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PORK UN MEJOR FUTURO

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Next Generation Sequencing Approaches for PRRSV Diagnostics and Beyond

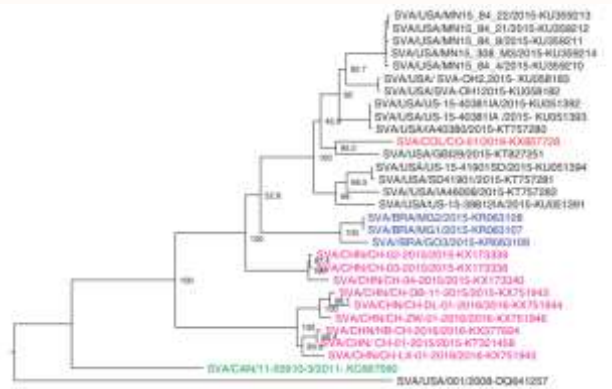
Asociación porkcolombia
FONDO NACIONAL DE LA PORCICULTURA

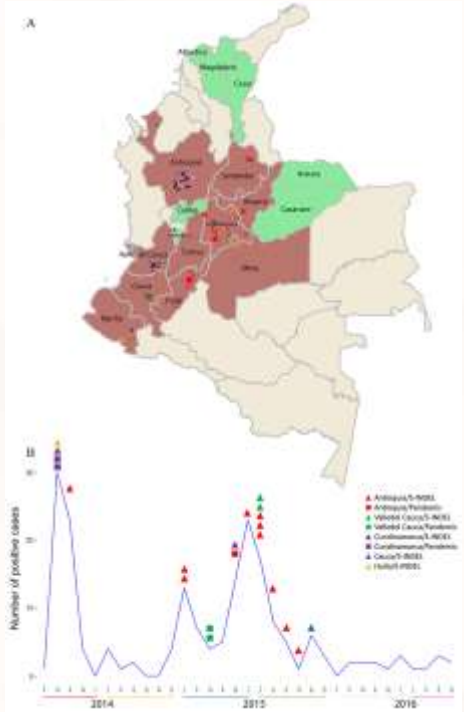
Thank you for the invitation!

- Director of Science and Technology for the Americas, Indical BioScience
- Over 20 years of diagnostic experience
  - Professorship at University of Minnesota and Kansas State University
  - Diagnostics and research groups focused on swine enteric pathogens and Strep suis.
  - Team used Next Generation Sequencing to understand molecular epidemiology, evolution and emergence of swine pathogens.
- Honor of supporting the Colombian swine industry to identify Senecavirus A and understand the Porcine Epidemic Diarrhea Virus 2014 and 2016 epidemic

Several lineages of PEDV in Colombia 2014 and 2016

Emergence of Senecavirus A





Collaboration with
Asociación Colombiana de Porcicultores
and
Instituto Colombiano Agropecuario ICA

Outline

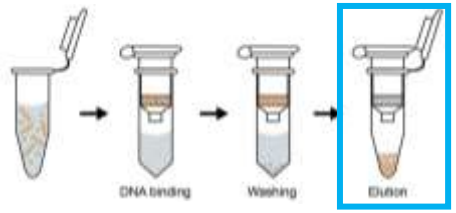
- Technologies
  - PCR technology
  - Sanger Sequencing
  - Second and Third Next Generation Sequencing (NGS)
- NGS applications for Diagnostics
  - Shotgun Metagenomes
    - PRRSV whole genome sequencing
    - Novel Pathogen detection
    - Pathogens in Feedback
  - Whole Genome Sequencing (WGS) – bacterial sequencing
    - Streptococcus suis

Realtime PCR technology

- Semi-quantitative measurement of the pathogen in the sample
- Fluorescent probes increases diagnostic sensitivity
- High Specificity – less false positives
- High throughput – multiple samples in a single PCR run
- Multiplexing – multiple pathogens detect in the same PCR reaction
- Fast results – same or next-day results

Extraction

=



+

= Ct values

Sanger Sequencing

- Traditional sequencing method
- A PCR product is created to sequencing
- Capillary electrophoresis
- One trace file per reaction
- ~500-1,000 nucleotides
- Gene Targeted approach
    - PRRSv ORF5, Influenza A HA
- Inexpensive and fast, with results in a few days
- Low sequence coverage

Extraction

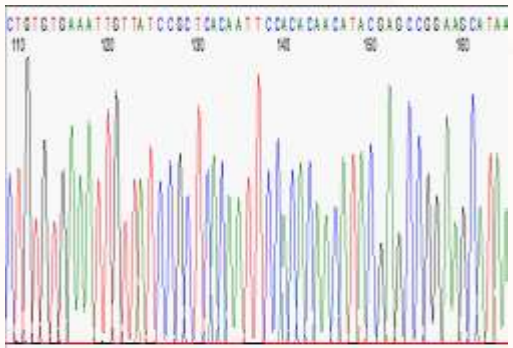
PCR Amplification

Cycle Sequencing PCR

PCR clean-up

Capillary electrophoresis sequencing

Sequence analysis and Interpretation



Next Generation Sequencing (NGS)

- High-throughput, parallel generation of genomic data (reads)
- Multiple samples sequenced with millions of reads
- Everything in the sample is sequenced – a broad approach
- Ton of data to analysis

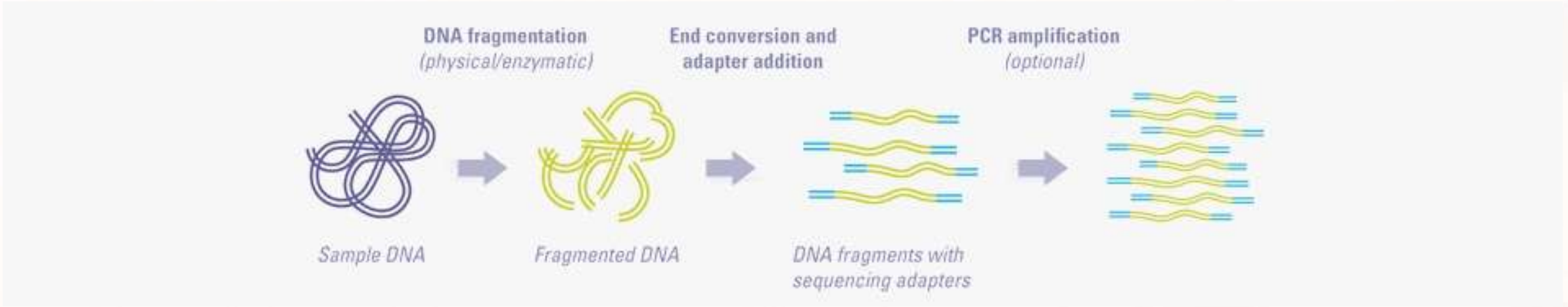
Second Generation – Short read

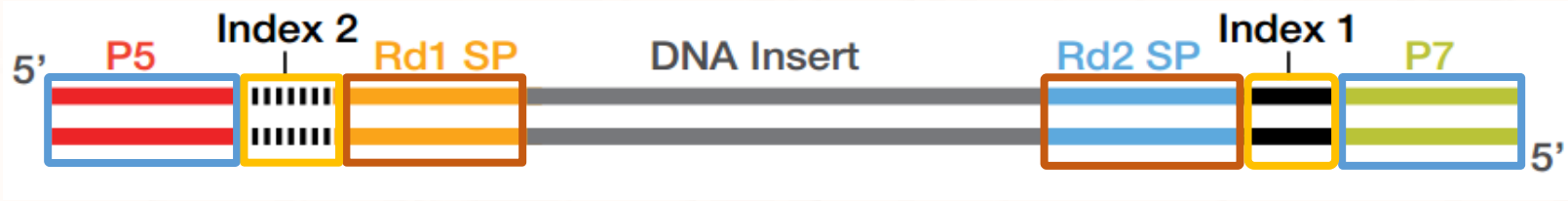
- 50-400 nucleotides per read
- High accuracy
- Longer run time
- 1-5 days, depends on data output
- Best known example:
  - Illumina - iSeq, MiSeq, NextSeq, NovaSeq

Third generation - Long Read

- 1,000->10,000 nucleotides per read
- Lower accuracy
- Faster run time
- <1-2 days
- Best known example:
  - Oxford Nanopore - MinION, GridION

