2N – oral fluid (OF) and faecal sock (FS) samples																			
Sampling date		September						October						November					
Days after insertion		9		37		65		10		24		38		2		29-36		48	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF	14.9	14.2		18.6			12.8	12.2	15.4	14.9	22.7	27.6				15.7	25.6	24.0
H1 (A(H1N1)pdm09)	OF																		
PRRSV type 1	OF																		
PRRSV type 2	OF																		
PCMV	OF	8.0	10.7	18.7	18.0		20.6	11.2	10.2	13.1	13.4	19.5	18.2			17.8	18.8	22.8	17.9
A. pleuropneumoniae	OF							24.0	24.3	24.4	25.2	24.7					23.4		
PCV2	OF	24.6		18.7	20.3	14.9	14.6					26.9				26.0			21.8
***S. suis* type 2**	OF	23.4	23.2	21.8	23.3	23.6	22.3	21.3	21.8	19.7	21.6	22.0	21.4			21.2	21.9	23.3	23.8
B. bronchiseptica	OF				25.7							26.6	25.8			25.6			
PCV3	OF			26.6	23.7	19.5	26.8			20.4	22.8					22.3	27.6	29.7	28.8
PPV	OF	26.2	27.4		25.76	18.7	17.9	28.4	30.3			27.1	30.7				32.3		
M. hyopneumoniae	OF																		
M. hyorhinis	OF	26.3	25.9	26.3		23.9		25.8	23.2	21.4	23.2	24.7	24.5			22.0	24.9	24.7	
P. multocida	OF							25.4		19.4							34.8		
B. pilosicoli	FS															21.6			
L. intracellularis	FS			21.6	20.5	20.7	16.8			24.9	26.2	22.4	15.4			12.1	10.2	19.0	18.8
***E. coli* F4**	FS	20.7	24.1					21.9	22.0					14.6	15.3				
***E. coli* F18**	FS	22.8						24.0	26.6	24.2									
PCV3	FS					27.9								24.9	23.1				
PCV2	FS					15.7	16.2												
Rotavirus A	FS	26.1	19.1	30.5	32.9	29.7		22.9	21.2	21.0	17.9	29.7		13.5	13.1	24.2	23.2	21.0	15.7

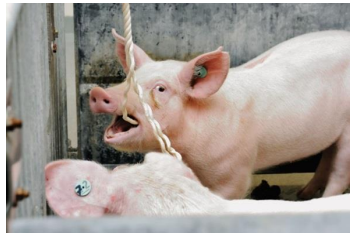
The overall prevalence of pathogens

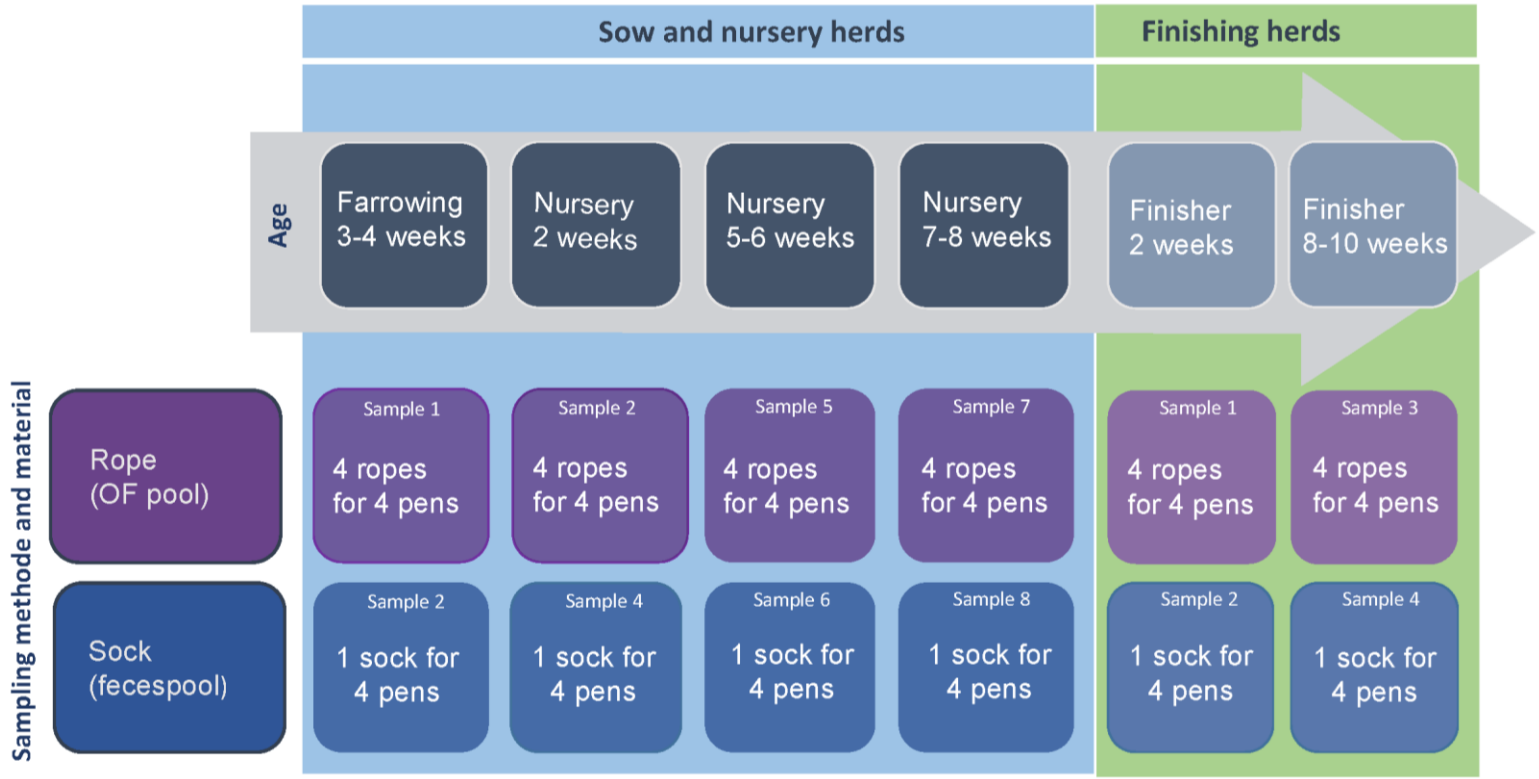
Pathogen	Material	Overall prevalence	8 Coughing events	p value	77 Diarrhoeic events	p value
swIAV	Oral fluid	34.9%	6/8	0.02		
PRRSV type 1	Oral fluid	0%				
PRRSV type 2	Oral fluid	0%				
PCMV	Oral fluid	92.4%	8/8 (Cq<20)	0.02		
A. pleuropneumoniae	Oral fluid	17.4%	3/8	0.15		
PCV2	Oral fluid	59.9%	4/8	0.72		
S. suis type 2	Oral fluid	98.8%	8/8	0.91		
B. bronchiseptica	Oral fluid	25.0%	0/8			
PCV3	Oral fluid	53.5%	5/8	0.73		
M. hyopneumoniae	Oral fluid	1.2%	0/8			
M. hyorhinis	Oral fluid	59.3%	7/8	0.08		
P. multocida	Oral fluid	15.1%	0/8			
B. pilosicoli	Faecal sock	39.7%			37/77 (Cq<26)	0.03
L. intracellularis	Faecal sock	40.8%			37/77 (Cq<24)	0.01
E. coli F4	Faecal sock	19.0%			12/77	0.43
E. coli F18	Faecal sock	28.7%			16/77	0.06
PCV3	Faecal sock	16.1%			13/77	-
PCV2	Faecal sock	40.2%			32/77	0.87
Rotavirus A	Faecal sock	54.0%			29/77	0.0002

