Combating Unknown Enemies with the Help of Molecular Diagnostics

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Boehringer Ingelheim

Metagenomics

NGS (Next Generation Sequencing)

RNA sequencing is key to diagnostics

RNA is produced by all viruses and bacteria

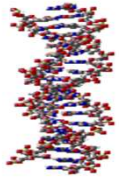
Total random RNA sequencing does not require a hypothesis

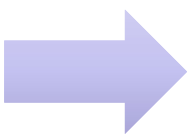
Sequencing provides unsurpassed specificity

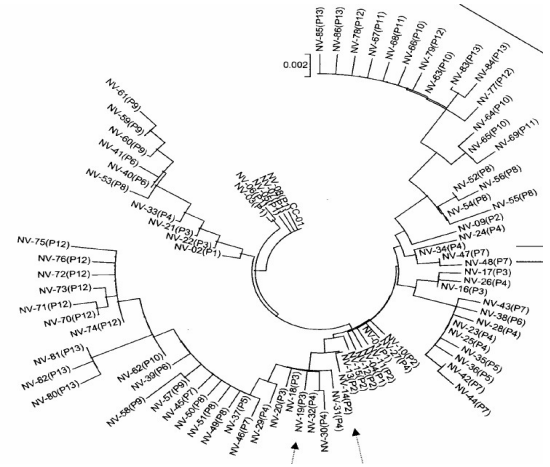
Proportion of reads may provide a “semi quantitative” representation

If random, it provides a representation of “all” agents present

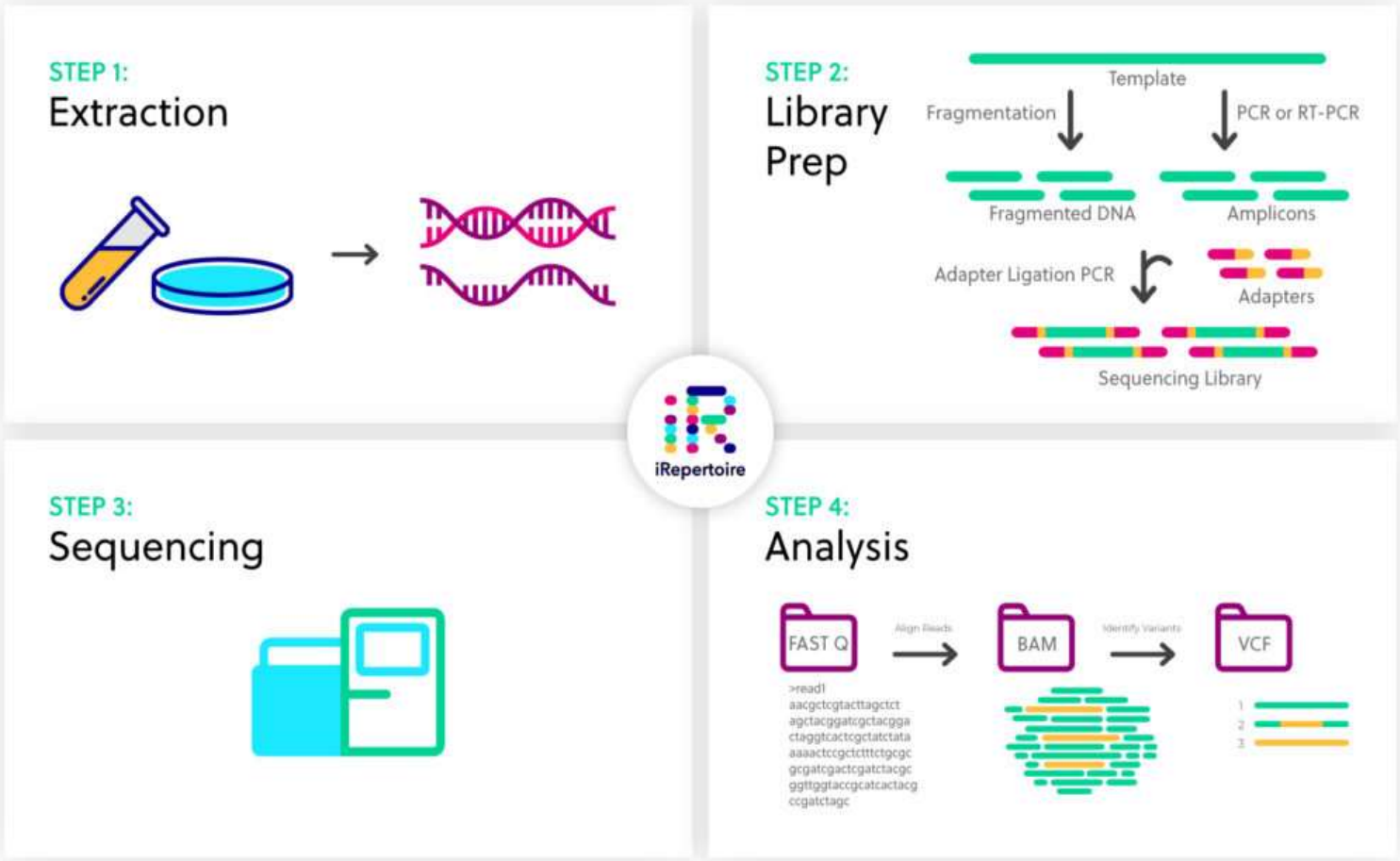
May detect mixed infections that are relevant to clinical diagnosis



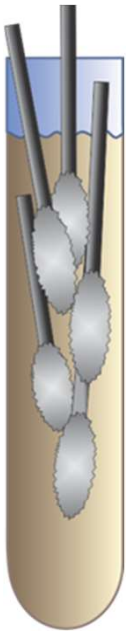




NGS (Next Generation Sequencing)