Library Prep allow for high level multiplexing





For clustering

Libraries must have P5 and P7 binding regions on either end of a library

For sequencing

Libraries must have sequencing primer binding regions

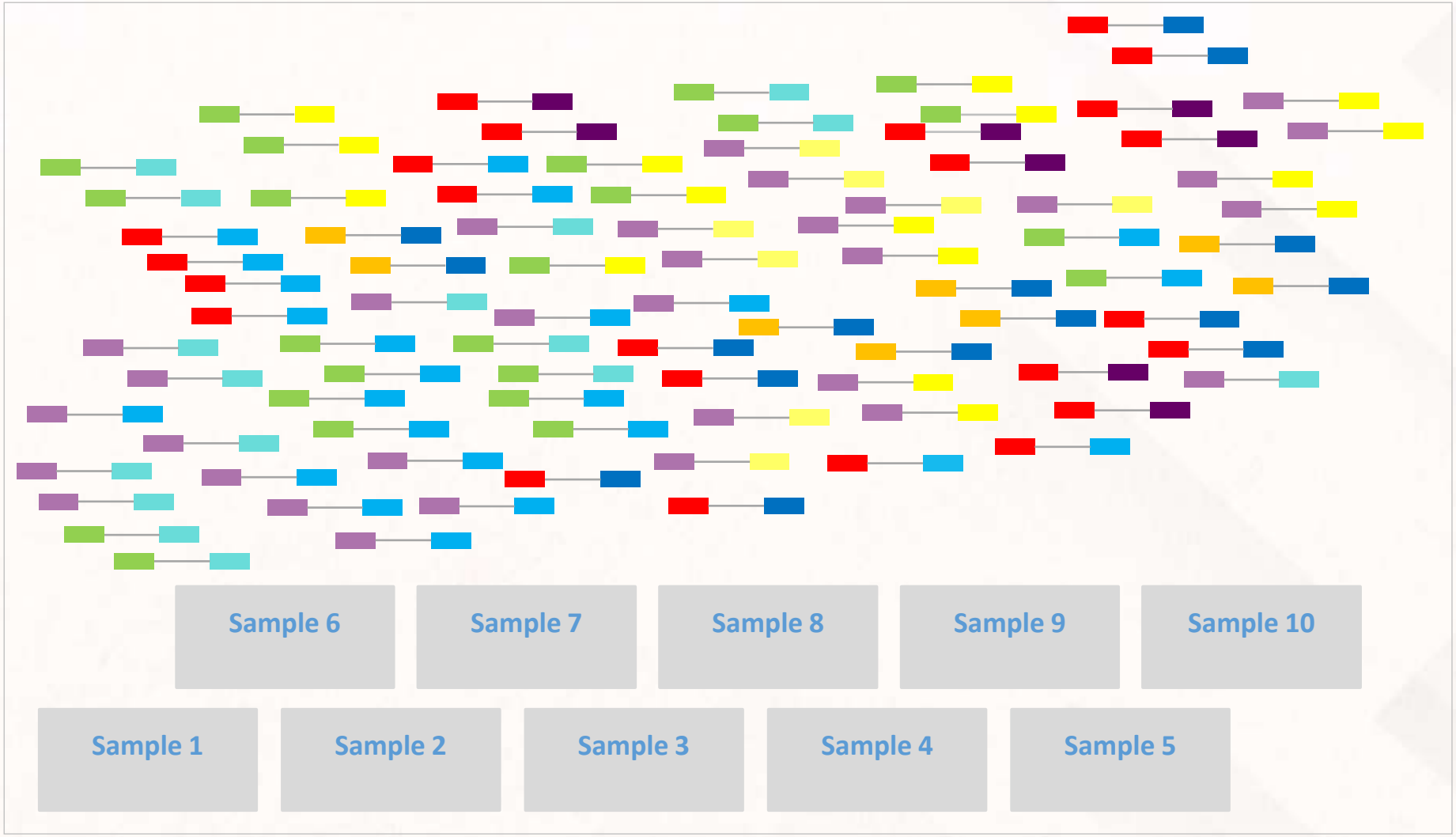
For mixing samples

Libraries must have a unique index or barcodes sequence

Courtesy of Illumina

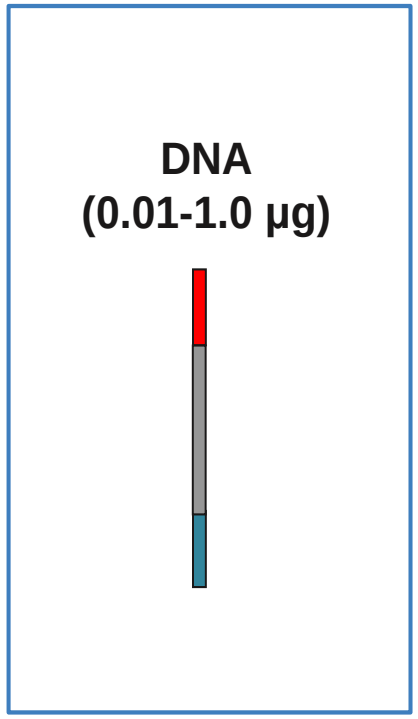
How and Why Samples can be multiplexed

…then sorted by index



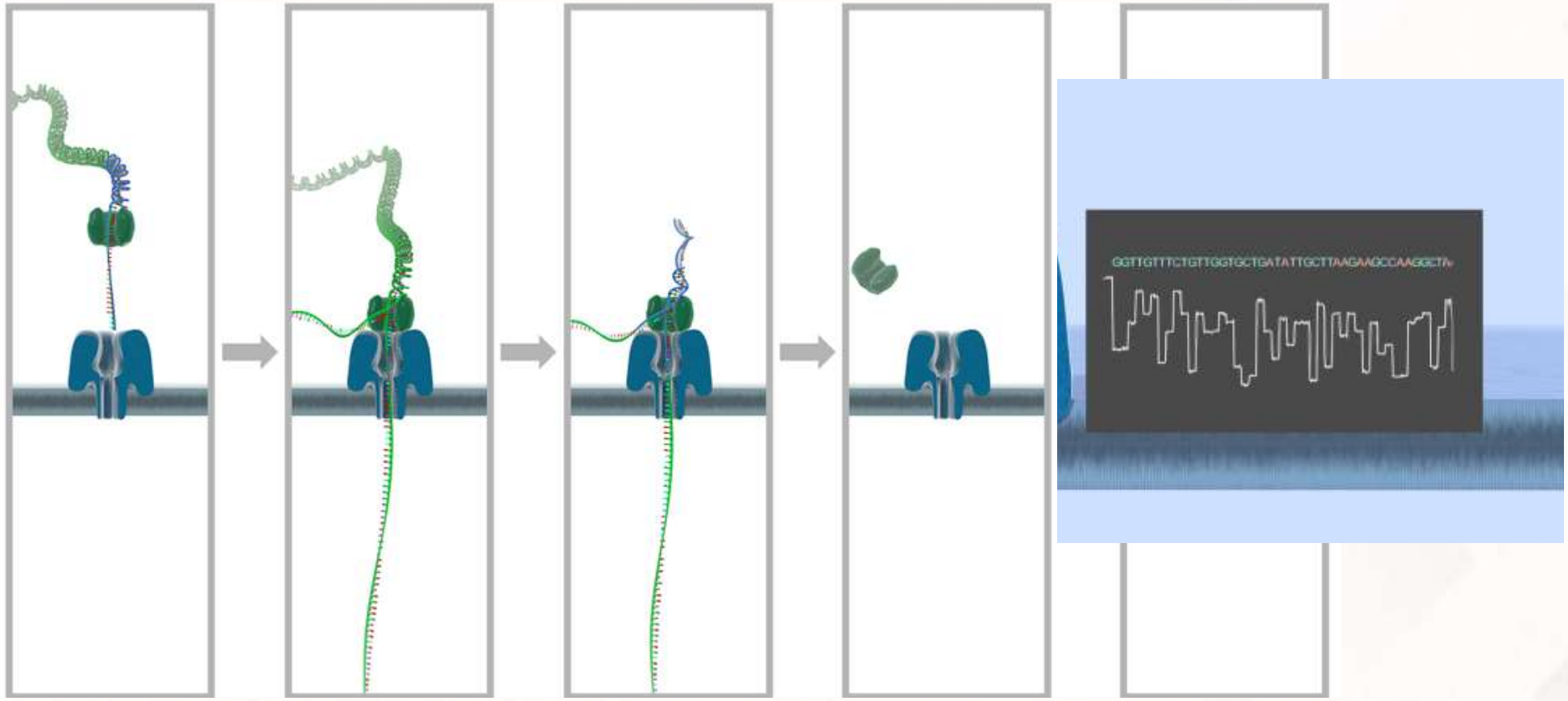
Courtesy of Illumina

Second Generation Sequencing (Illumina)



Library Preparation

Third Generation Sequencing (Oxford Nanopore)



NGS applications for Diagnostics

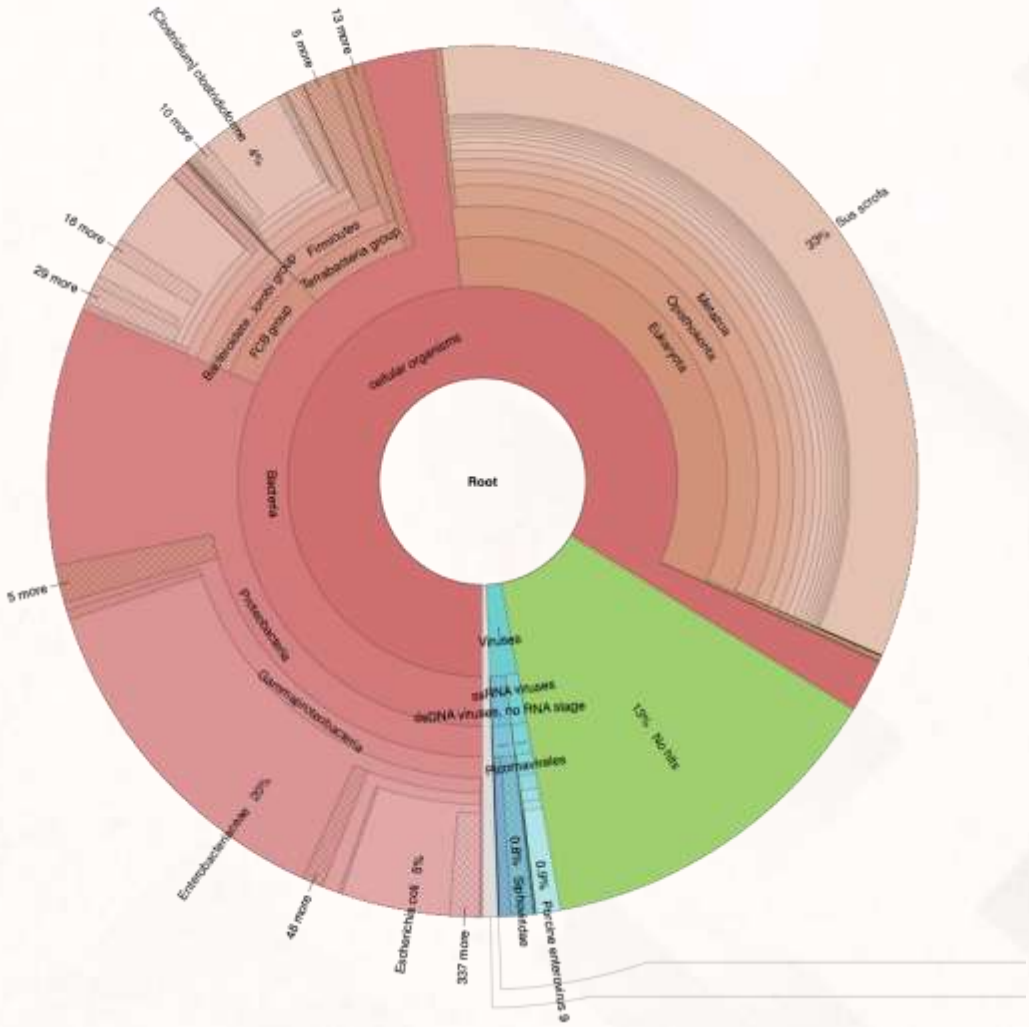
Shotgun Metagenomics	Whole Genome Sequencing (WGS)	Amplicon Sequencing
Sequencing **"everything"** in a sample	Sequencing a **cultured bacterial isolate**	Sequencing **specific genes** for taxonomy
• Sequencing from field samples or isolates ◦ PRRSV, PCV2, PEDV • Novel pathogen detection causing disease • Selection or updating of bacterial/viral strains for autogenous vaccines	• Identify the epidemiological link between strains • Serotype, MLST, virulence factors, antimicrobial resistant genes, and other genetic markers of interest • Selection or updating of autogenesis vaccines	• 16s metagenomics – bacterial differences between health and sick pigs. • Influenza A genome • Rotavirus genome