SOS "Swine, Objective Surveillance"

The concept behind SOS:

- Herds are sampled at regular intervals throughout the year
- Common guidelines for all herds
- The farmer is responsible for the sampling





The pathogens surveilanced in SOS

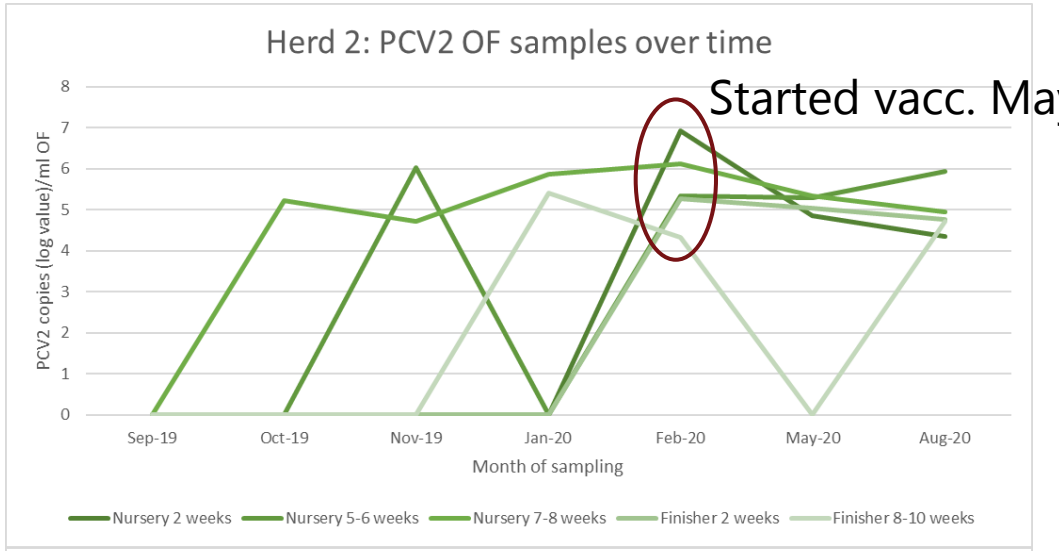
Oral fluid samples:

- Influenza A virus
  - Pandemic H1 (H1pdm)
- Porcine Circovirus Type 2 (PCV2)
- Porcine Circovirus Type 3 (PCV3)
- Porcine Cytomegalovirus (PCMV)
- *Streptococcus suis type 2*
- *Haemophilus parasuis*
- *Pastuerella multocida*
- *Actinobacillus pleuropneumoniae*
- *Mycoplasma hyopneumonia*
- *Mycoplasma hyorhinis*
- *Bordetella bronchiseptica*