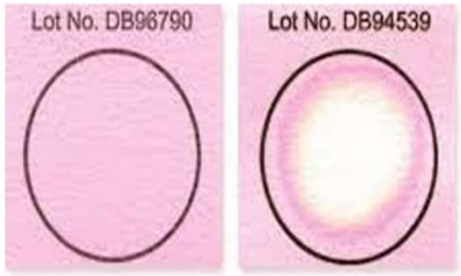


Manual for sample collection

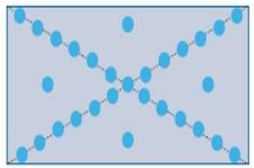


A

B

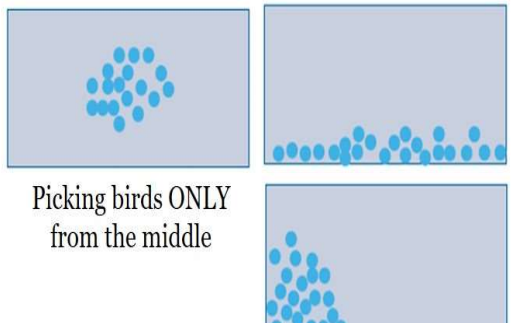


Recommended method



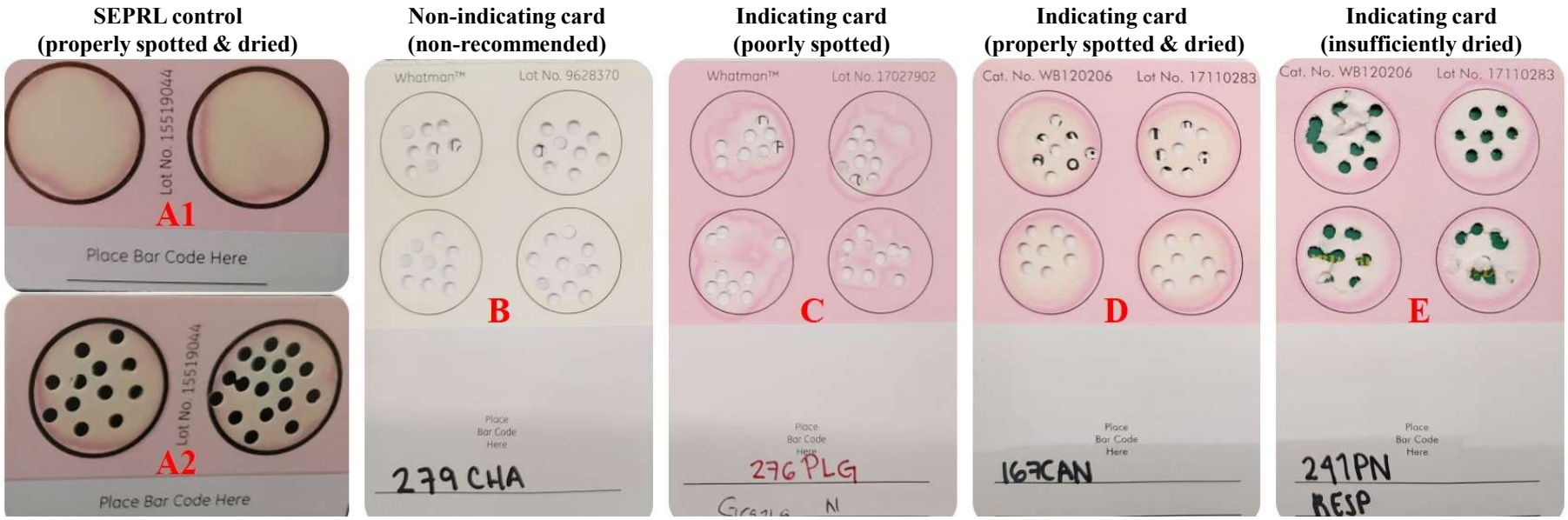
Select the birds randomly

Non-recommended method



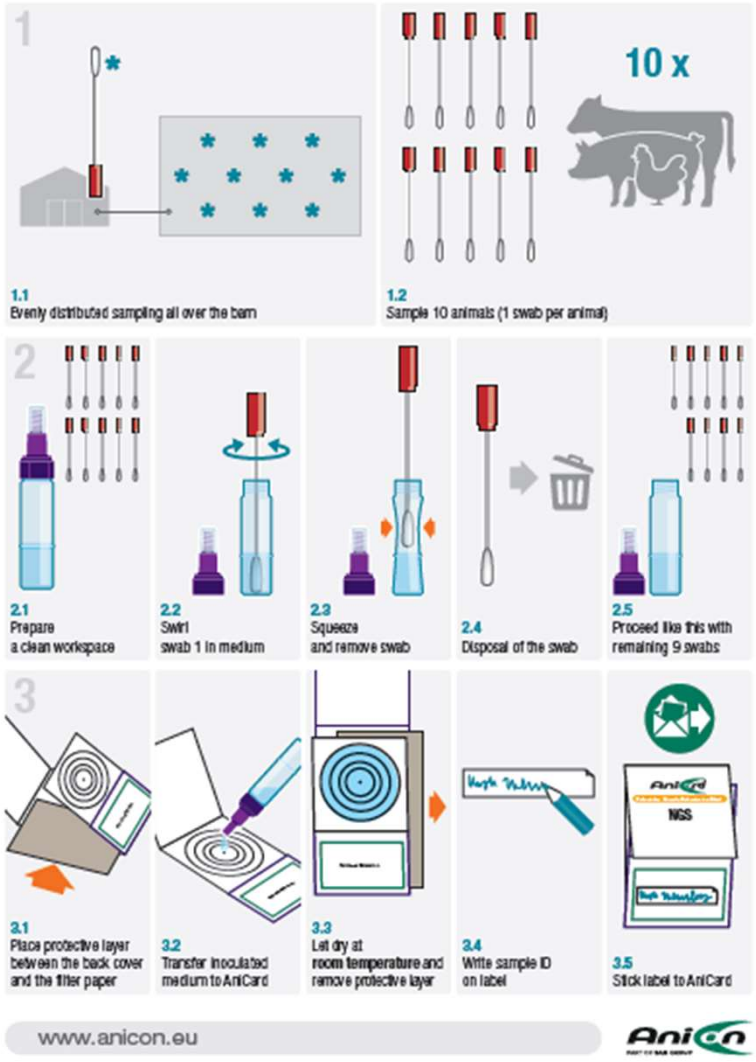
Picking birds ONLY from the middle

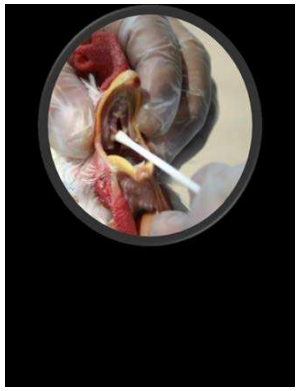
Manual for sample collection

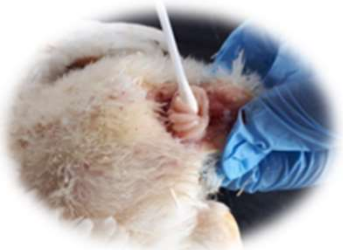


QC of FTA cards. These are Among the key aspects to pay attention to during sample preparation in the field. Panels A and D illustrates FTA cards (from SEPRL control sample and BAH field sample, respectively) that are properly spotted (with the recommended volumes of samples, i.e., approximately 125 µL) and sufficiently dried. Panel B illustrates non-indicating FTA card in which it is difficult to locate where the samples were spotted or verify if sufficient sample volumes were spotted. Panel E shows an insufficiently dried (damp) FTA card spotted with a field sample, which are difficult to punch out discs for subsequent extraction of nucleic acids.

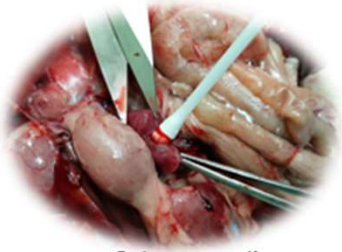
Manual for sample collection







Bursa sampling

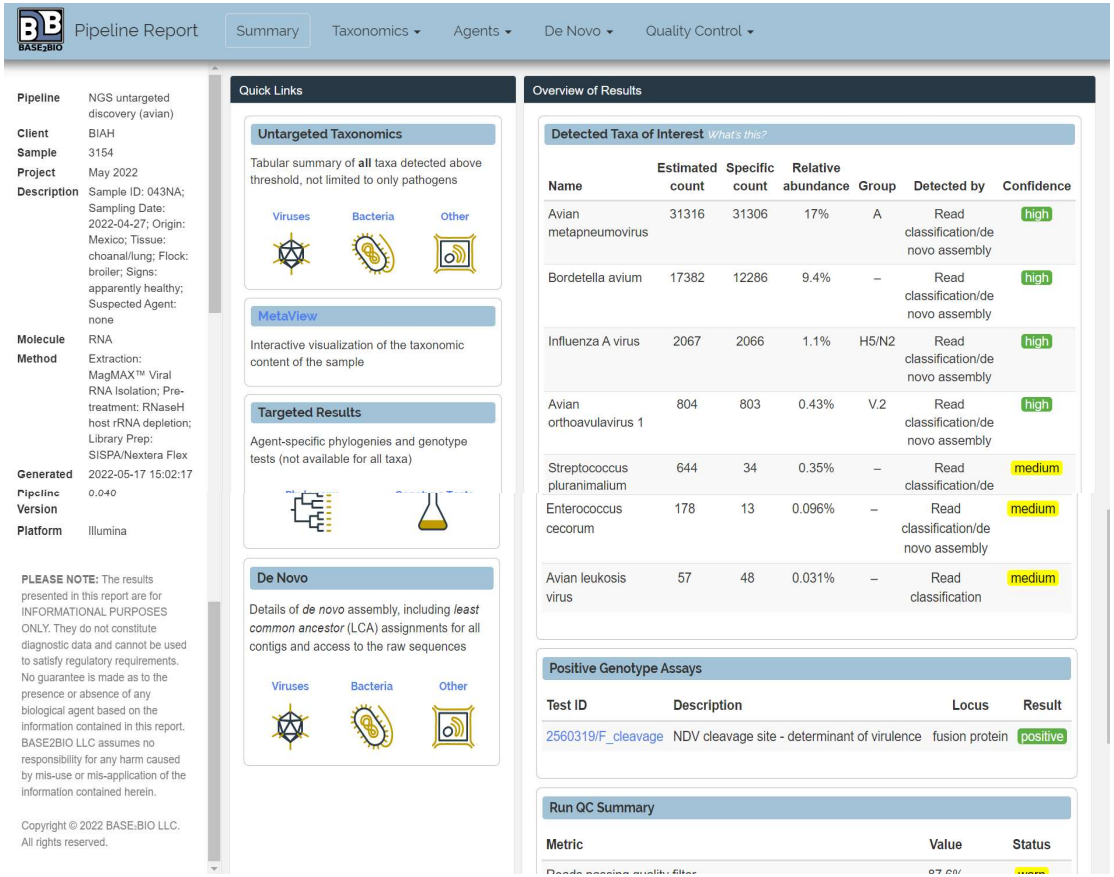


Spleen sampling

Metagenomics

Broiler flock with respiratory clinical signs, increased mortality, some with nervous signs. The Vet in charge is convinced this is a VVND case and is assuming the mortality Is associated with his vaccination program vs ND. He is thinking on changing the vaccination program vs ND.

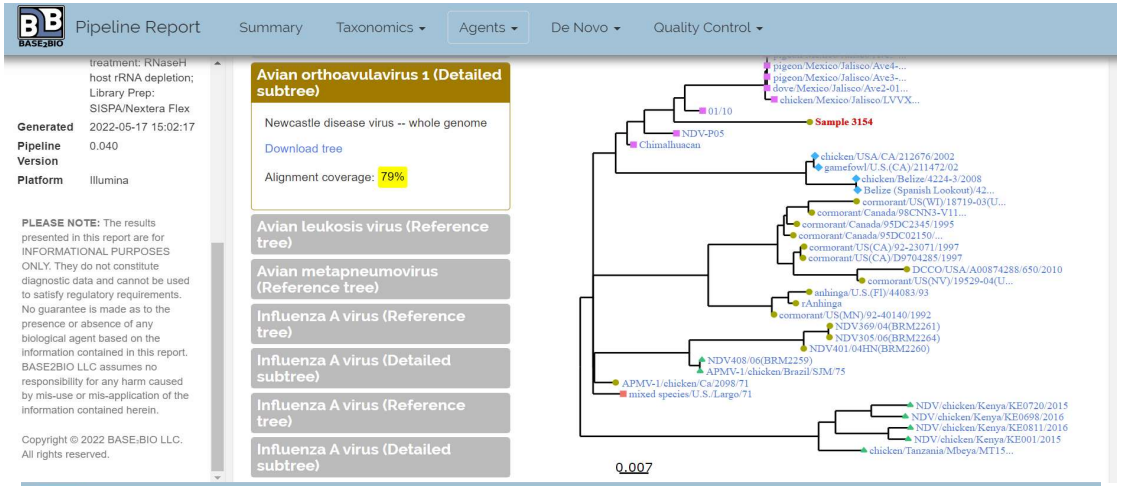
To unveil hidden pathogens:.

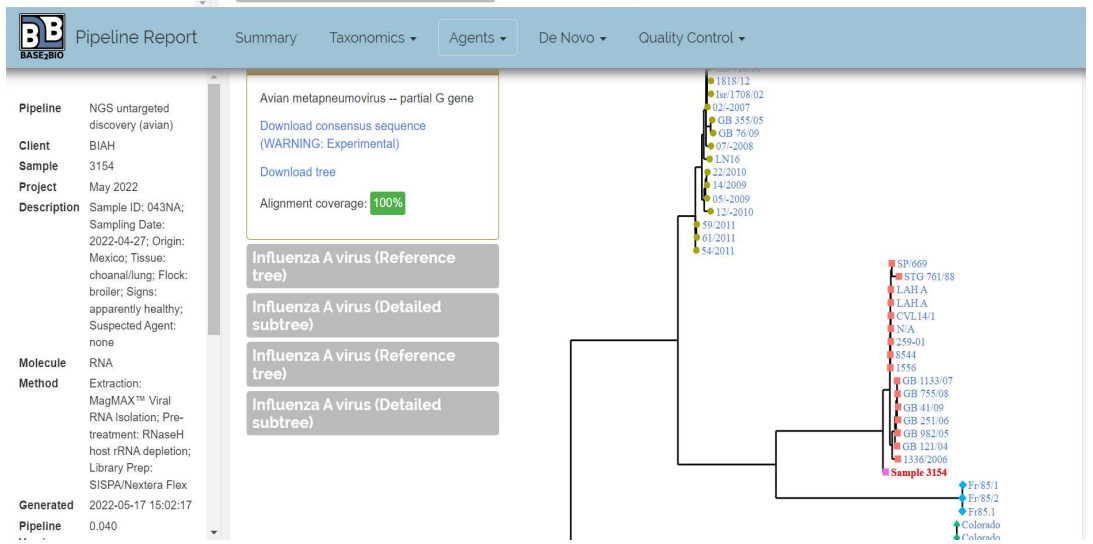


Metagenomics

To unveil hidden pathogens:

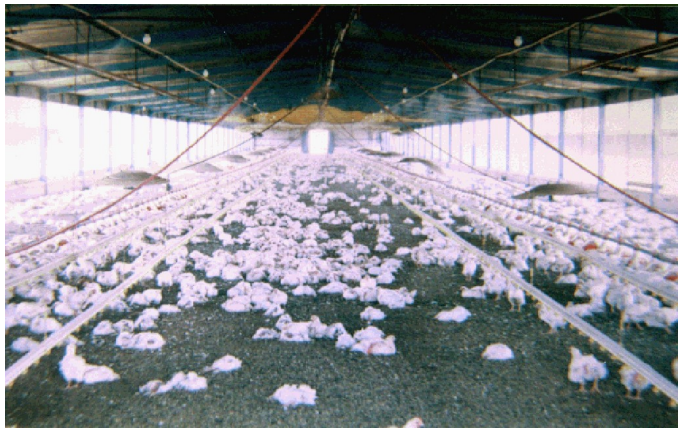
Some nervous signs
Torticolis caused by SHS