NGS applications for Diagnostics

Shotgun Metagenomics

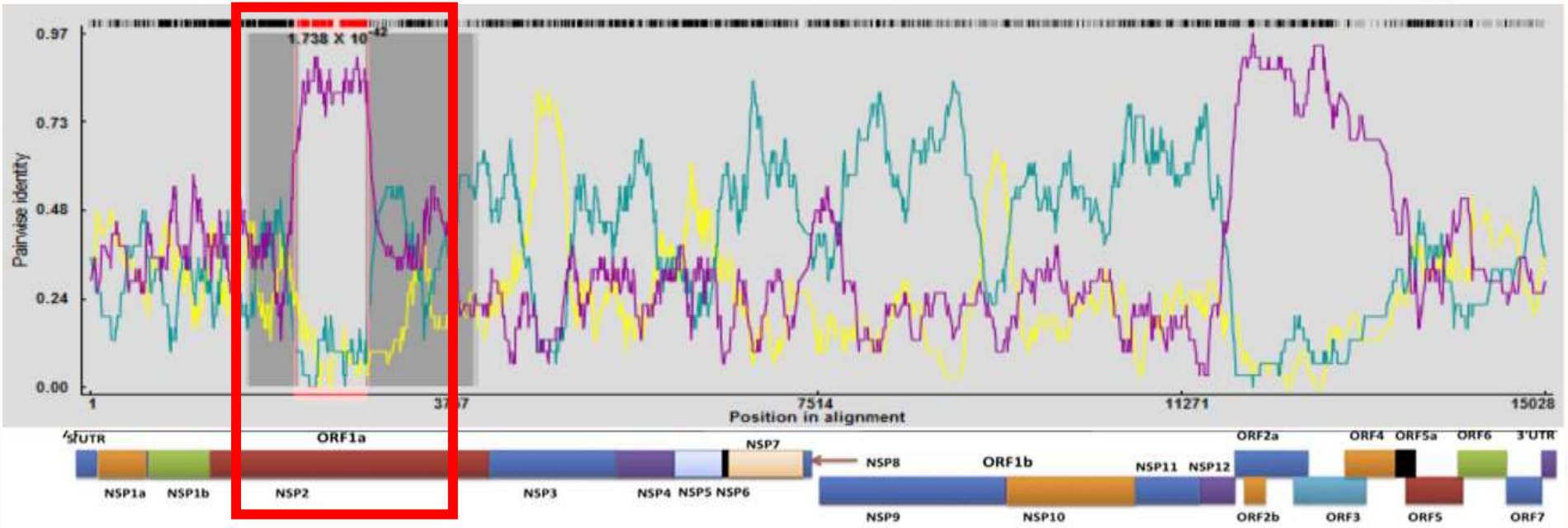
Sequencing **“everything”** in a sample

- PRRSV sequencing, differentiation, and comparing strains from field samples
- Metagenomic viral complex
  - PCV2 and virome
- Novel pathogen detection causing disease



Metagenomics - PRRS Investigation
Comparing 1-7-4 PRRSV strains

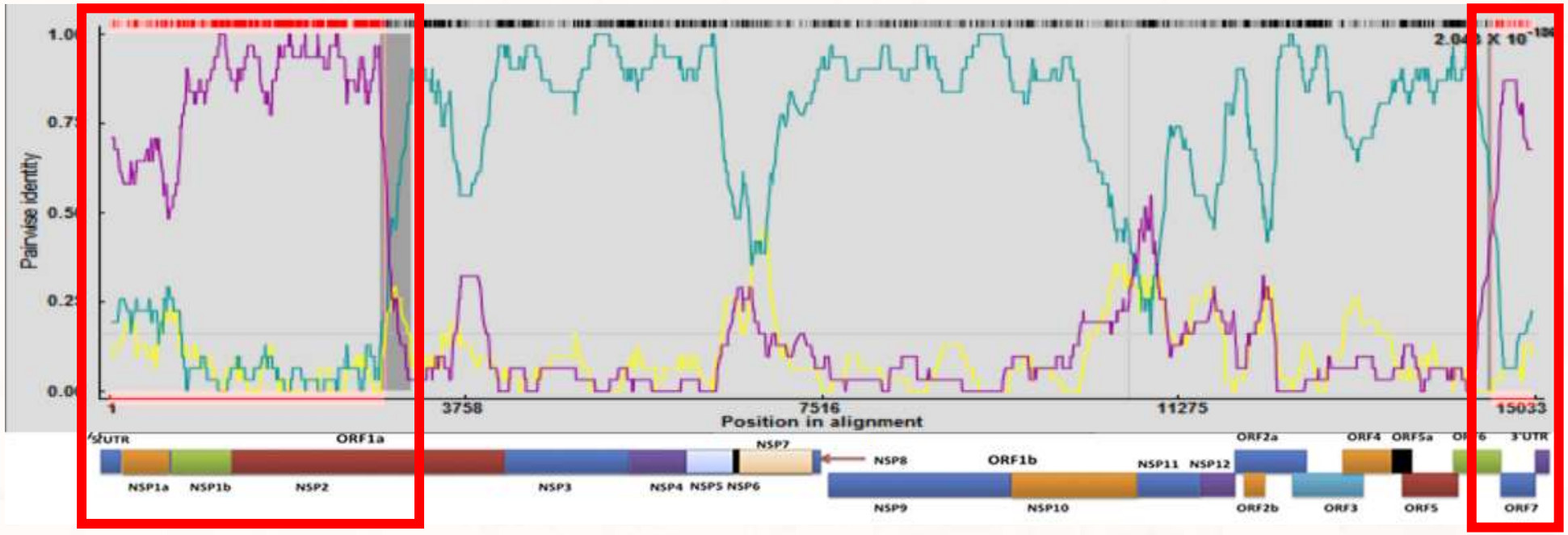
- 1-7-4 strains 2 strains from Minnesota and 1 single strain from North Carolina
  - ORF5 = 98.67% nucleotide identity
  - Whole Genome = 97.09% nucleotide identity



- Major insertions and deletions (INDEL) in NSP2
  - NSP2 plays a role in reducing pig's immune response

Metagenomics - PRRS Investigation:
Comparing 1-3-4 PRRSV strains

- 1 pathogenic strains from Minnesota in an immunized herd, 25% piglet mortality
- 1 pathogenic strains from North Carolina, up 42% mortality in finishers
  - ORF5 = 98.34% nucleotide identity
  - Whole Genome = 92.72% nucleotide identity

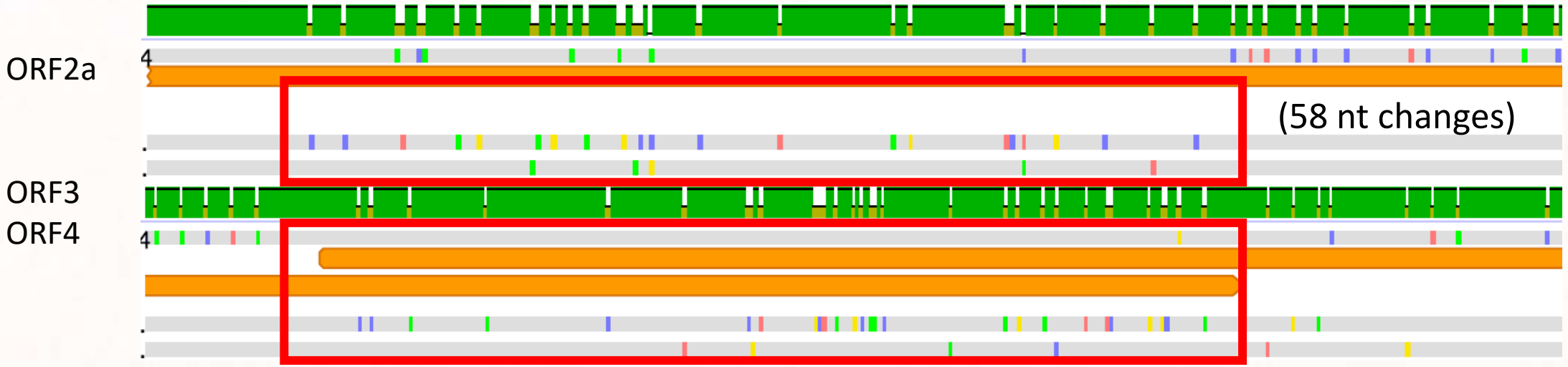


Metagenomics - PRRS Investigation:
Comparing 1-7-4 and 1-3-4 PRRSV strains

- Same insert and deletions (INDELs) as the North Carolina's 1-7-4 strains
  - The insertion is evolutionary important
  - New NSP2 ribosomal shift was identified

Metagenomics - PRRS Investigation:
Mixture of PRRSV strains in a sample

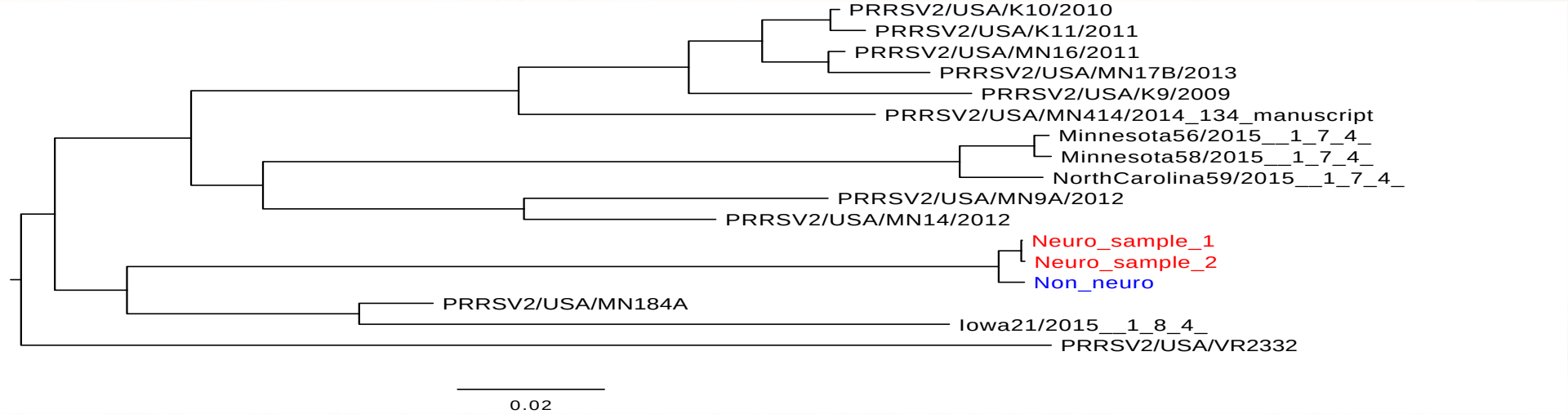
- Case from Minnesota
  - ORF5 = 100% nucleotide identity
  - Whole Genome = 99.61-99.84
  - Two variable regions (ORF2a, ORF3, ORF4)
    - 94.99% nucleotide percent identity



- Like ORF5, ORF2a, ORF3, and ORF4 elicit neutralizing antibodies
  - May be involved in viral entry into the cell

Metagenomics - PRRS Investigation:
Comparing PRRSV strains

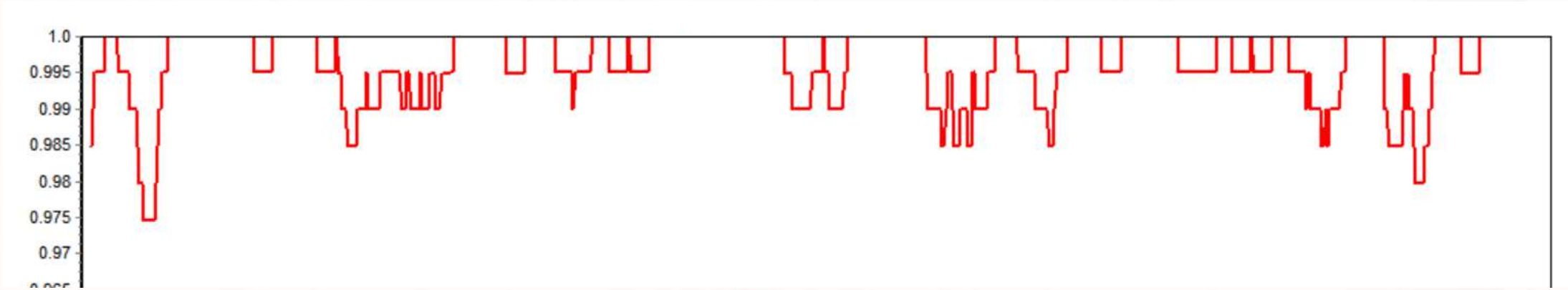
- Neurological case of PRRS
  - Virus was introduced from another system
  - ORF5 =100% nucleotide identity
  - Whole Genome = 99.33% nucleotide identity (~100 nt and 19 aa changes)



- What's causing neurological signs?
  - Undiscovered protein, amino acid changes in the PRRSV?