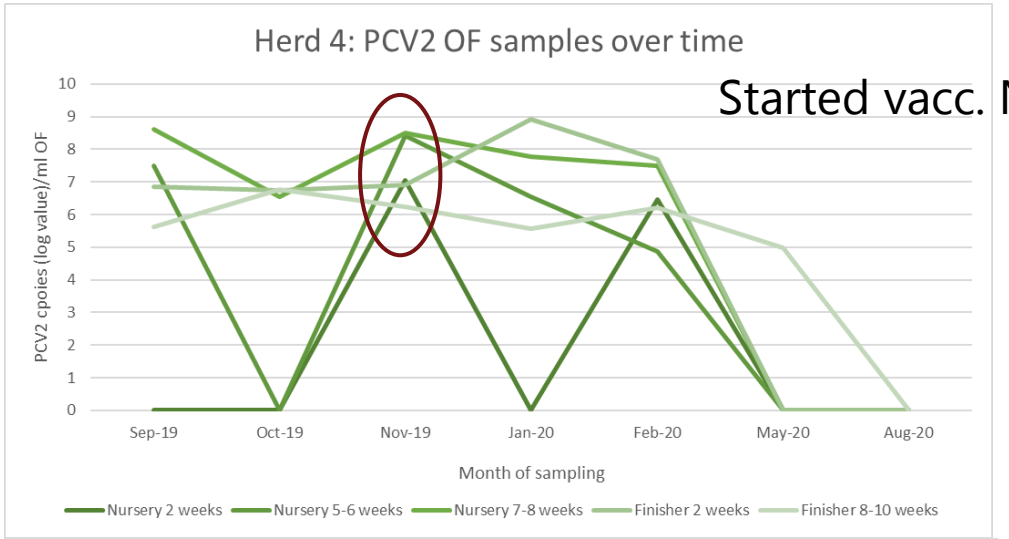
Sock samples:

- *Escherichia coli F4*
- *Escherichia coli F18*
- *Lawsonia intracellularis*
- *Brachyspira pilosicoli*
- Rotavirus A
- Porcine Circovirus Type 2 (PCV2)
- Porcine Circovirus Type 3 (PCV3)

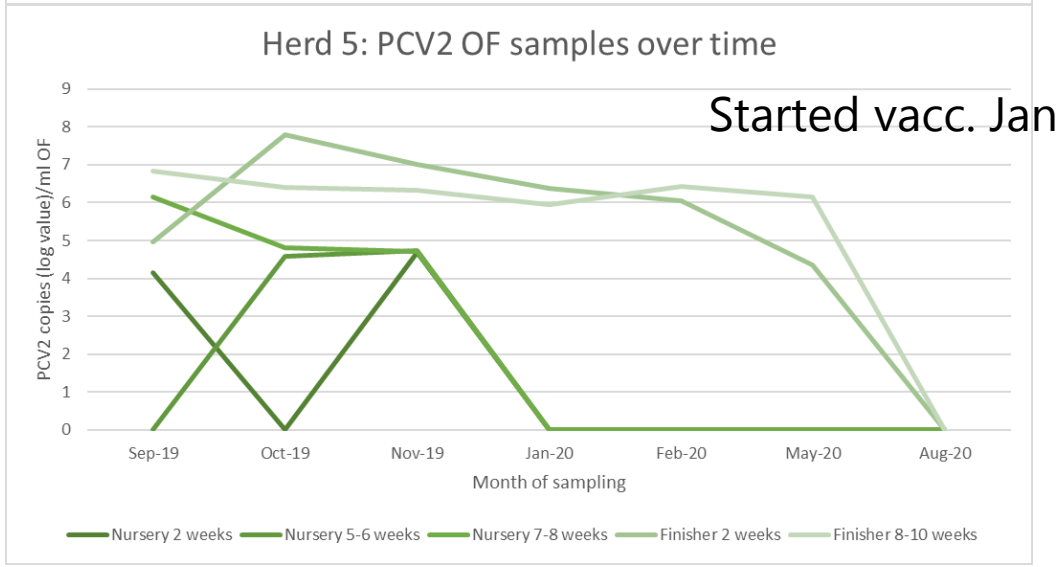
PCV2 – Examples of results from four herds