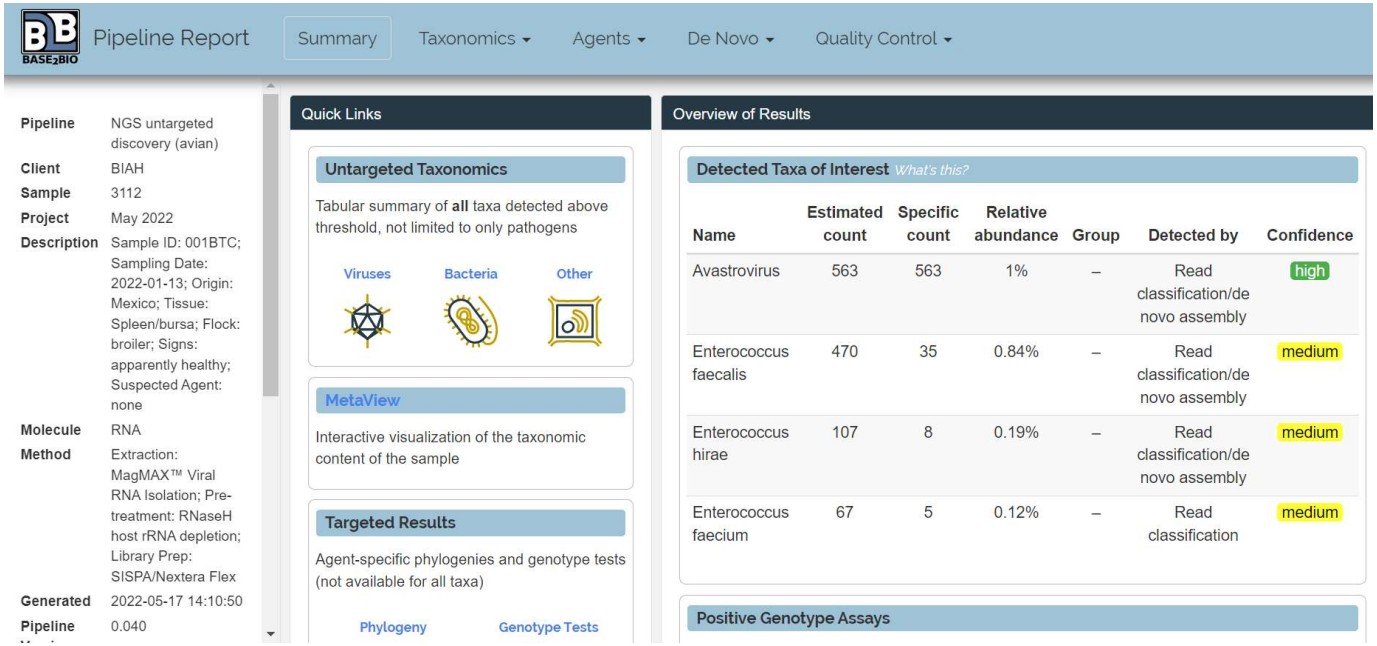


Metagenomics

To unveil hidden pathogens:



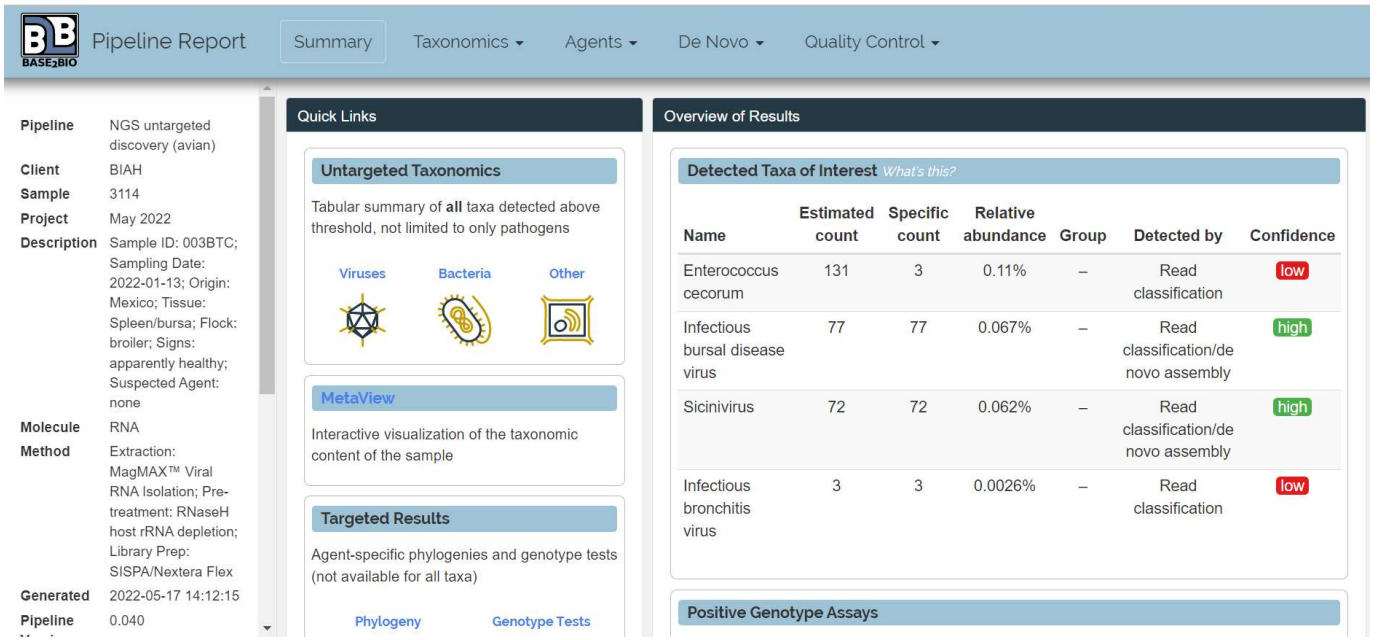
Broiler flock which experienced a curve of mortality starting the second week of age. Lesions in liver, kidneys and diarrhea are the main findings. Vet is suspecting on Malabsorption syndrome caused by Reovirus and is asking to include the Reo fraction vs malabsorption in the breeders.



Metagenomics

To measure vaccination program efficacy:

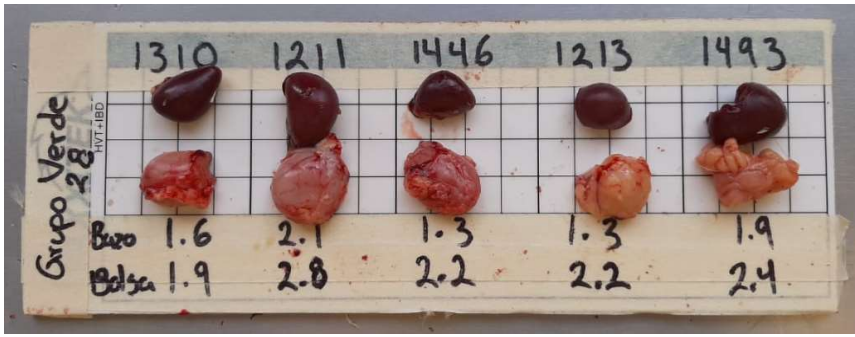
Broiler flock with normal mortality and no clinical signs. The Vet is asked to take samples for PCR testing before slaughter age. The report is positive to IBD Virus. The Vet is recommended to modify the vaccination program.

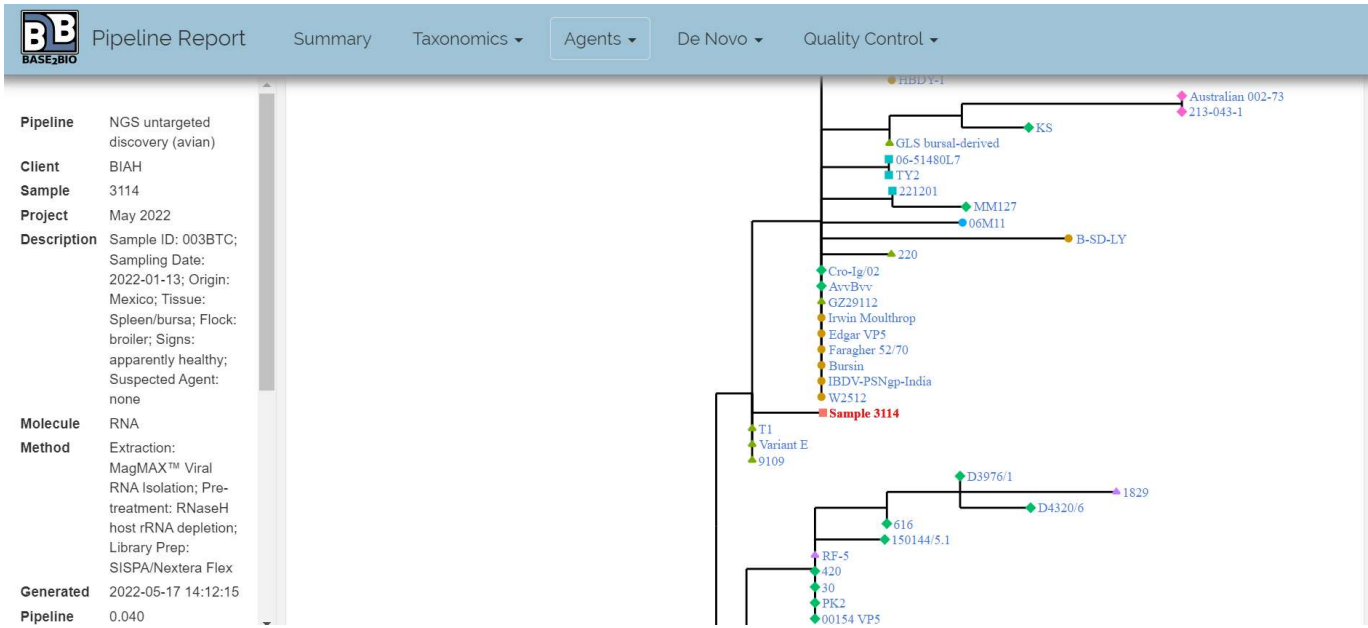


Metagenomics

To measure vaccination program efficacy:

IBD variant E





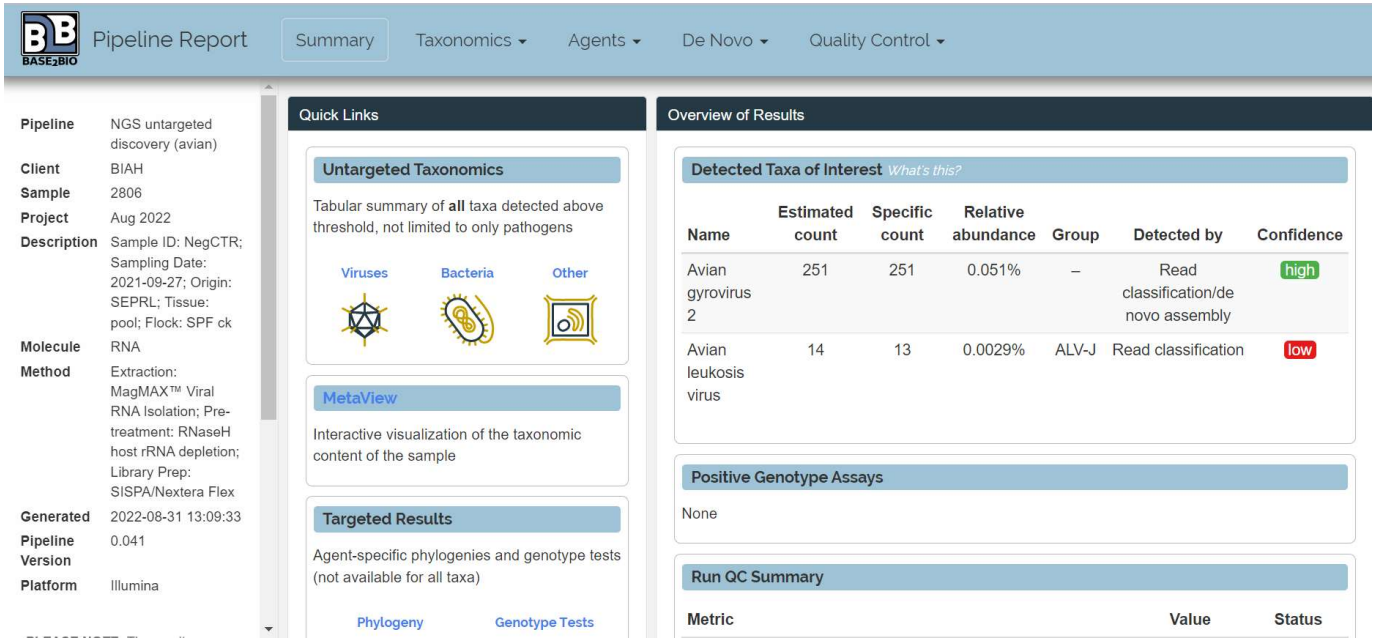
Metagenomics

To detect infectious causes of immune suppression:

Causes of bursal athrophy

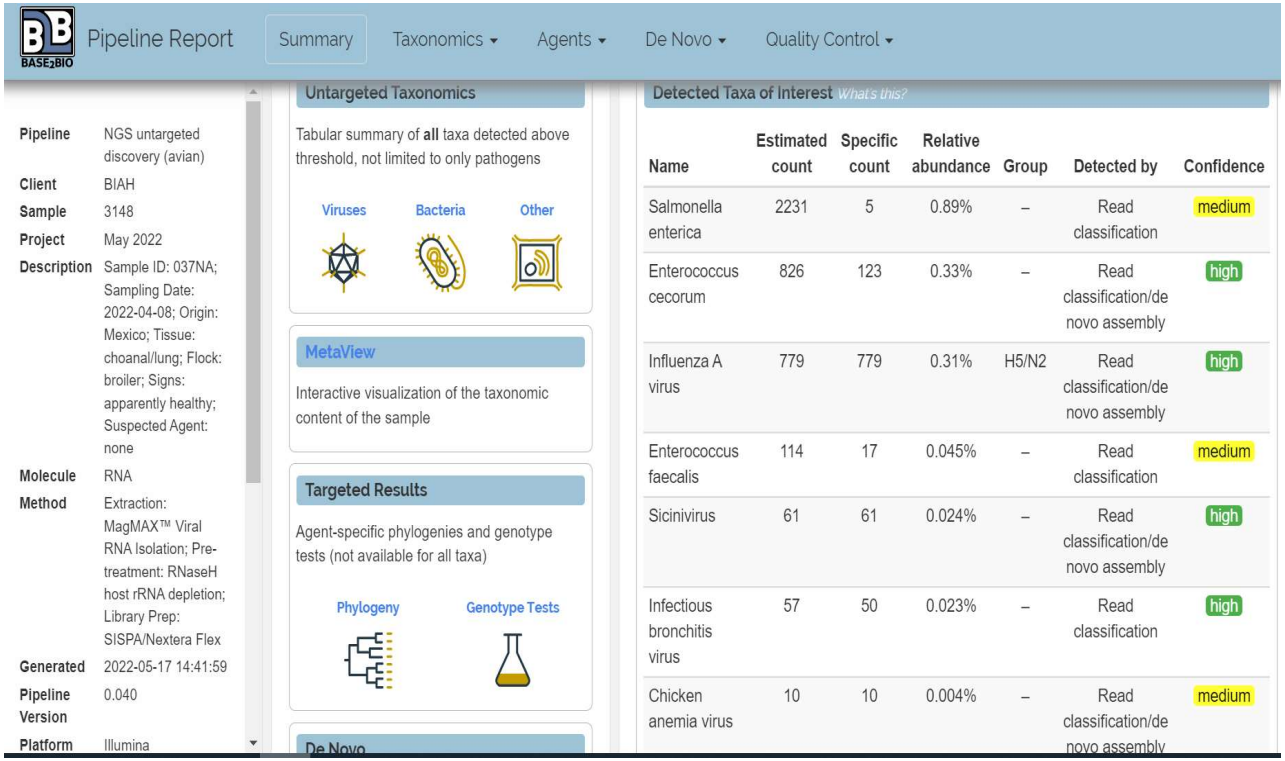
Not all is about IBD

CAV
Reo
Adeno
Marek
AI
VVND
Mycotoxins
Stress



Metagenomics

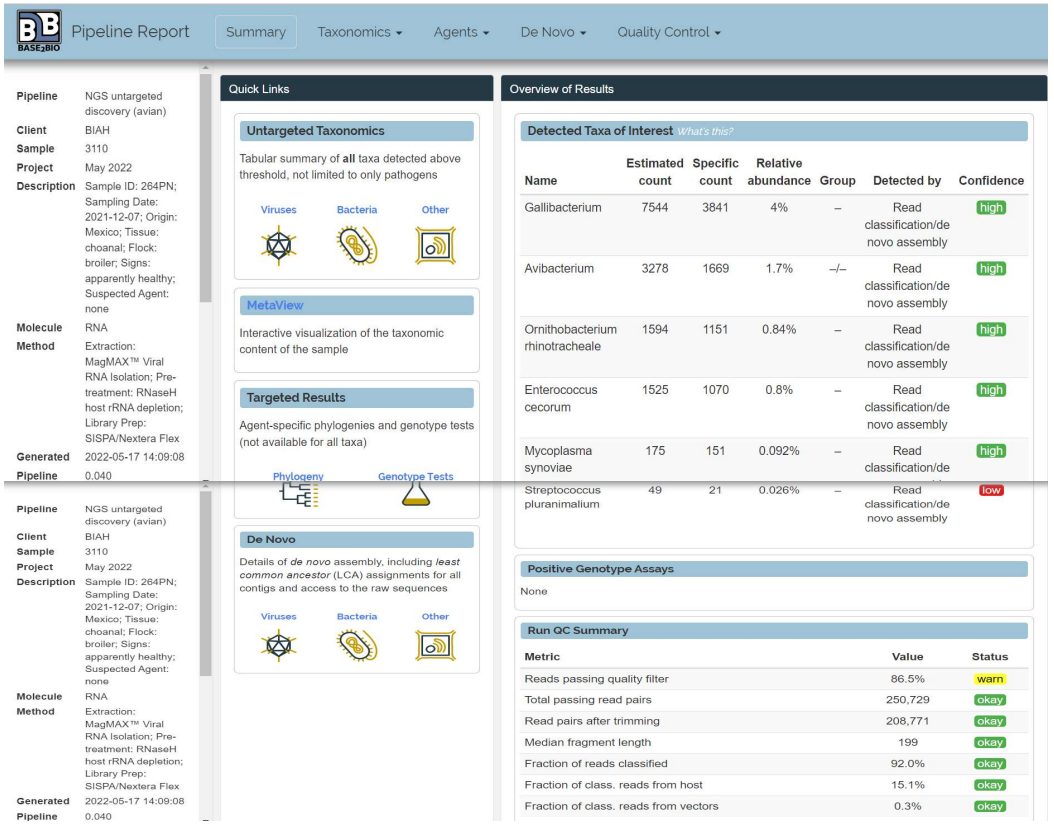
To investigate mixed infections:



Avian Influenza +
Infectious Bronchitis
+
CAV
+
Bacteria

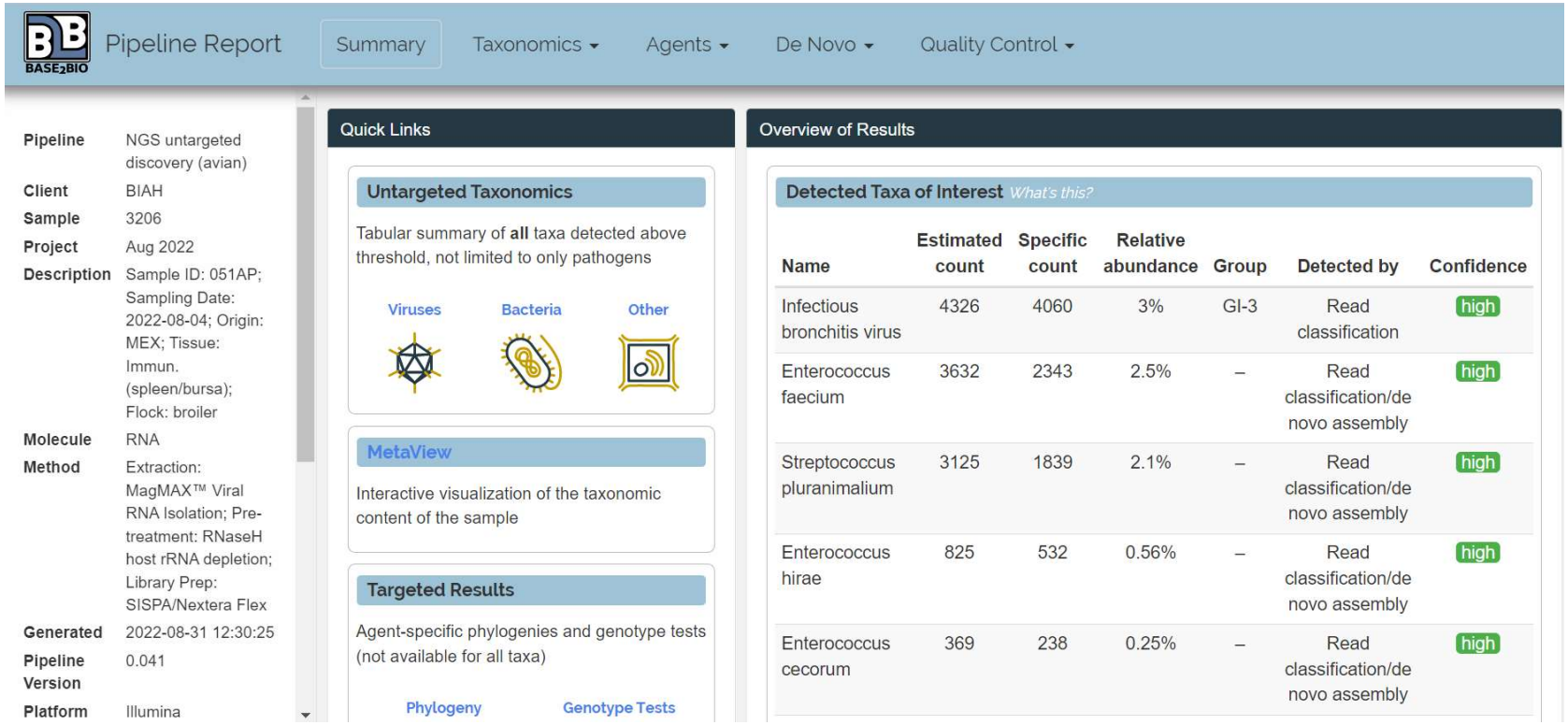
Metagenomics

To investigate mixed infections:



Metagenomics

To understand the epidemiology:



Name	Estimated count	Specific count	Relative abundance	Group	Detected by	Confidence
Infectious bronchitis virus	4326	4060	3%	GI-3	Read classification	high
Enterococcus faecium	3632	2343	2.5%	–	Read classification/de novo assembly	high
Streptococcus pluranimalium	3125	1839	2.1%	–	Read classification/de novo assembly	high
Enterococcus hirae	825	532	0.56%	–	Read classification/de novo assembly	high
Enterococcus cecorum	369	238	0.25%	–	Read classification/de novo assembly	high

Metagenomics

To understand the epidemiology:

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Resource Announcements

Check for updates

| Virology | Announcement

Coding-complete genome sequence of a GI-13 infectious bronchitis virus from commercial chicken in India

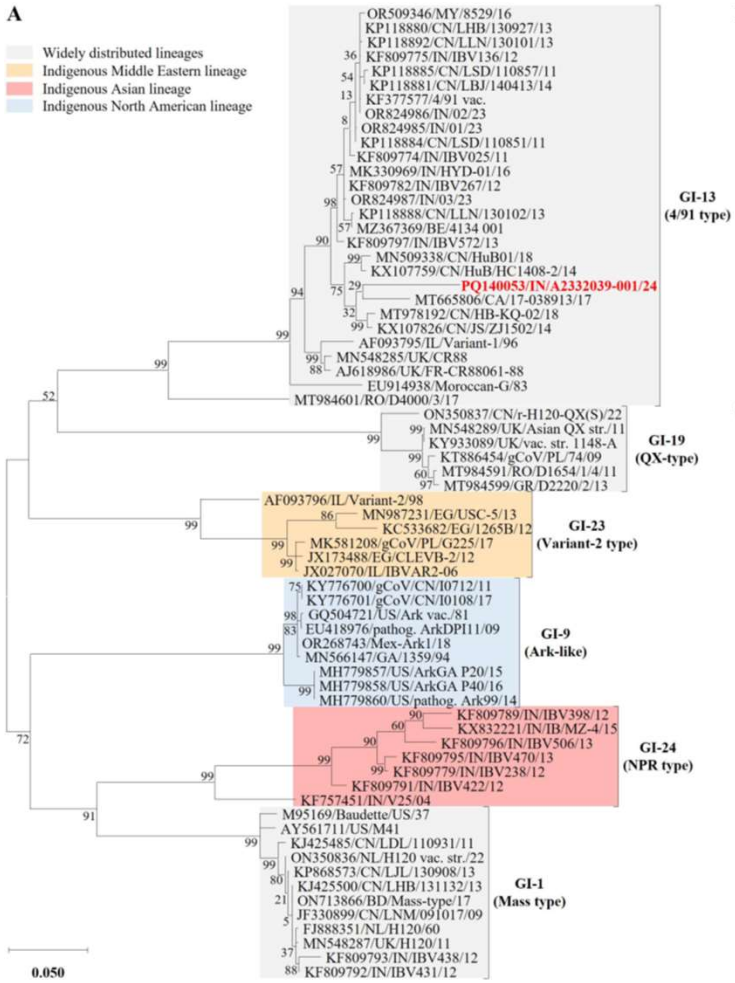
Henry M. Kariithi,[1] Jeremy D. Volkening,[2] Claudio L. Afonso,[2] Mohamed Helmy,[3] Pushparaj P. Chaudhari,[4] Eduardo L. Decanini[3]

AUTHOR AFFILIATIONS See affiliation list on p. 3.

ABSTRACT Infectious bronchitis virus (IBV) causes a highly contagious, acute upper respiratory disease in chickens characterized by nasal discharge, coughing, and rales. Here, the complete genome sequence of a recombinant GI-13 IBV strain ck/IN/A2332039-001/24 was sequenced from a choanal sample of a commercial broiler chicken in India using nontargeted next-generation sequencing.

Metagenomics

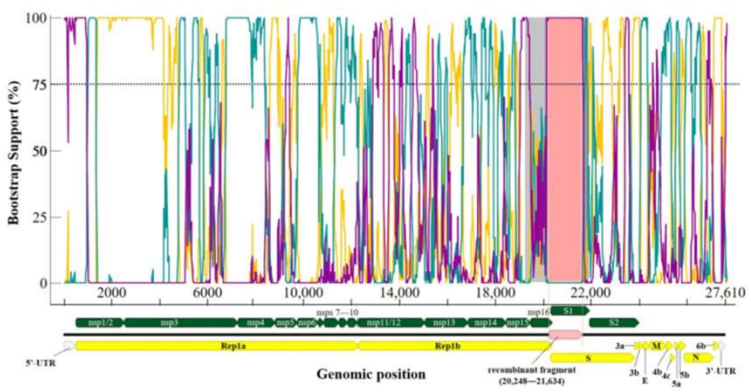
To understand the epidemiology:



B

Lineage	Strain	Complete genome	Gene 1 Rep1a	Gene 1 Rep1ab	Gene 2 S	Gene 2 S1	Gene 3 (3a-3b-E)	Gene 4 (M-4b-4c)	Gene 5 (5a-5b)	Gene 6 (N)
GI-13	MT984601/RO/D4000/3/17	**92.28**	93.42	93.8	84.66	82.87	88.63	**92.9**	90.52	91.95
	MT665806/CA/17-038913/17	89.87	89.12	90.56	89.33	92.39	86.1	89.86	91.87	93.17
	KP118892/CN/LLN/130101/13	90.98	90.87	91.52	89.73	93.57	86.41	88.52	92.1	90.98
	OR824986/IN/02/23	91.09	90.9	91.53	**89.81**	93.69	86.41	90.72	92.1	90.98
	OR824985/IN/01/23	91.09	90.89	91.53	89.79	93.57	86.41	90.81	92.1	90.89
	KF809775/IN/IBV136/12	-	-	-	-	93.63	-	-	-	-
	KF809797/IN/IBV572/13	-	-	-	-	93.57	-	-	-	-
	KF809774/IN/IBV025/11	-	-	-	-	93.54	-	-	-	-
	MN509338/CN/HuB01/18	-	-	-	-	**94.19**	-	-	-	-
GI-1	KF809793/IN/IBV438/12	-	-	-	-	77.08	-	-	-	-
	KF809792/IN/IBV431/12	-	-	-	-	78.42	-	-	-	-
	MG763935/IN/53/13	91.94	93.43	93.97	82.17	78.42	86.73	92.05	95.37	93.82
	KJ425485/CN/LDL/110931/11	92.08	93.42	93.96	82.75	78.42	89.89	90.72	**95.94**	93.5
	ON350836/NL/H120 vaccine/22	92.19	93.38	93.93	82.2	78.54	86.73	92.63	95.49	**94.39**
	MN548287/UK/H120/11	91.93	93.44	**93.98**	82.15	78.35	86.73	92.63	95.49	93.74
GI-9	GQ504720/US/Ark DPI/81	91.65	**94.08**	93.42	82.11	77.63	91	90.62	95.71	93.25
	MH779858/US/ArkGA P40/16	89.71	89.82	90.79	81.68	77.33	89.73	90.62	95.71	93.33
	MH779860/US/Ark99/14	89.72	89.83	90.79	81.71	77.33	89.73	90.72	95.71	93.33
	KY776700/gCoV/CN/I0712/11	91.78	94	93.52	82.39	77.76	**91.15**	90.62	95.49	93.25

C



Plot legend

- 95-99% breakpoint confidence intervals
- Track sequence with a recombinant origin
- Plot of major parent (GQ504720/Ark DPI/81 {GI-9}) & rec. str. PQ140053/IN/A2332039-001/2024 {GI-13}
- Plot of minor parent (KP118892/LLN/130101/13 {GI-13}) & rec. str. PQ140053/IN/A2332039-001/2024 {GI-13}
- Plot of major parent (GQ504720/Ark DPI/81 {GI-9}) & minor parent (KP118892/CN/LLN/130101/13 {GI-13})

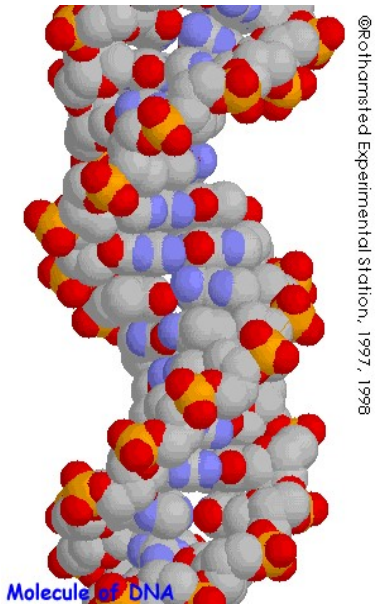
Recombination event confirmation

algorithm	# seqs detected in	*p*-value
RDP	1	1.068×10^{-74}
GENECOV	1	2.460×10^{-93}
BootScan	1	1.923×10^{-18}
MaxChi	1	9.226×10^{-26}
Chimaera	1	7.433×10^{-21}
SiScan	1	8.741×10^{-37}
3Seq	1	3.799×10^{-13}

Targeted PCR & Sequencing

PCR (Polymerase Chain Reaction)

Gene are on nucleic acid chains (DNA, RNA)



DNA

DNA viruses (Herpesvirus, Adenovirus, ...)