Metagenomics - PRRS Investigation
NGS cannot answer every question, yet

- PRRS break in non-immunized herd
  - Severe clinical signs in piglets (Ct values = 6-11)
  - ORF5 =99.17% nucleotide identity to vaccine strain
  - Whole Genome = 99.6% nucleotide identity (61 nt changes)

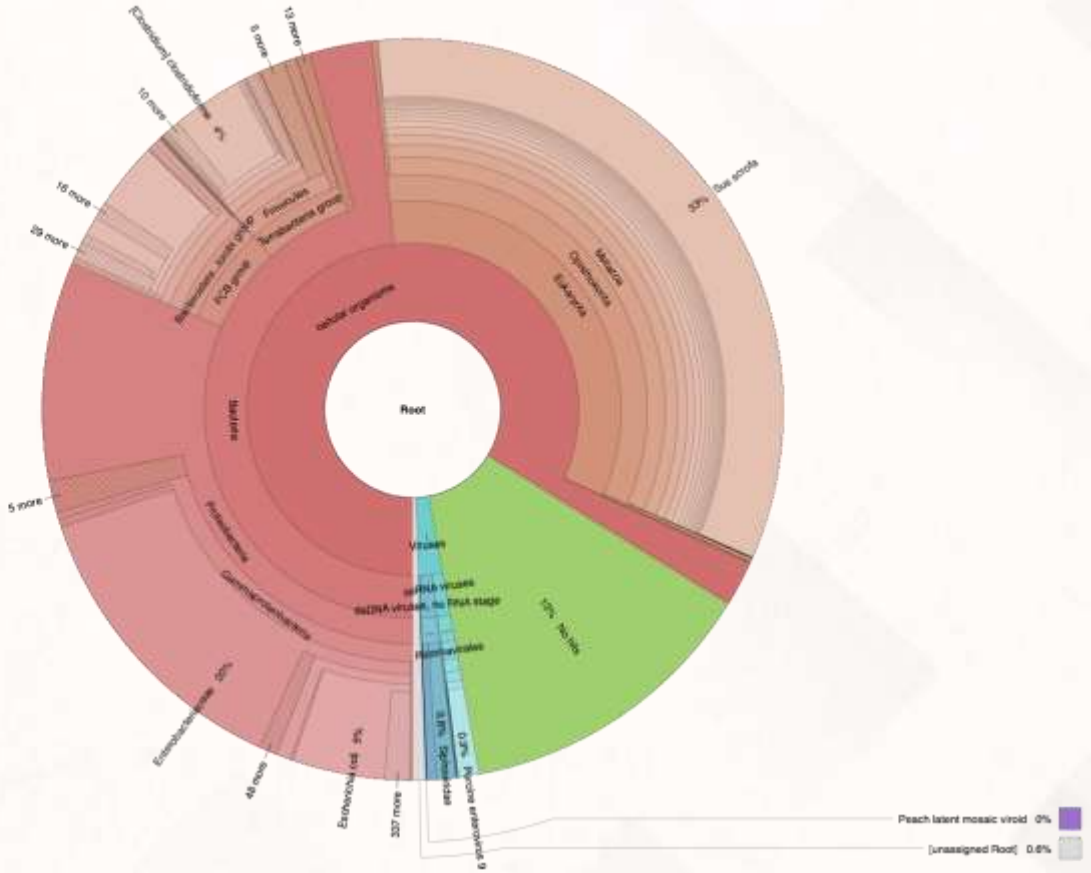


- Revertant or ancestral relationship of the vaccine strain

Metagenomics – Novel Pathogen Discovery
Unknown cause of enteric disease

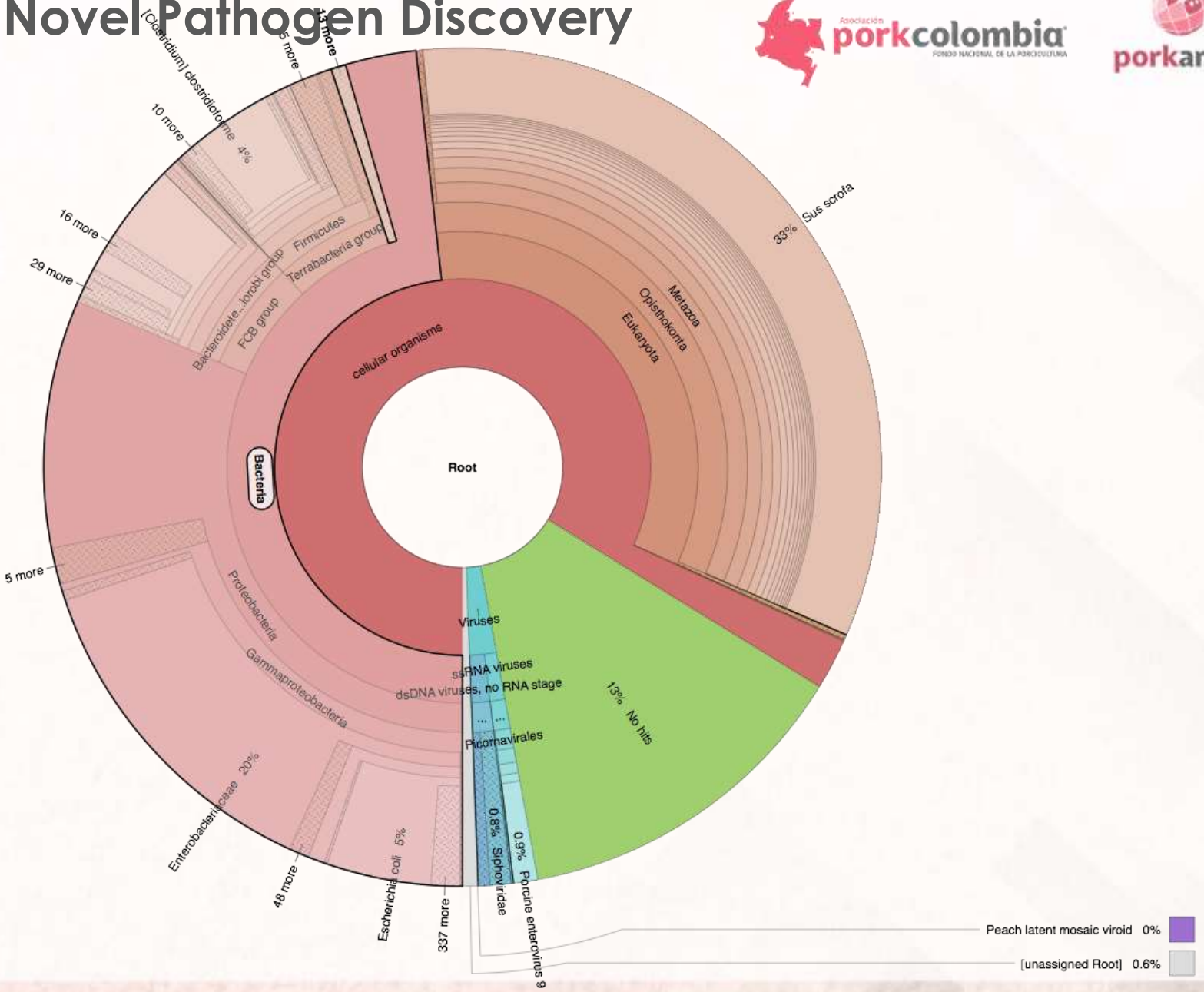
Unknown cause of enteritis

- 12 porcine fecal samples from Texas, USA
- Pigs presenting with diarrhea
- Bacterial cultures lacked grow any pathogenic strains
- Viral qPCR testing was negative for porcine coronaviruses and rotaviruses
- Extracted total RNA for NGS and analysis

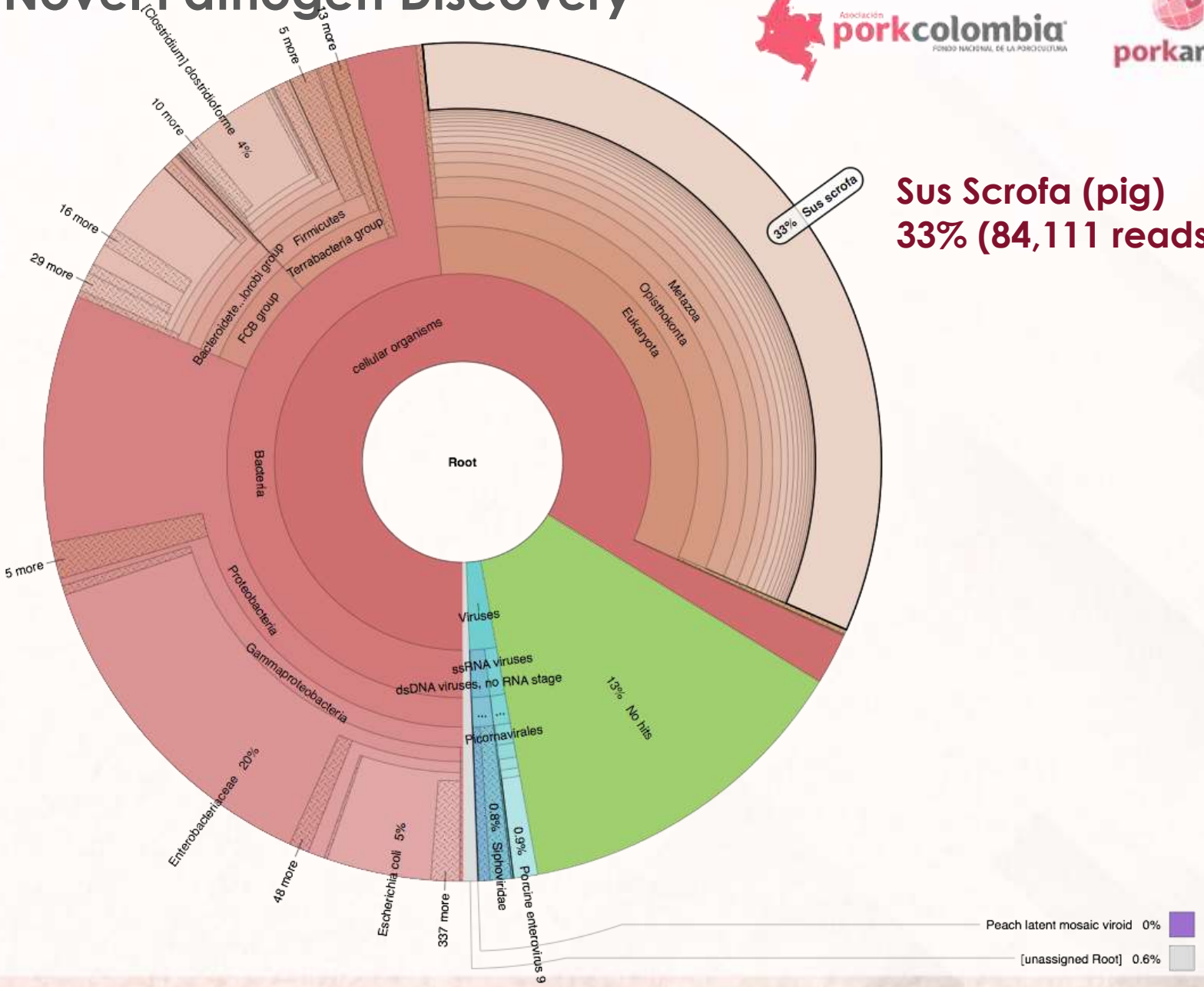


Metagenomics – Novel Pathogen Discovery

Bacteria 48% (122,663 reads)