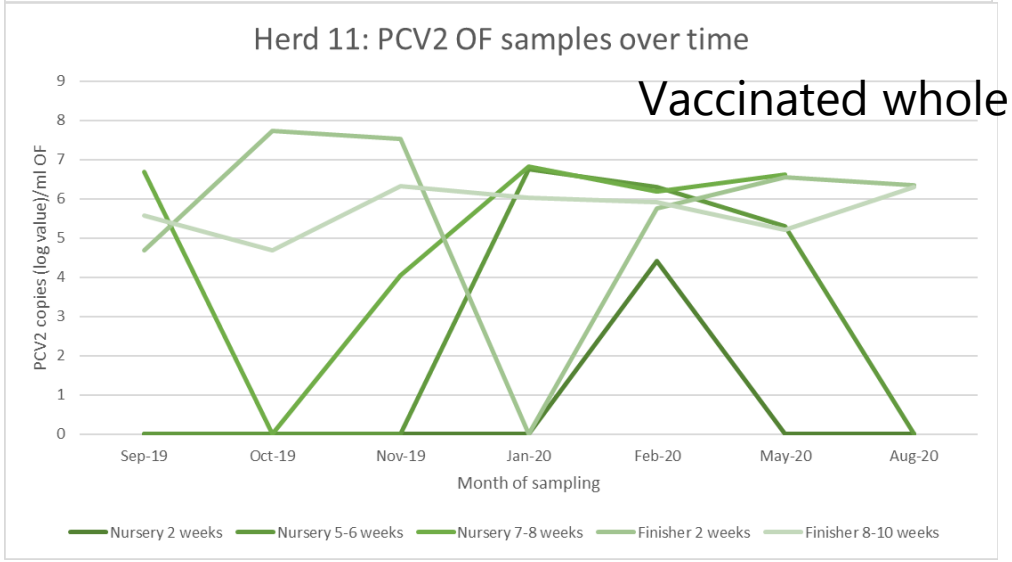
Started vacc. May'20



Started vacc. Nov'19



Started vacc. Jan'20



Vaccinated whole period

The outcome of SOS

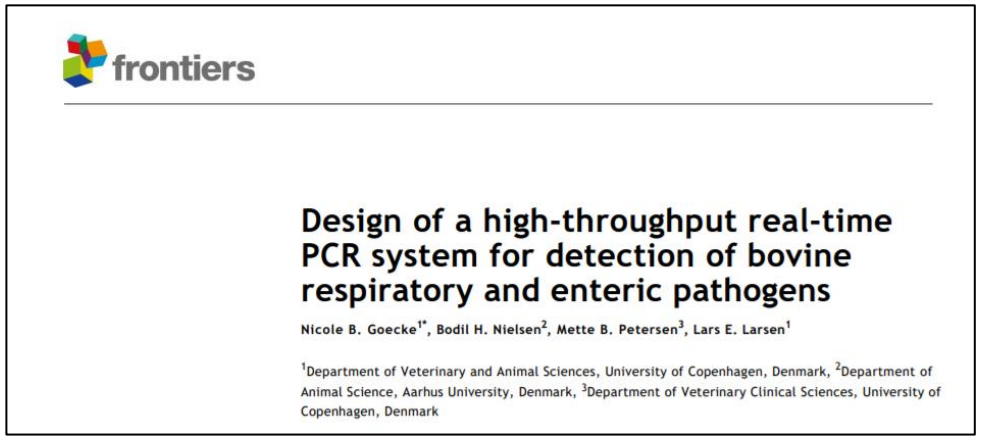
- Create an overview of the pathogen dynamics in the herd
- Monthly monitoring of pathogens provides information on:
  - The distribution of pathogens in a healthy status
  - An outbreak situation → benchmarking the findings of pathogens
    → More specific treatment

- Enables follow-up on interventions and change in management

The bovine high-throughput real-time PCR system

Development of a bovine high-throughput system

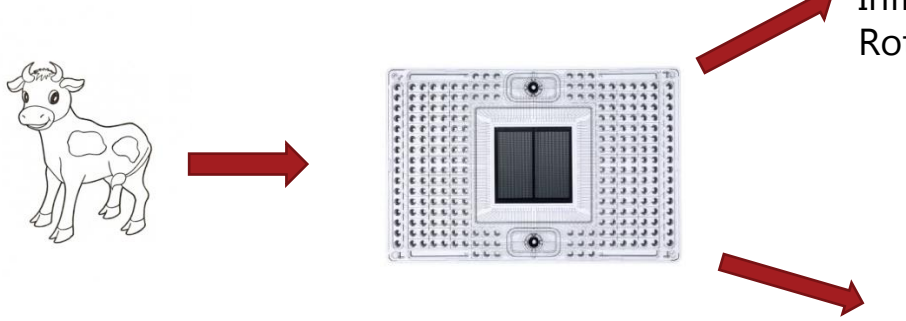
- Used for detecting of 11 different bovine viruses and bacteria
- All the relevant pathogens causing respiratory and intestinal diseases in calves are included
  - Reduce the risk of not detecting the relevant pathogen(s)


frontiers

Design of a high-throughput real-time PCR system for detection of bovine respiratory and enteric pathogens

Nicole B. Goecke[1*], Bodil H. Nielsen[2], Mette B. Petersen[3], Lars E. Larsen[1]

[1]Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark, [2]Department of Animal Science, Aarhus University, Denmark, [3]Department of Veterinary Clinical Sciences, University of Copenhagen, Denmark



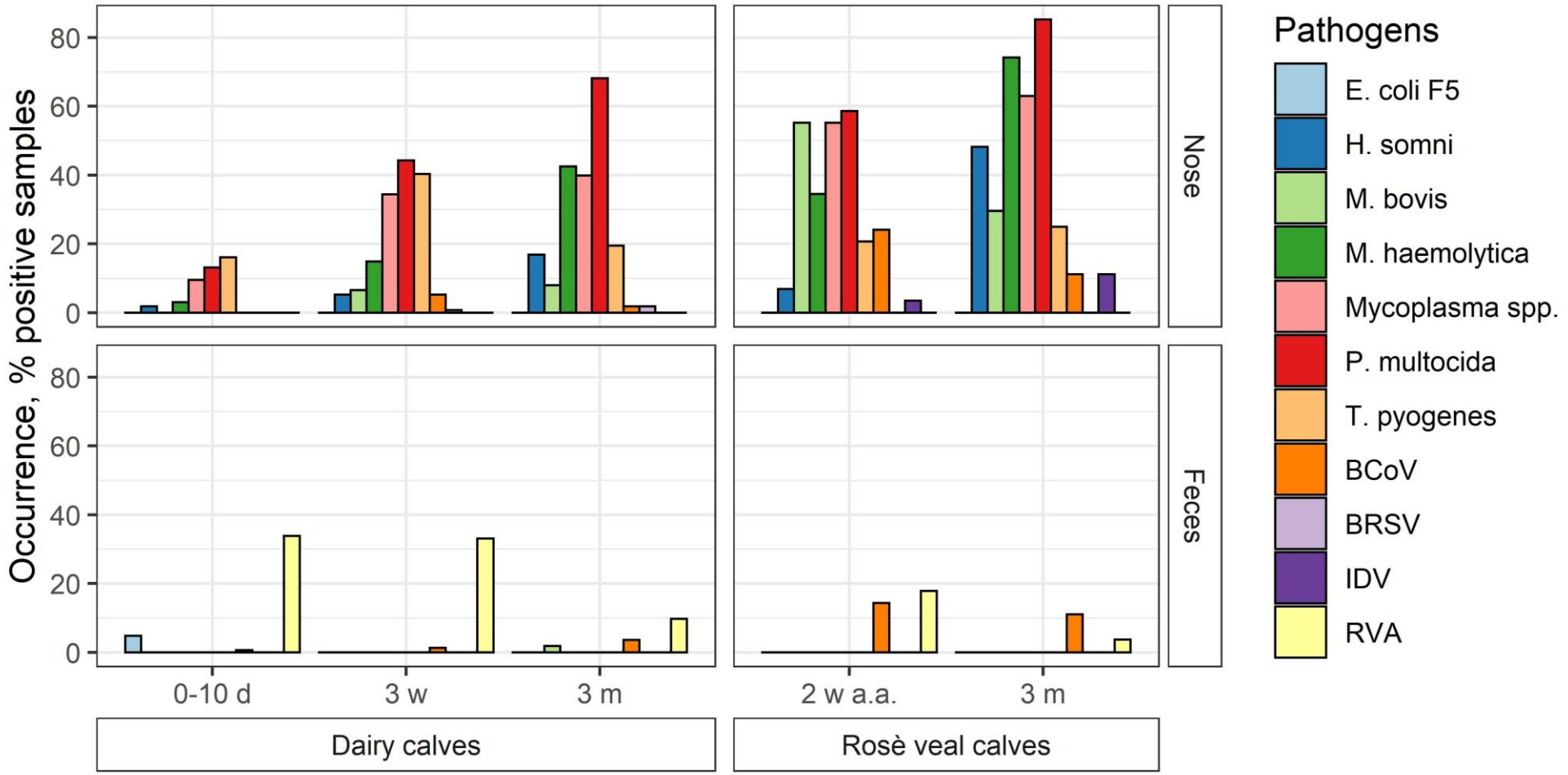
Viruses
Bovine coronavirus (BCoV)
Bovine respiratory syncytial virus (BRSV)
Influenza D virus (IDV)
Rotavirus A (RVA)