Bacteria (plasmids, nucleus)

Eucaryotes (nucleus)

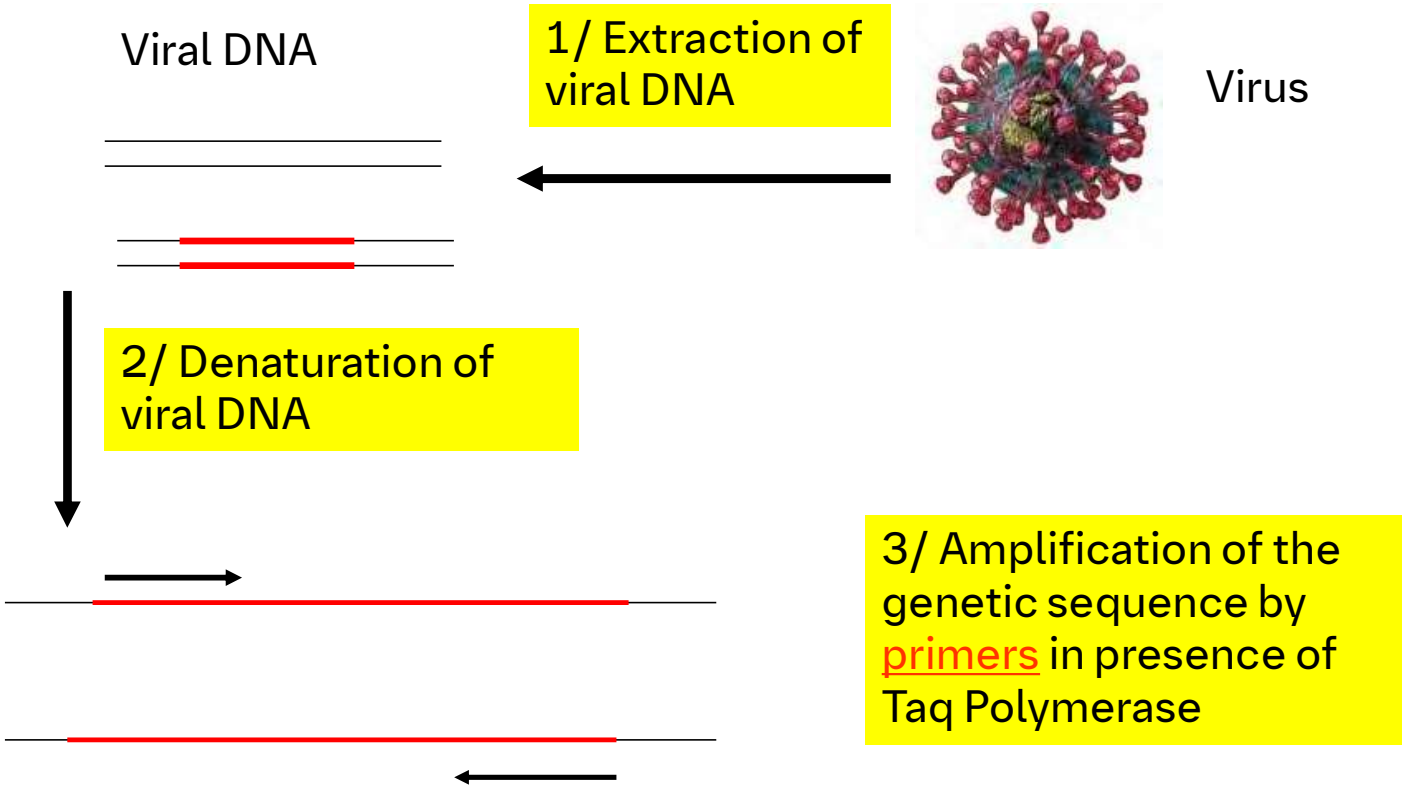
RNA

RNA viruses (Ortho- & Para-myxovirus, Metapneumovirus, ...)

Bacteria

Eucaryotes: RNAm, RNAt, RNAr

PCR (Polymerase Chain Reaction)



PCR (Polymerase Chain Reaction)

The «billion» amplified genes are then detectable using two types of PCR

Conventional PCR

Amplification **then** reveal by **Electrophoresis**

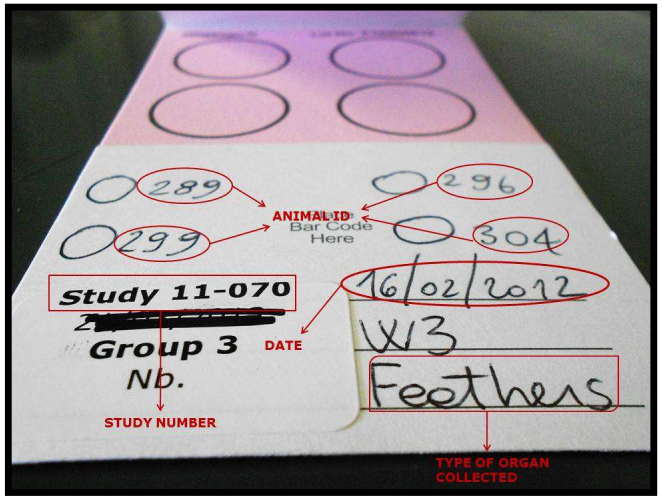
Real – Time PCR

Amplification **and** reveal **simultaneously** by

Fluorescence reads

Manual for sample collection

FTA cards



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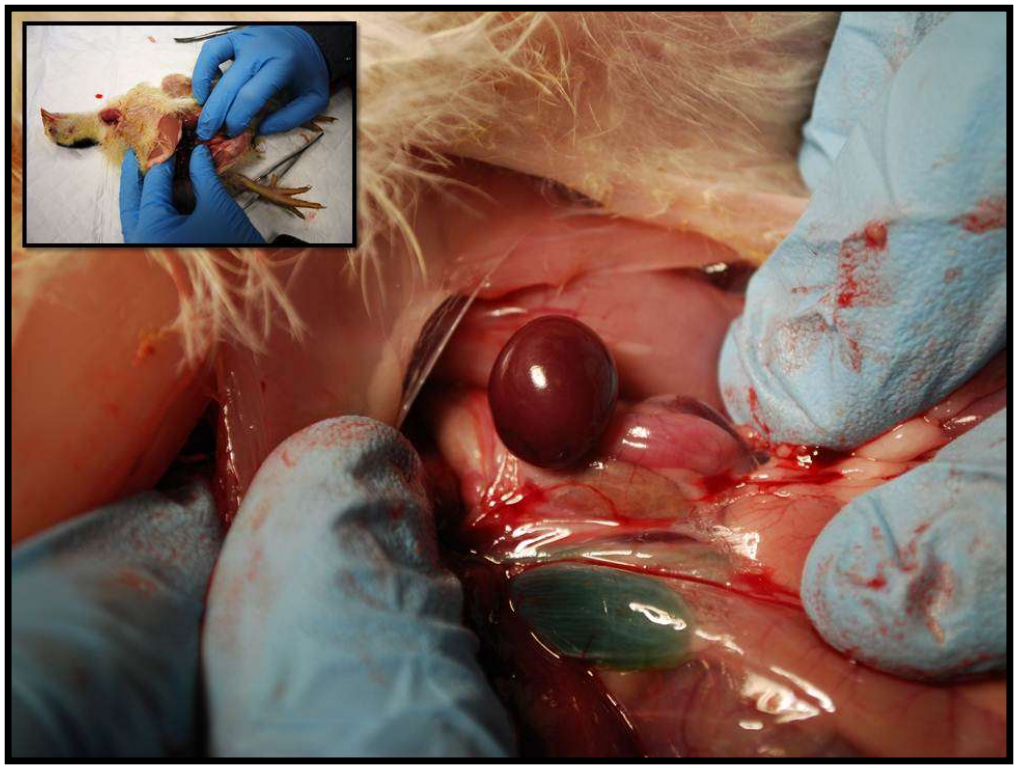
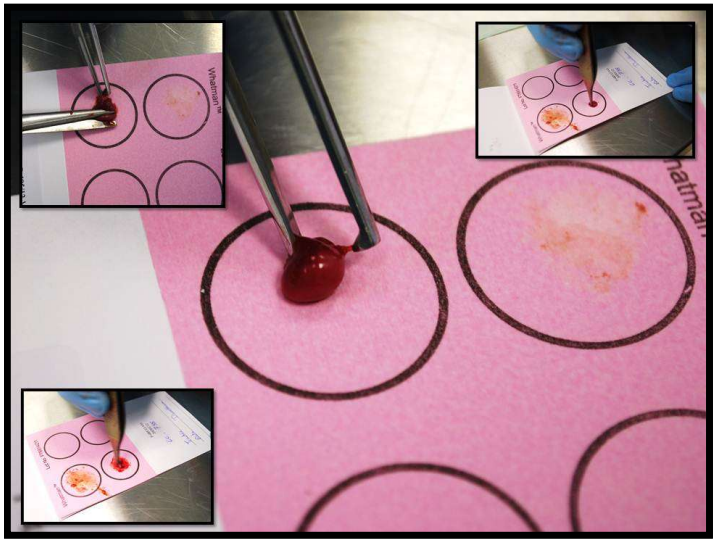


FTA cards

Manual for sample collection



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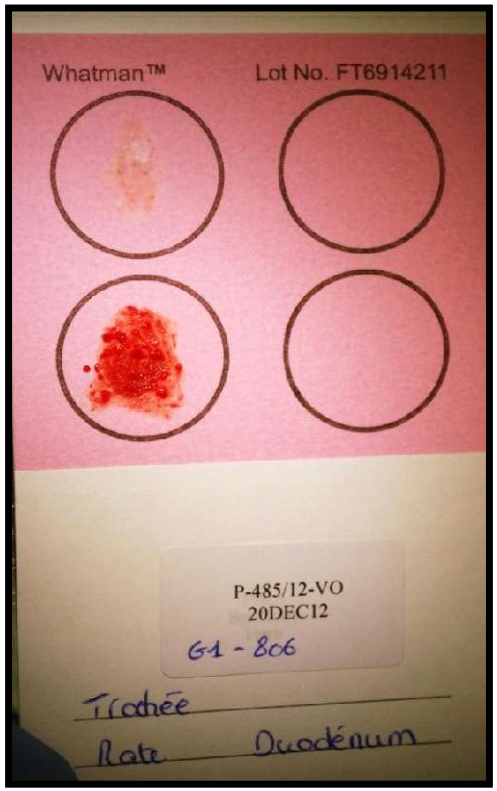