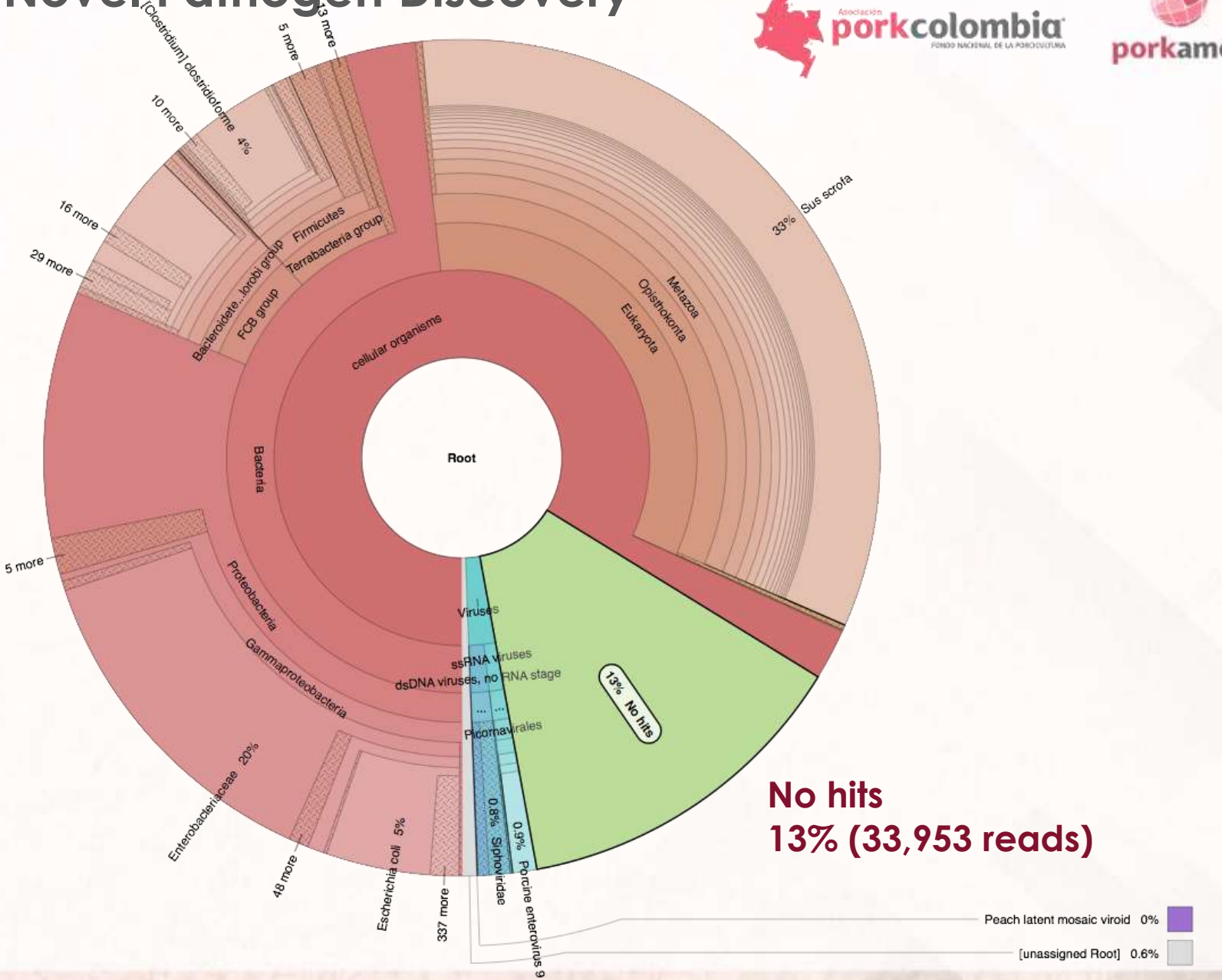


Metagenomics – Novel Pathogen Discovery



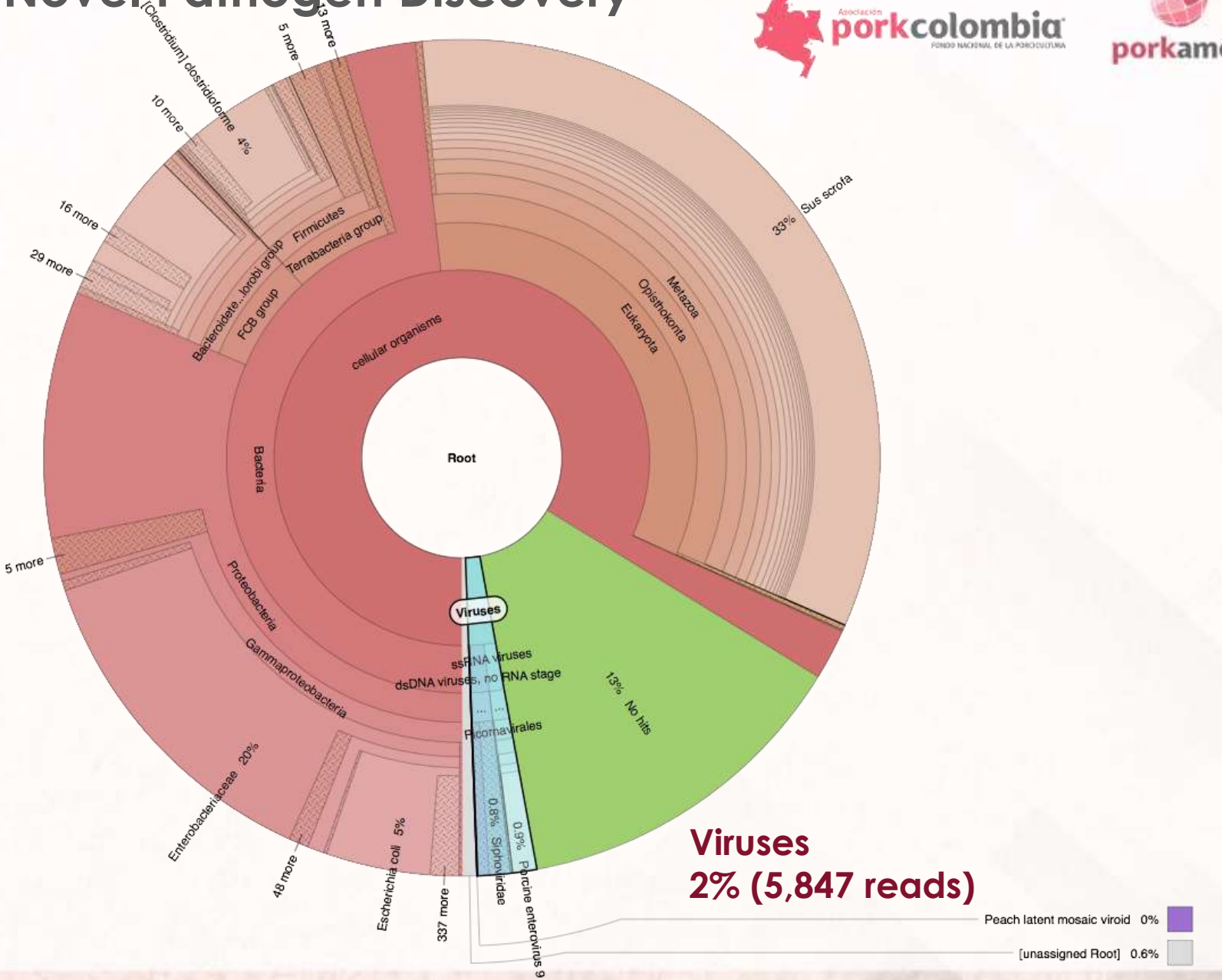
Sus Scrofa (pig)
33% (84,111 reads)