Bacteria
Mannheimia haemolytica
Pasteurella multocida
Histophilus somni
Mycoplasma spp.
Mycoplasma bovis
Trueperella pyogenes
Escherichia coli F5

Study design - Calf project 2018-2021

- Sampling
  - Nasal swab, serum and faecal samples were collected from 100 Danish herds (83 dairy and 17 slaughter herds)
    - Dairy herds: three age groups (0-10 days, 3 weeks and 3 months of age)
    - Slaughter herds: two age groups (2 weeks after arrival and 3 months of age)
  - Sample periods: September-April 2018-2019 and 2019-2020
- Clinical observations
  - Rectal temperature, coughing, nasal and eye discharge, diarrhea, hair coat, joints, body condition

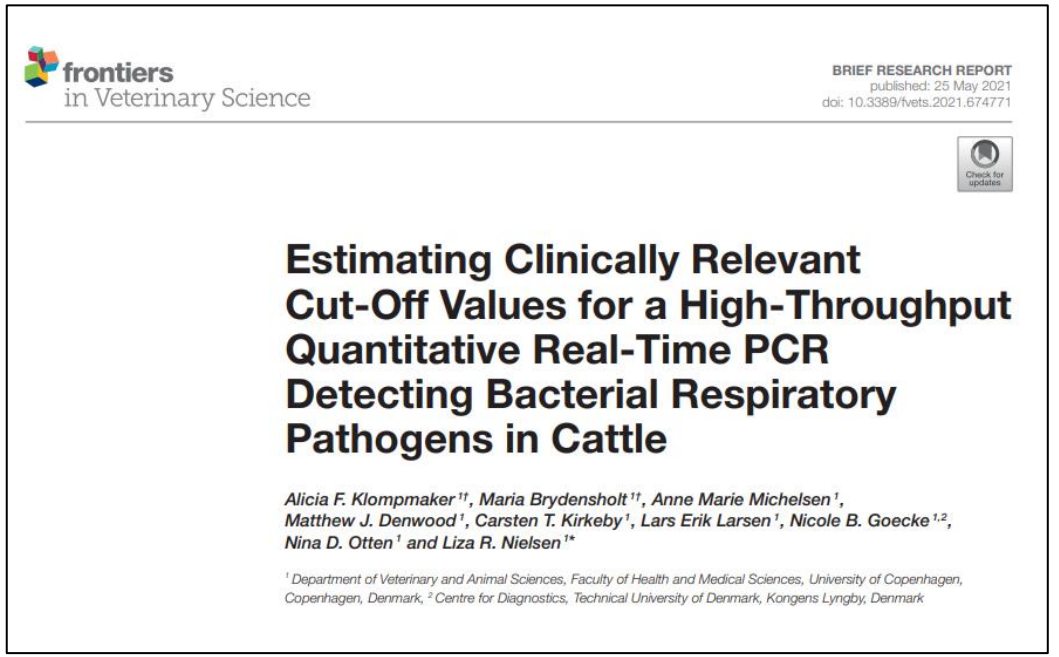
Occurrence of respiratory and enteric pathogens in pools



Clinically relevant cut-offs

- Association between PCR result and clinical signs
- Analysis of 340 calves
  - Clinical signs:
    - Temperature
    - Coughing
    - Nasal discharge
    - Eye discharge

    } Healthy vs. sick
  - Respiratory pathogens:
    - *Mannheimia haemolytica*
    - *Mycoplasma bovis*
    - *Pasteurella multocida*
    - *Histophilus somni*
    - *Trueperella pyogenes*



frontiers in Veterinary Science

BRIEF RESEARCH REPORT
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Estimating Clinically Relevant Cut-Off Values for a High-Throughput Quantitative Real-Time PCR Detecting Bacterial Respiratory Pathogens in Cattle

Alicia F. Klompmaker[1†], Maria Brydensholt[1†], Anne Marie Michelsen[1], Matthew J. Denwood[1], Carsten T. Kirkeby[1], Lars Erik Larsen[1], Nicole B. Goecke[1,2], Nina D. Otten[1] and Liza R. Nielsen[1]*