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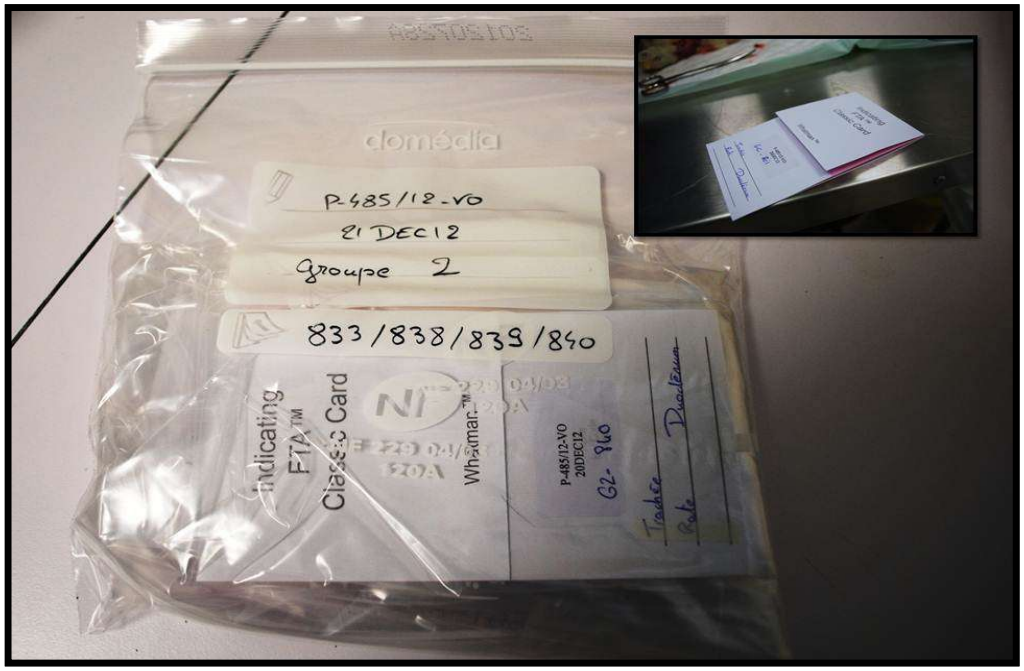


FTA cards

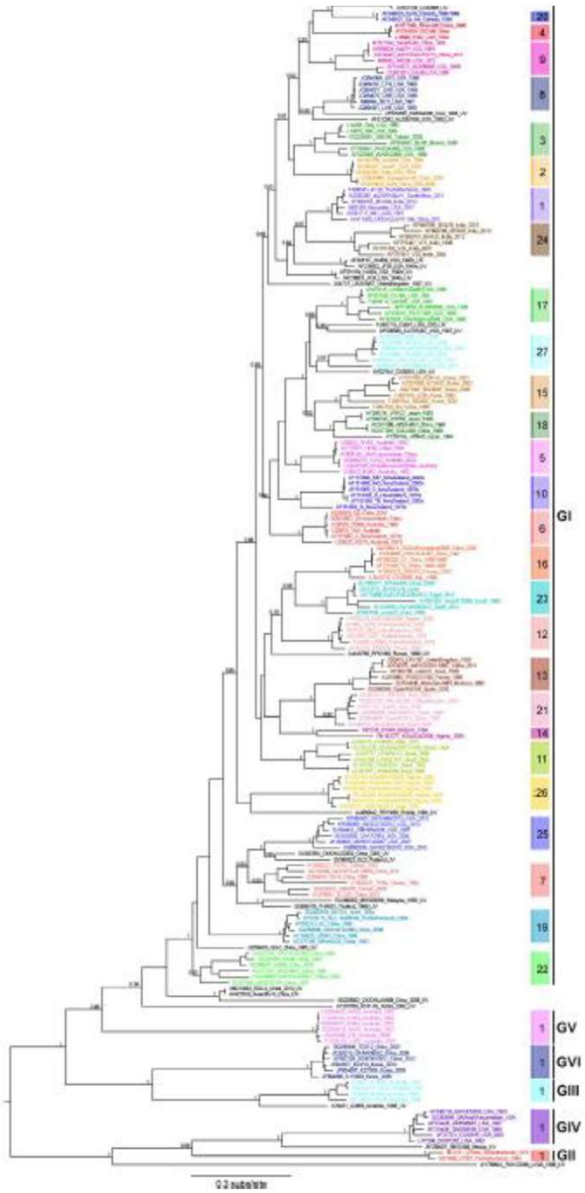
Infectious Bronchitis

Genotypes GI –GVI (nowadays GIX or GX)

<u>Differentiation into lineages</u>:

GI: 27 lineages (e.g.GI-1: Mass; GI-13: 793b; …)

~30 lineages to date



[Valastro et Al, 2016]

Infectious Bronchitis

Screening PCR (for Avian Coronavirus)

Typing PCRs

Sequencing (partial S1 gene) including most variable regions

Next generation sequencing (IBV directed - whole S1 gene)

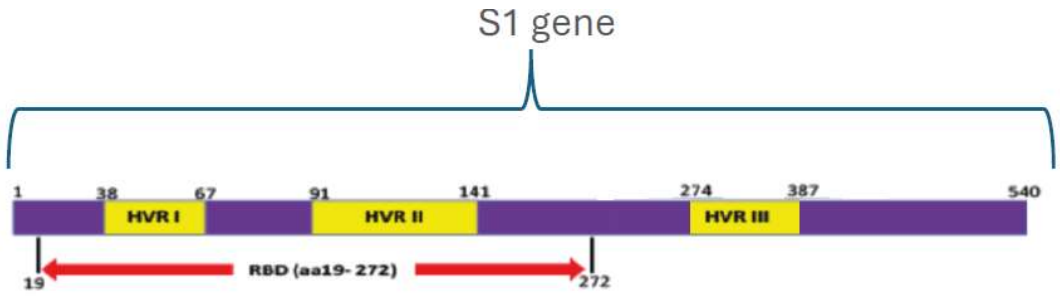


Table 2. Sequence alignment of hypervariable regions (HVRs) amino acid sequences for four isolates in this study compare to their parental origin 4/91 genotype (793/B serotype).

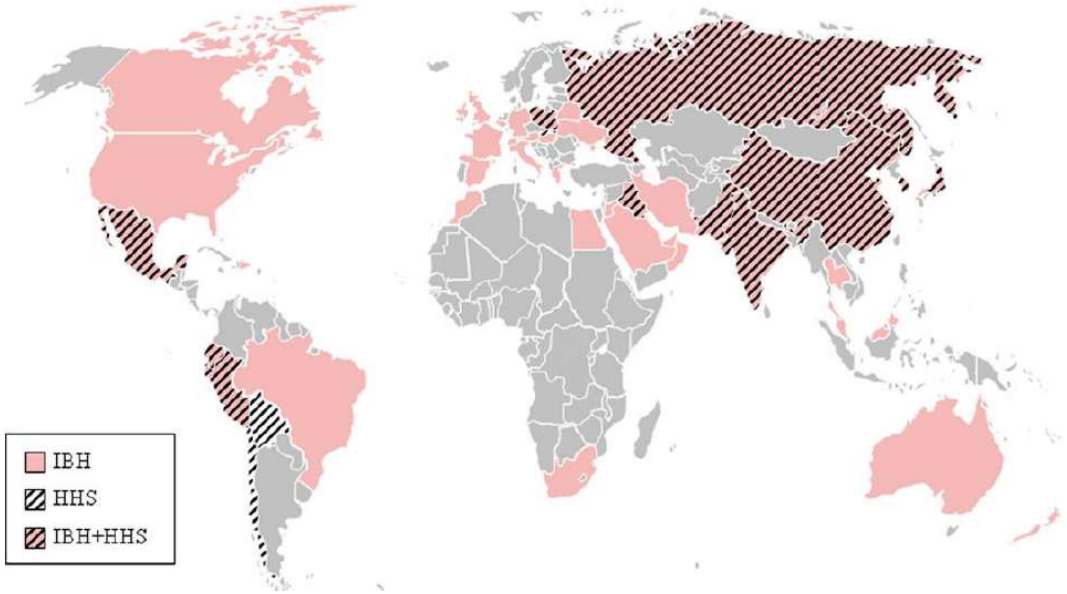
Strain	HVR1 (60–88)	HVR2 (115–140)	HVR3 (275–292)
4/91	VSVSDCTAGTFYESYNISAASVAMT VPPA	FKSQQGSCPLTGMIPQNHIRISA MRS	TFTNVSNASPNSGGVD TF
CR88	G. A . . . . . H. . . . S . . . . . HN	. . N . L . . . . D	S . . . . .
SA/1/2019	. . AG . . . . . S . . . . K	. . . . . Y	. . . . .
SA/2/2019	. . AG . . . . . F . . . . . A . . .	. . . . . L	. . . . .
SA/3/2019	. . AGE . . . . . F . . S . . . . . A . . .	. . . . . Y	. . . . .
SA/4/2019	. . AGQ . . . . S.	. . . . .	. . . . .

A dot indicates an identical amino acid.

[Rohaim et Al, 2020]

Fowl Adenovirus

Disease	Mostly types
Inclusion Body Hepatitis	FAdV-2, -8a, -8b (species E) FAdV-11 (species D)
Gizzard Erosions	FAdV-1 (species A)
(Hepatitis-) Hydropericardium Syndrome	FAdV-4 (species C)



[Schachner et Al, 2018]

Fowl Adenovirus

Screening PCR

Determination of serotype by sequencing of Hexon gene

Infectious Bursal Disease

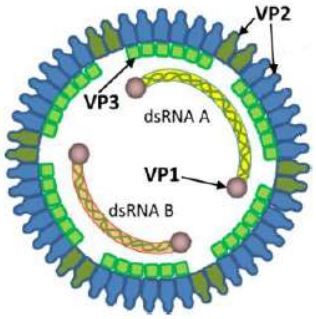
VP2 (segment A)	
A1	Classical virulent/attenuated
A2	US antigenic
A3	Very virulent
A4	dIBDV
A5	Mexican
A6	Italian
A7	Early Australian
A8	Australian variant
A9	Portugal
A0	Serotype 2

+

VP1 (segment B)	
B1	Classical like (incl. serotype 2)
B2	Very virulent-like
B3	Early Australian-like
B4	Polish/Tanzanian
B5	Nigerian

=

Genotypes
(e.g. A1B1, A3B2, ...)