Metagenomics – Novel Pathogen Discovery

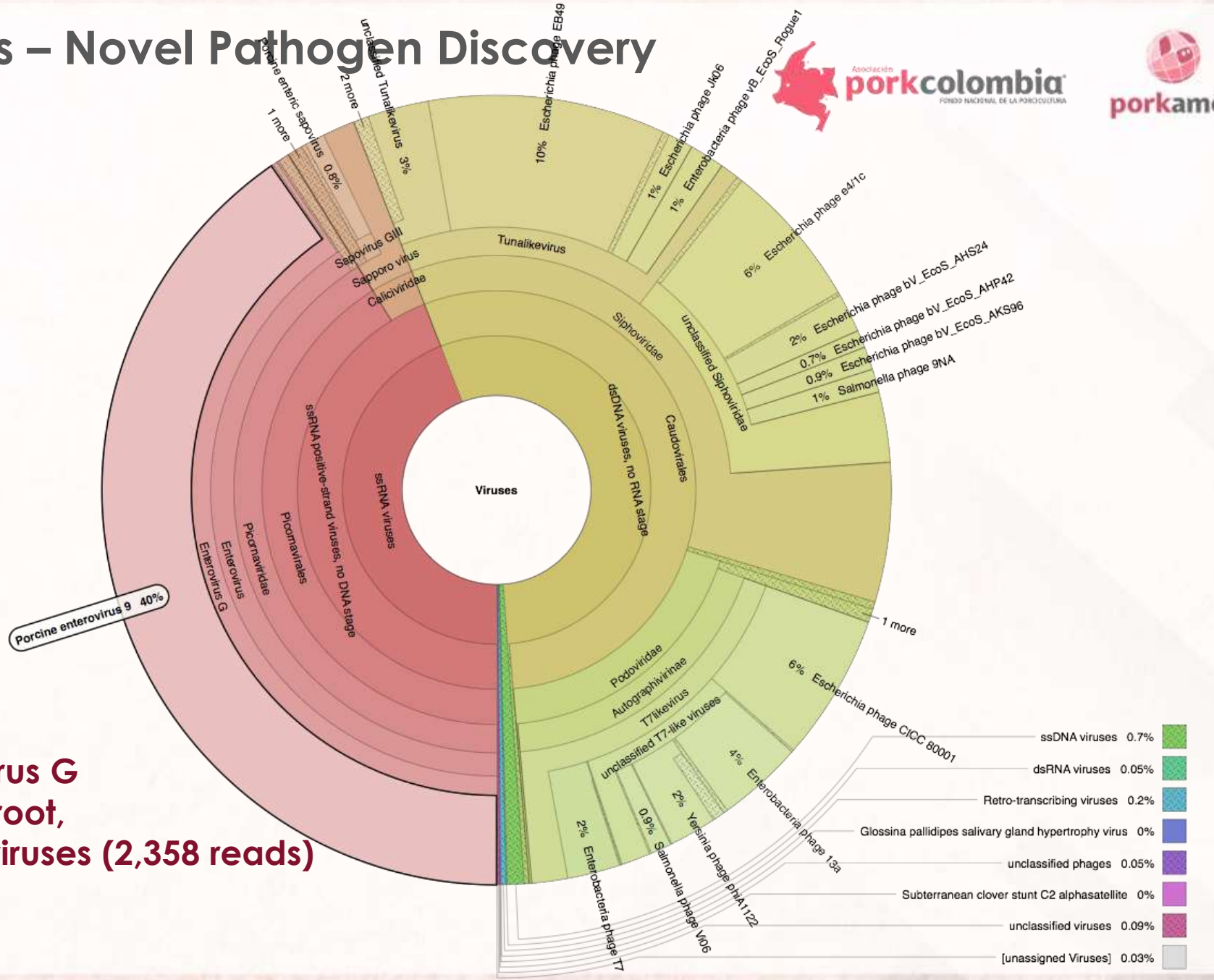


No hits
13% (33,953 reads)

Metagenomics – Novel Pathogen Discovery



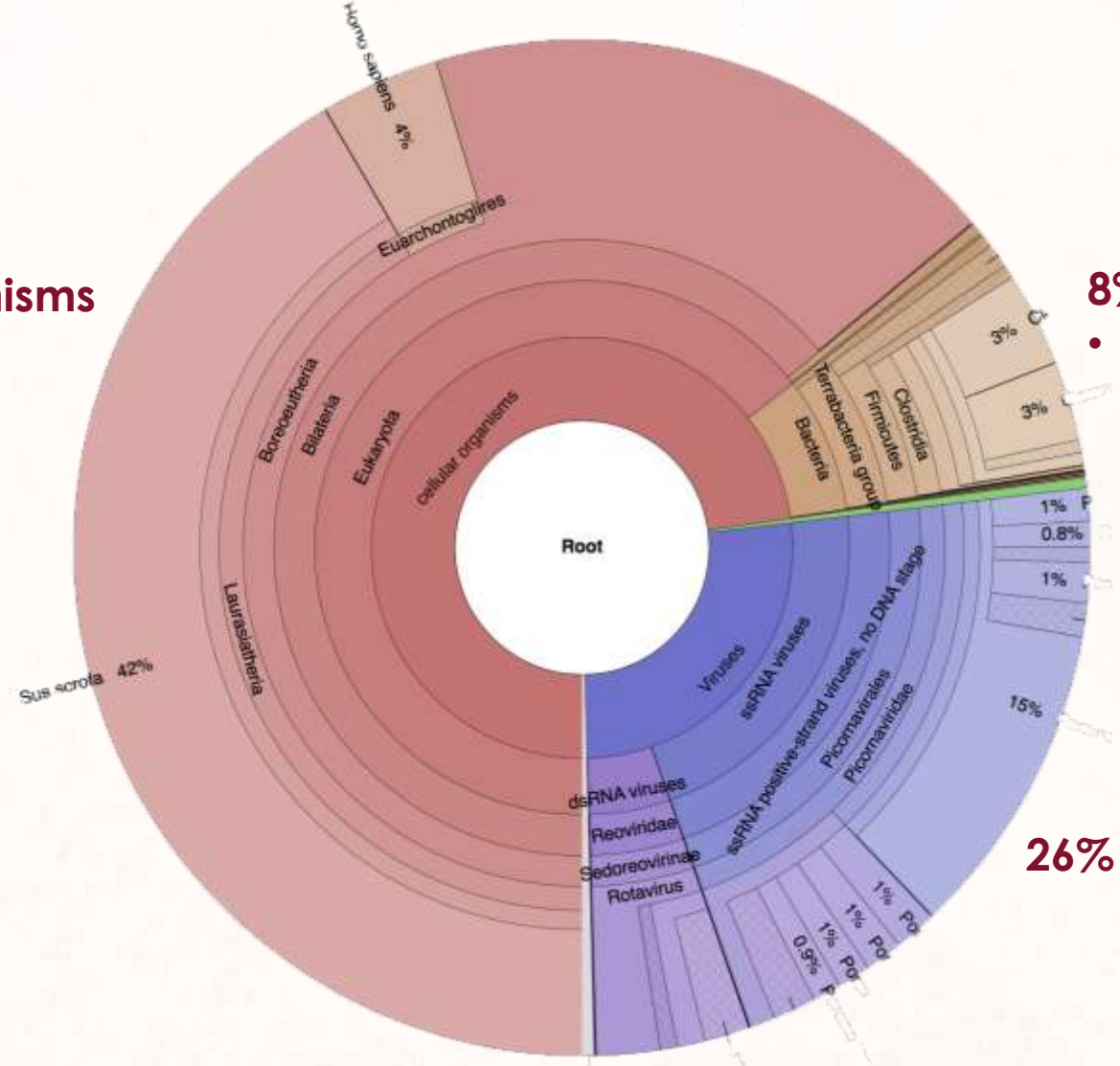
Metagenomics – Novel Pathogen Discovery



Enterovirus G
0.9% of root,
40% of viruses (2,358 reads)

Metagenomics – Feedback
What are we really feeding sows?

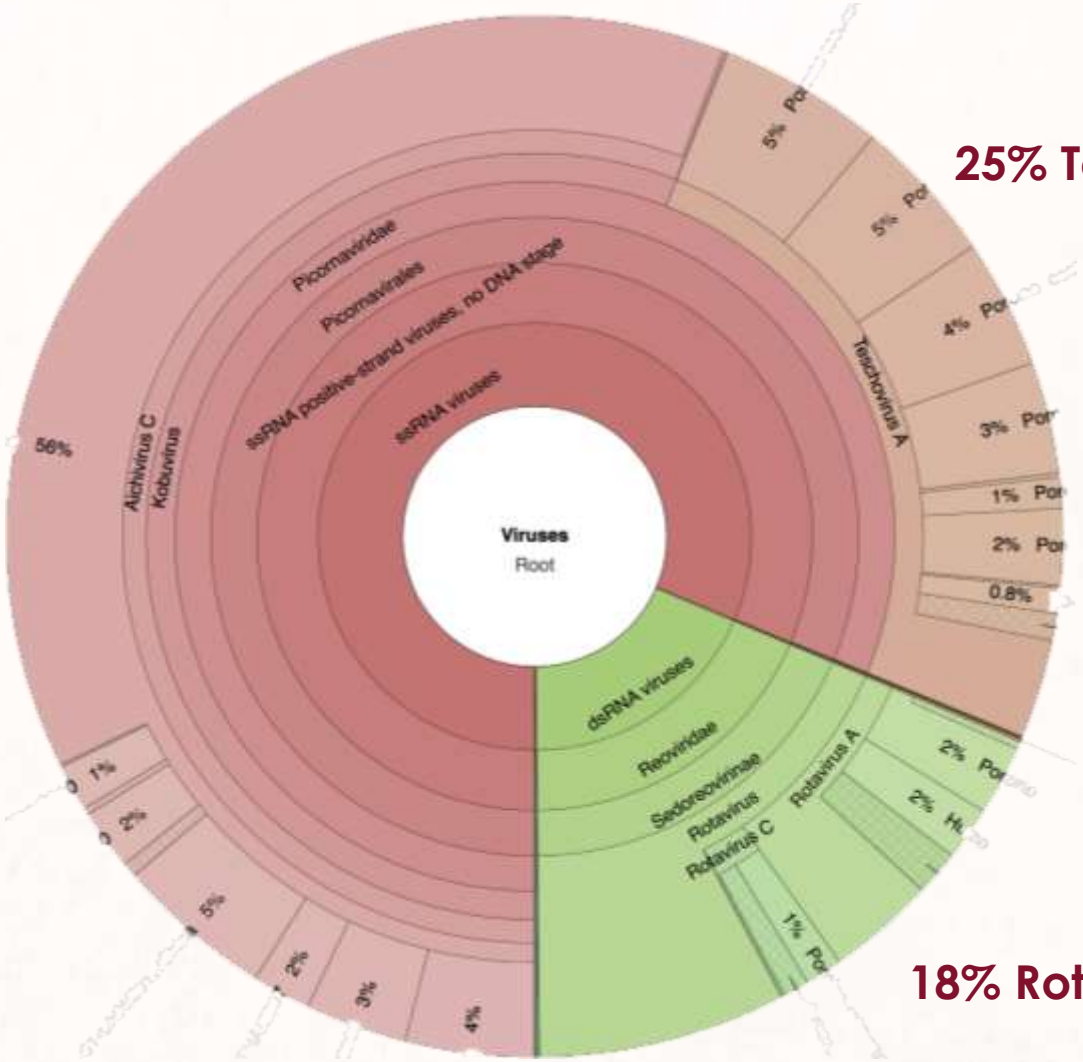
64% cellular organisms



8% bacteria
- Clostridium identified

26% viruses

Metagenomics – Feedback
What are we really feeding sows?