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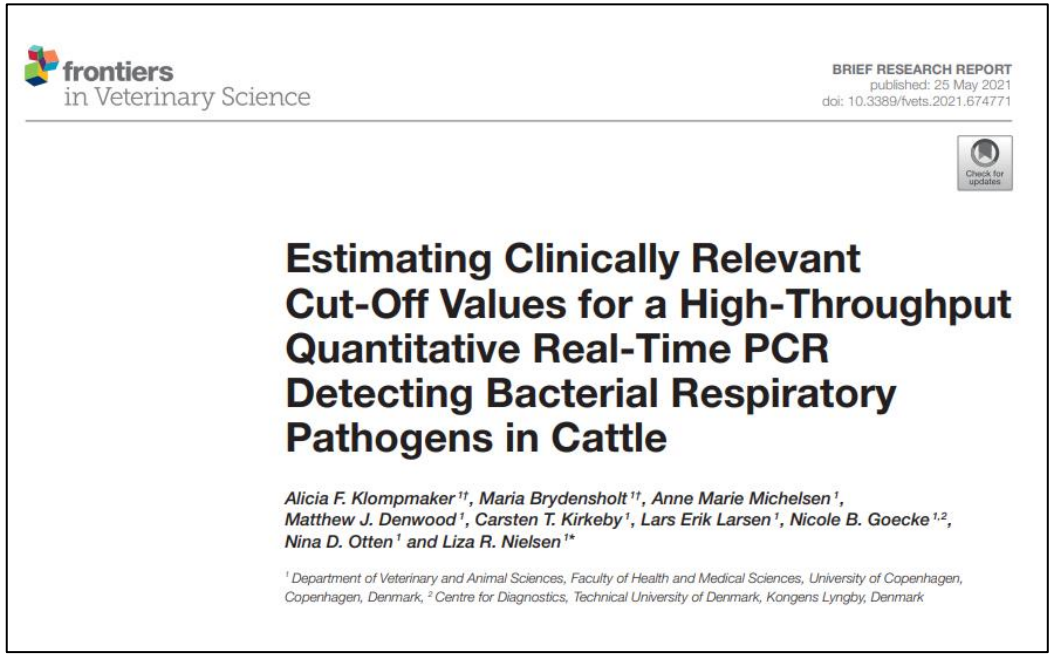
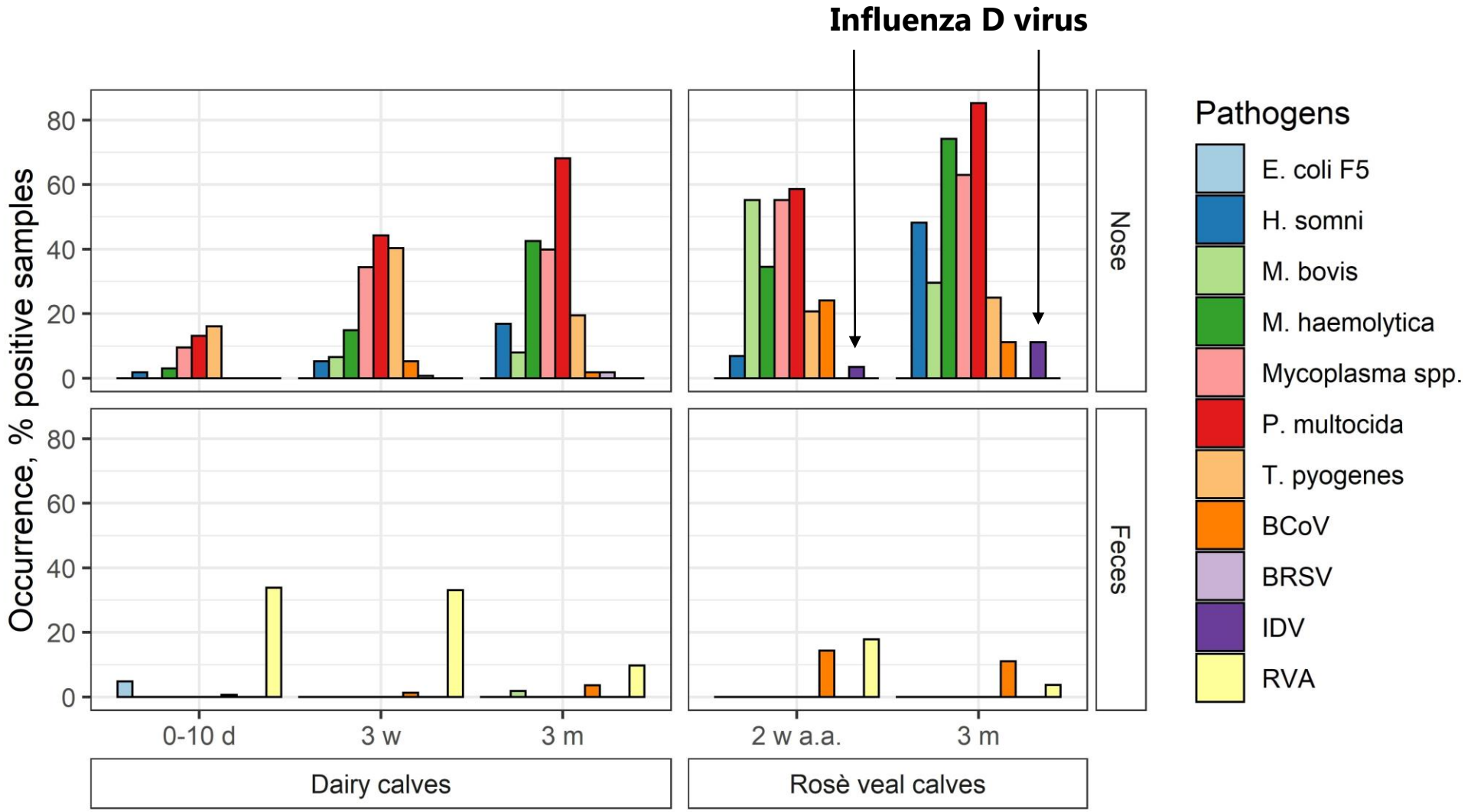
Clinically relevant cut-offs