25% Teschovirus

56% Kobuvirus

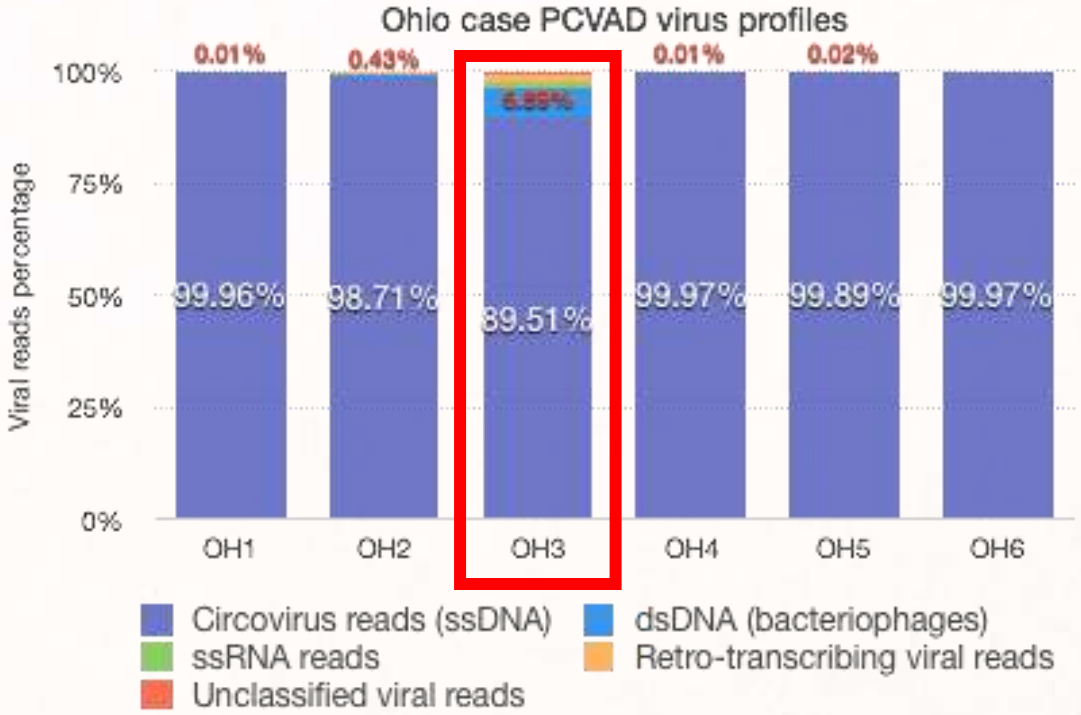
18% Rotaviruses

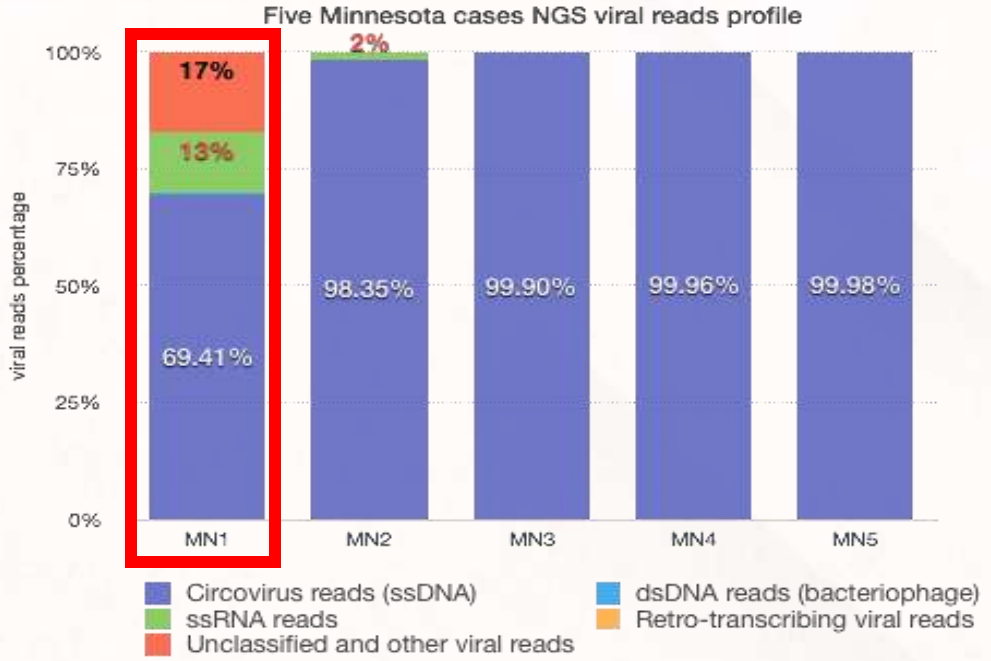
Metagenomics

Investigating Porcine Circovirus Associated Disease

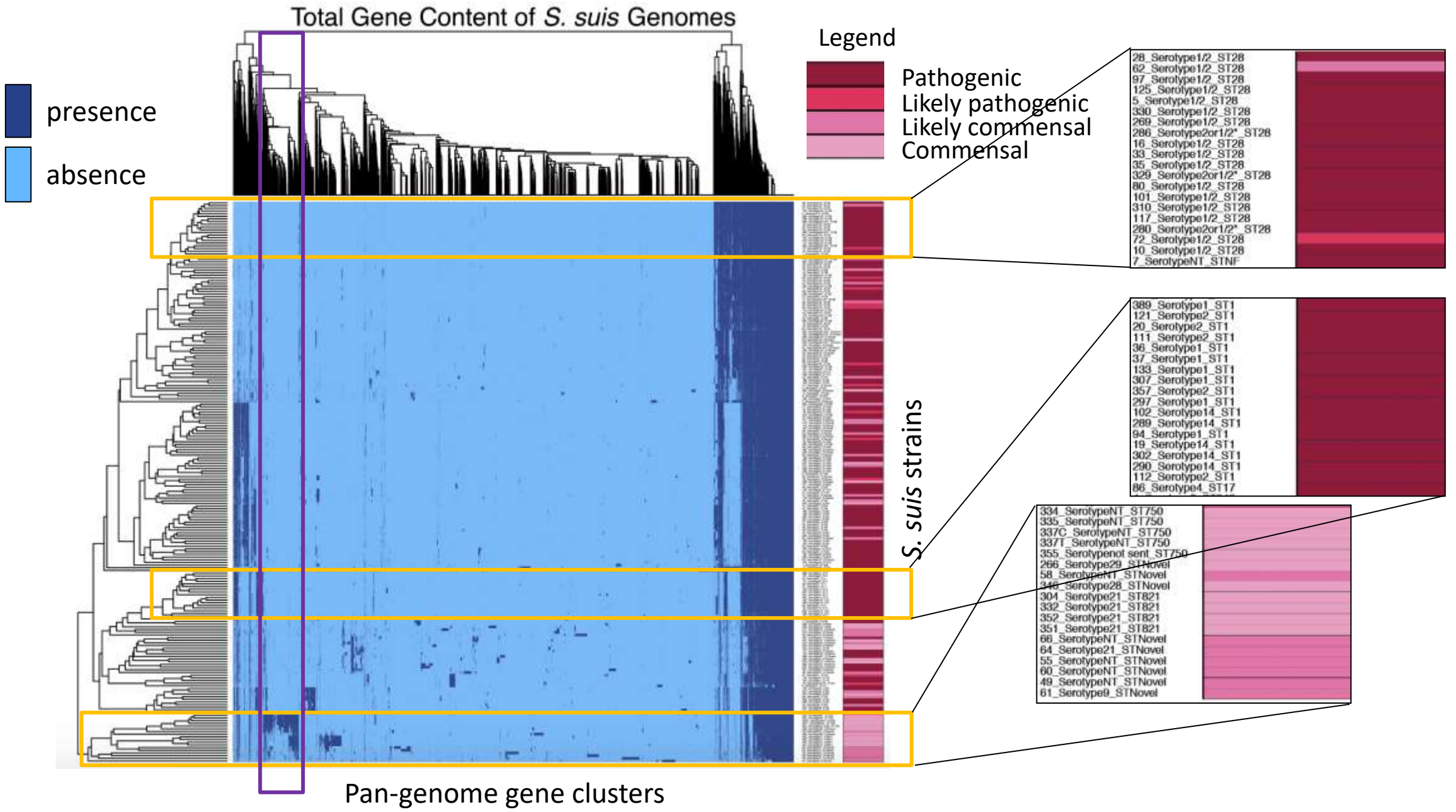
- Objective: To investigate the involvement of other viruses with Porcine Circovirus Associated Disease
  - Single case from Ohio, 6 samples
  - Five cases from Minnesota

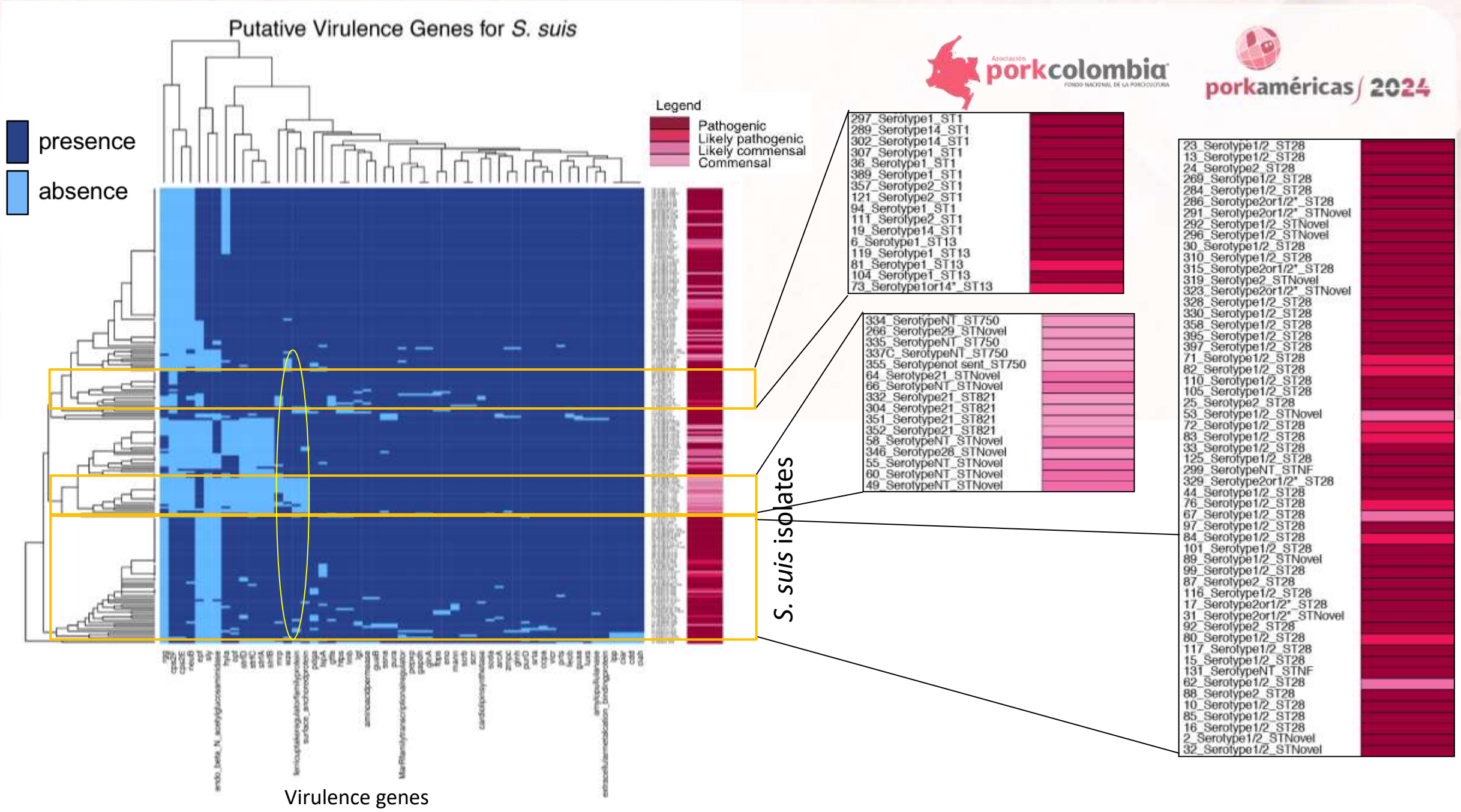
Metagenomics:
NGS Results



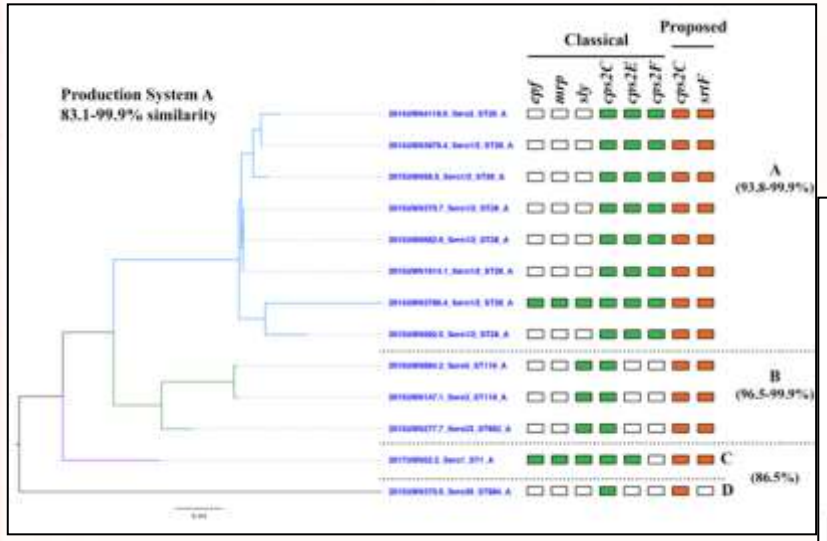


- Additional clinically important viruses were not identified in the Ohio samples
- Pasivirus A and Porcine parvovirus 7 were identified in MN1

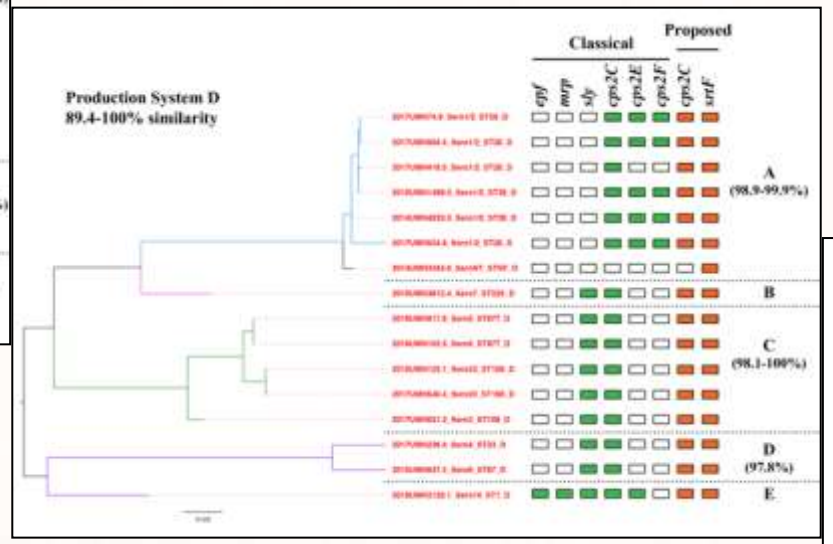

Total Gene Content of S. suis Genomes
presence
absence
Legend
Pathogenic
Likely pathogenic
Likely commensal
Commensal
28_Serotype1/2_ST28
62_Serotype1/2_ST28
97_Serotype1/2_ST28
125_Serotype1/2_ST28
5_Serotype1/2_ST28
330_Serotype1/2_ST28
269_Serotype1/2_ST28
286_Serotype2or1/2*_ST28
16_Serotype1/2_ST28
33_Serotype1/2_ST28
35_Serotype1/2_ST28
329_Serotype2or1/2*_ST28
80_Serotype1/2_ST28
101_Serotype1/2_ST28
310_Serotype1/2_ST28
117_Serotype1/2_ST28
280_Serotype2or1/2*_ST28
72_Serotype1/2_ST28
10_Serotype1/2_ST28
7_SerotypeNT_STNF
389_Serotype1_ST1
121_Serotype2_ST1
20_Serotype2_ST1
111_Serotype2_ST1
36_Serotype1_ST1
37_Serotype1_ST1
133_Serotype1_ST1
307_Serotype1_ST1
357_Serotype2_ST1
297_Serotype1_ST1
102_Serotype14_ST1
289_Serotype14_ST1
94_Serotype1_ST1
19_Serotype14_ST1
302_Serotype14_ST1
290_Serotype14_ST1
112_Serotype2_ST1
86_Serotype4_ST17
334_SerotypeNT_ST750
335_SerotypeNT_ST750
337C_SerotypeNT_ST750
337T_SerotypeNT_ST750
355_Serotypenot sent_ST750
266_Serotype29_STNovel
58_SerotypeNT_STNovel
346_Serotype28_STNovel
304_Serotype21_ST821
332_Serotype21_ST821
352_Serotype21_ST821
351_Serotype21_ST821
66_SerotypeNT_STNovel
64_Serotype21_STNovel
55_SerotypeNT_STNovel
60_SerotypeNT_STNovel
49_SerotypeNT_STNovel
61_Serotype9_STNovel
S. suis strains
Pan-genome gene clusters


Putative Virulence Genes for S. suis
presence
absence
Legend
Pathogenic
Likely pathogenic
Likely commensal
Commensal
297_Serotype1_ST1
289_Serotype14_ST1
302_Serotype14_ST1
307_Serotype1_ST1
36_Serotype1_ST1
389_Serotype1_ST1
357_Serotype2_ST1
121_Serotype2_ST1
94_Serotype1_ST1
111_Serotype2_ST1
19_Serotype14_ST1
6_Serotype1_ST13
119_Serotype1_ST13
81_Serotype1_ST13
104_Serotype1_ST13
73_Serotype1or14*_ST13
334_SerotypeNT_ST750
266_Serotype29_STNovel
335_SerotypeNT_ST750
337C_SerotypeNT_ST750
355_Serotypenot sent_ST750
64_Serotype21_STNovel
66_SerotypeNT_STNovel
332_Serotype21_ST821
304_Serotype21_ST821
351_Serotype21_ST821
352_Serotype21_ST821
58_SerotypeNT_STNovel
346_Serotype28_STNovel
55_SerotypeNT_STNovel
60_SerotypeNT_STNovel
49_SerotypeNT_STNovel
S. suis isolates
23_Serotype1/2_ST28
13_Serotype1/2_ST28
24_Serotype2_ST28
269_Serotype1/2_ST28
284_Serotype1/2_ST28
286_Serotype2or1/2*_ST28
291_Serotype2or1/2*_STNovel
292_Serotype1/2_STNovel
296_Serotype1/2_STNovel
30_Serotype1/2_ST28
310_Serotype1/2_ST28
315_Serotype2or1/2*_ST28
319_Serotype2_STNovel
323_Serotype2or1/2*_STNovel
328_Serotype1/2_ST28
330_Serotype1/2_ST28
358_Serotype1/2_ST28
395_Serotype1/2_ST28
397_Serotype1/2_ST28
71_Serotype1/2_ST28
82_Serotype1/2_ST28
110_Serotype1/2_ST28
105_Serotype1/2_ST28
25_Serotype2_ST28
53_Serotype1/2_STNovel
72_Serotype1/2_ST28
83_Serotype1/2_ST28
33_Serotype1/2_ST28
125_Serotype1/2_ST28
299_SerotypeNT_STNF
329_Serotype2or1/2*_ST28
44_Serotype1/2_ST28
76_Serotype1/2_ST28
67_Serotype1/2_ST28
97_Serotype1/2_ST28
84_Serotype1/2_ST28
101_Serotype1/2_ST28
89_Serotype1/2_STNovel
99_Serotype1/2_ST28
87_Serotype2_ST28
116_Serotype1/2_ST28
17_Serotype2or1/2*_ST28
31_Serotype2or1/2*_STNovel
92_Serotype2_ST28
80_Serotype1/2_ST28
117_Serotype1/2_ST28
15_Serotype1/2_ST28
131_SerotypeNT_STNF
62_Serotype1/2_ST28
88_Serotype2_ST28
10_Serotype1/2_ST28
85_Serotype1/2_ST28
16_Serotype1/2_ST28
2_Serotype1/2_STNovel
32_Serotype1/2_STNovel
Virulence genes

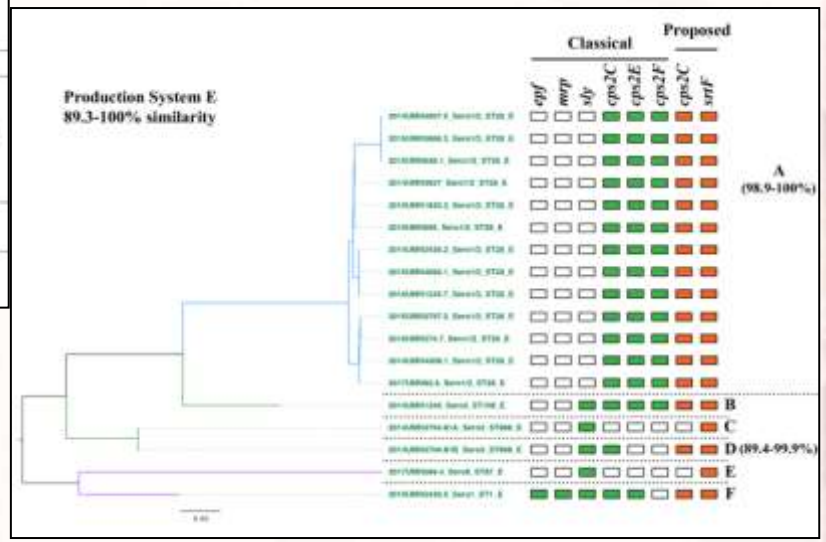
Production System – vaccine selection



- Possibly 3 strains
- Single strain from A, B, and C
- Strain D likely not pathogenic



- Possibly 5 strains
- Single strain for each grouping
- Or 4 vaccines, combine B and C



- Possibly 2-3 strains
- Single strain for grouping A

Conclusion

- Next Generating Sequencing is a powerful tool for making better-informed decisions about pathogens' differences.

- Shotgun Metagenomics allows for
  - Sequence comparison between viral strains.
  - Selection of bacterial and viral strains for autogenous vaccines
  - Novel pathogens detection causing clinical disease

- Complete genome sequencing of known and unknown pathogens allows further identification of strain variation to address emerging and re-emerging viruses.

- Whole Genome Sequencing of bacterial isolates identifies
  - serotype, MLST, virulence factors, and genetic markers

  to understand the diversity within a production system, allowing better guidance for selecting autogenous vaccine strain.

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¡GRACIAS!

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