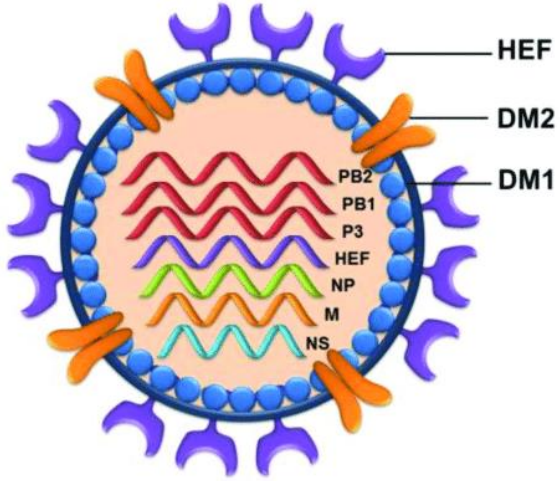
- Association between PCR result and clinical signs
- Analysis of 340 calves
  - Clinical signs:
    - Temperature
    - Coughing
    - Nasal discharge
    - Eye discharge
  - Respiratory pathogens:
    - *Mannheimia haemolytica* – ?
    - *Mycoplasma bovis* – Cq $\leq$ 21.3
    - *Pasteurella multocida* – Cq $\leq$ 21.3
    - *Histophilus somni* – Cq $\leq$ 17.4
    - *Trueperella pyogenes* – ?



Occurrence of respiratory and enteric pathogens in pools



Influenza D virus



- The family Orthomyxoviridae
  - Influenza A virus – 8 gene segments
  - Influenza B virus – 8 gene segments
  - Influenza C virus – 7 gene segments
  - Influenza D virus – 7 gene segments - encodes only one glycoprotein (HEF)

- Isolated for the first time in 2011 in the US (Oklahoma)
- Detected in Asia, Europe and the US
- Detected in bovine, pigs, horses, sheep and goats

Influenza D virus – HEF gene

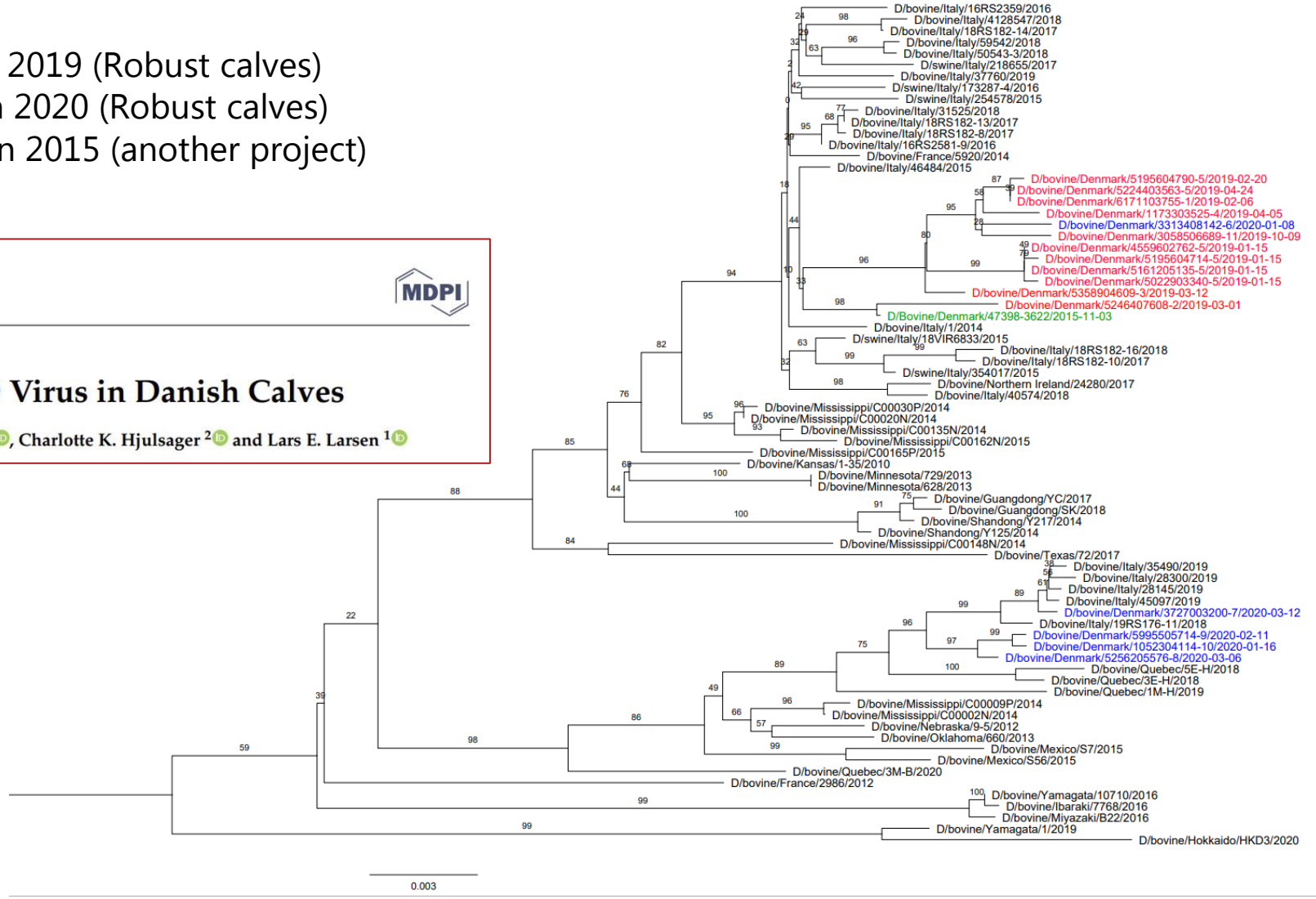
Red: Samples collected in 2019 (Robust calves)
Blue: Samples collected in 2020 (Robust calves)
Green: Sample collected in 2015 (another project)

viruses MDPI

Article

Characterization of Influenza D Virus in Danish Calves

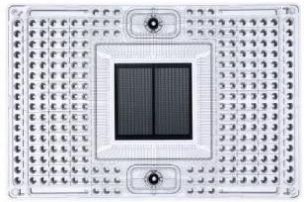
Nicole B. Goecke [1,*,†], Yuan Liang [1,†], Nina D. Otten [1], Charlotte K. Hjulsager [2] and Lars E. Larsen [1]



Bovine high-throughput system

- Calf project 2018-2021
  - 100 Danish calf herds (83 dairy and 17 veal herds)

- Calf project 2022
  - 6 Danish calf herds (veal)



Viruses
Bovine coronavirus (BCoV)
Bovine Parainfluenza 3 (BPi3)
Bovine respiratory syncytial virus (BRSV)
Influenza D virus (IDV)
Rotavirus A (RVA)

Parasites
Cryptosporidium
Eimeria
Giardia intestinalis

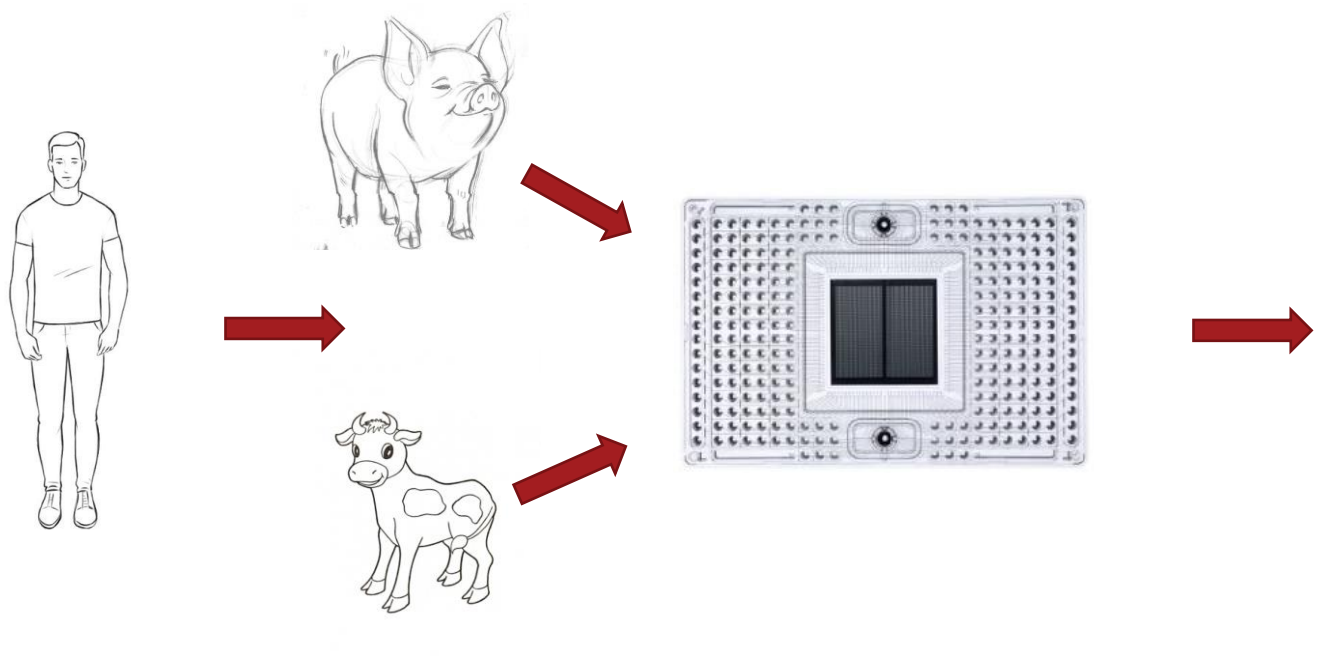
Bacteria
Mannheimia haemolytica
Pasteurella multocida
Histophilus somni
Mycoplasma spp.
Mycoplasma bovis
Trueperella pyogenes
Escherichia coli F5

SOS

" Swine, Objective Surveillance"

An AMR high-throughput real-time PCR system

- AMR: Antimicrobial resistance genes



Antimicrobial classes (55 genes)

Aminoglycoside (5)
Macrolide (4)
Phenicol (3)
Sulphonamide (2)
Tetracycline (5)
Trimethoprim (3)
Vancomycin (2)
Beta-Lactamase (4)
Low affinity PBPs (2)
ESBL (7)
Quinolone (6)
Ampc Beta-Lactamase (3)
Carbapenemase (6)
Colistin (2)
Fosfomycin (1)

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