[Islam et Al, 2021]

Infectious Bursal Disease

Screening PCR

Typing PCRs (A1, A3, A7)

Partial VP1 and VP2 sequencing

IBDV directed NGS for whole VP1&VP2 sequencing

Consider recombinant vaccine strains (HVT vector vaccines or DIVA PCRs)

Marek's Disease

Meleagrid aplhaherpesvirus 1 (= Herpesvirus of turkey (HVT) non-pathogenic)

Gallid alphaherpesvirus 3 (non-pathogenic) – SB1

Gallid alphaherpesvirus 2 (= Marek's Disease Virus)

Meq protein has a key role in oncogenicity, is involved in neurovirulence, is relevant for virulence among others: proline rich repeats (PRRs; 4-proline stretches)

The more proline repeats the lower the virulence

Marek's Disease

Screening PCR

DIVA PCR (CVI988 – RN1250 possible with CVI988 detection system)

Sequencing of meq gene for number of proline repeats

HVT recombinant vaccine strains: HVT-based PCRs (HVT screening or DIVA PCRs based on inserts or overlapping genetic sequence 'unique fingerprint of the construct')

Possible qPCR with proper quantification at day 21 – Gimeno's Technique

[Gimeno I et Al, 2008]

Newcastle Disease

Patho-genotyping?

Jaganathan *et al. BMC Veterinary Research* (2015) 11:219
DOI 10.1186/s12917-015-0537-z

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Observation of risk factors, clinical manifestations and genetic characterization of recent Newcastle Disease Virus outbreak in West Malaysia

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Background: Newcastle disease virus remains a constant threat in commercial poultry farms despite intensive vaccination programs. Outbreaks attributed to ND can escalate and spread across farms and states contributing to major economic loss in poultry farms.

Results: Phylogenetic analysis in our study showed that eleven of the samples belonged to genotype VIId. All farms were concurrently positive with two immunosuppressive viruses; Infectious Bursal Disease Virus (IBDV) and Marek's Disease Virus (MDV). Amino acid sequence analysis confirmed that eleven of the samples had sequence motifs for velogenic/mesogenic strains; three were lentogenic.

Conclusion: In conclusion, no new NDV genotype was isolated from the 2011 NDV outbreak. This study suggests that the presence of other immunosuppressive agents such as IBD and MDV could have contributed to the dysfunction of the immune system of the chickens, causing severe NDV outbreaks in 2011. Risk factors related to biosecurity and farm practices appear to have a significant role in the severity of the disease observed in affected farms.

Newcastle Disease

Patho-genotyping?

Fig. 1 Clinical signs observed from the outbreak. **a** A typical torticollis is shown. These symptoms normally occur 7 to 10 days after a complaint of high mortality is reported. **b** In severely affected birds, mild swollen head and dyspnea with profuse secretions in the trachea were found. **c** & **d** Hemorrhagic & necrosis of intestines especially the caecal tonsils & peyer's patches were found. **e** Upon PM, the trachea was severely congested and late in the disease stages pericarditis, perihepatitis and caseous air sacculitis were observed. **f** Proventicular hemorrhages were consistent. **g**, **h** & **i** Bursa atrophy was also commonly found in the outbreak. The cut surfaces of the bursa were hemorrhagic – quite atypical from ND infection which prompted us to look for other infectious agents. Not shown above was atrophic thymus

Table 7 The F cleavage site and it's pathotypes from the Malaysian isolates

Isolate	Genbank® accession no	F cleavage site	Genotype	Pathotype	Source
F1	JN613112	RRRKRF	VIId	Velogenic/Mesogenic	This study
F2	JN613113	RRRKRF	VIId	Velogenic/Mesogenic	This study
F3	JN613114	RRRKRF	VIId	Velogenic/Mesogenic	This study
F4	JN613115	RRRKRF	VIId	Velogenic/Mesogenic	This study
F5	JN613116	GRQGRL	II	Lentogenic	This study
F6	JN613117	GRQGRL	II	Lentogenic	This study
F7	JN613118	GKQGRL	I	Lentogenic	This study
F8	JN613119	RRRKRF	VIId	Velogenic/Mesogenic	This study
F9	JN613120	RRRKRF	VIId	Velogenic/Mesogenic	This study
F10	JN613121	RRRKRF	VIId	Velogenic/Mesogenic	This study
F11	JN613122	RRRKRF	VIId	Velogenic/Mesogenic	This study
F12	JN613123	RRRKRF	VIId	Velogenic/Mesogenic	This study
F13	JN613124	RRRKRF	VIId	Velogenic/Mesogenic	This study
F14	JN613125	RRRKRF	VIId	Velogenic/Mesogenic	This study

Newcastle Disease

Patho-genotyping?

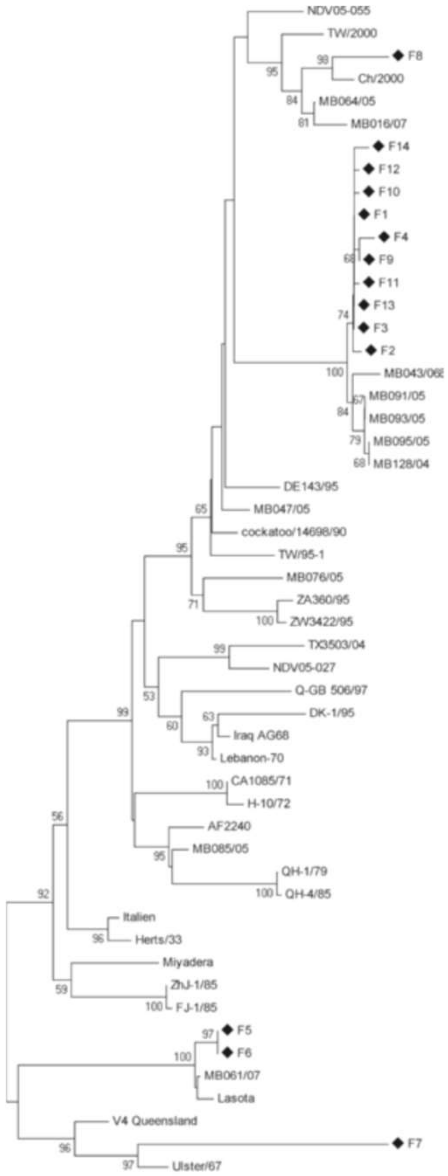
Table 5 Zootechnical results, clinical and necropsy findings

Risk factor	Affected farms	Negative controls
Age of first occurrence of disease	15.9[a]	28.0[a]
Age of clinical and necropsy examination	24.8[a]	32.5[a]
Mortality at grow-out, %	32.3 %[a]	4.5 %[b]
Presence of respiratory disease	100 %[a]	100 %[a]
Presence of enteric disease	100 %[a]	0 %[b]
Torticolis and neurological signs	91.7 %[a]	0 %[b]
Haemorrhages in more than 1 visceral organ	83.3 %[a]	0 %[b]
Thymus atrophy	100 %[a]	0 %[b]
Bursal atrophy	75 %[a]	50 %[b]
Ascites	8.3 %[a]	50 %[a]
Air-sacculitis, perihepatitis and peritonitis	16.7 %[a]	50 %[b]
Clouded air sacs	50 %[c]	100 %[d]

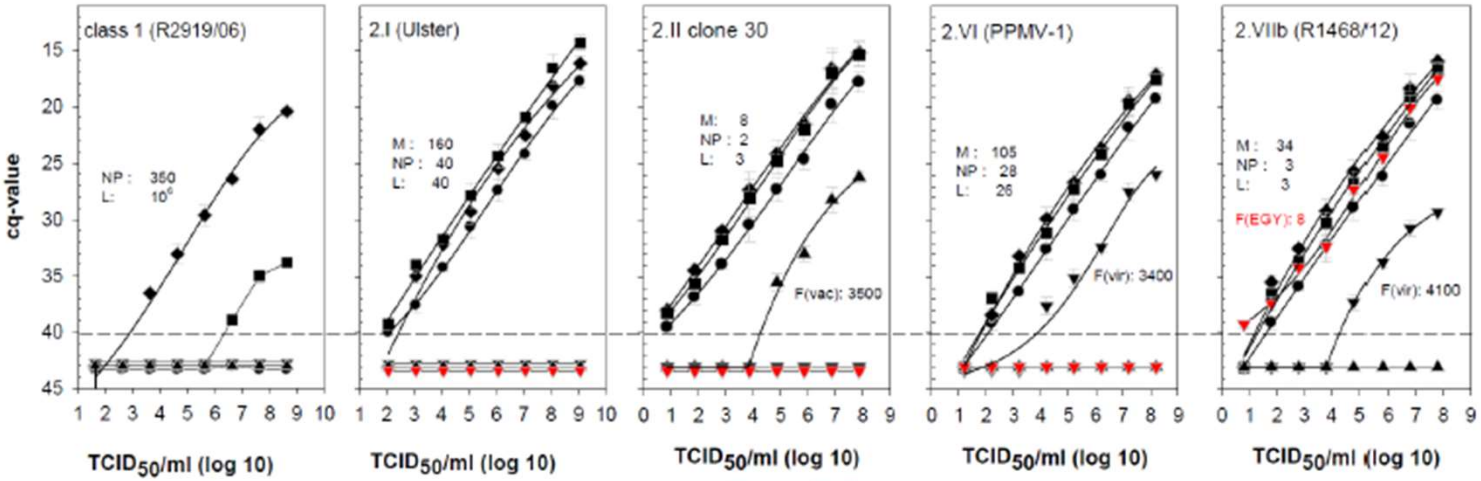
Note: [ab]Values in different columns bearing different superscripts are significantly different ($P < 0.05$), [cd]Values in different columns bearing different superscripts are significantly different ($P < 0.01$)

Table 6 Detection of infectious agents concurrently with NDV positive samples

Presence of concurrent infectious agent in disease farms	Frequency of detection
IBD	83.0 %
MD	83.0 %
IBD+MD	75.0 %
MD Serotype 1	58.0 %
MD Serotype 2	75.0 %
MD Serotype 3	67.0 %



Newcastle Disease



- Limited sensitivity of pathotype specific RT-qPCR
- Adapted reagents close gap in sensitivity between generic and pathotype specific F-RT-qPCR

Special case of WOAH list A Avian Influenza – HPAI H5Nx

Example of surveillance program (EU – France – Vaccinated Duck Population)

The objective of surveillance is to **ensure that there's no viral circulation amongst the vaccinated flocks**, This is to be made in all epidemiological units (one epidemiological unit is one farm)

1/ Event based surveillance is to ensure earliest detection of viral circulation, **any abnormal behavior or clinical signs** are to be declared without delay to veterinarian.

2/Enhanced passive surveillance: The sampling protocol involves the taking by the farmer or a technical worker of **tracheal or oropharyngeal swabs** from recently deceased birds up to a maximum of **five dead birds per week** to perform RT-PCR.

3/Active surveillance: objective is to detect low level circulation, to be **made by an official vet**, is based on a **clinical examination**, with the **evaluation of the zoo-technical criteria**, completed with a **monthly virological surveillance**. samples are taken from **60 vaccinated birds for PCR and 20 samples for NP ELISA serology**.

HPAI post-vaccination enhanced surveillance and the serological survey		
Parameters	Enhanced passive surveillance	Active surveillance
Where?	The epidemiological unit	
Who?	Farmer or technical worker	Official veterinary
Frequency?	Weekly	Every 30 days: virological testing On batch completion: serological testing
How?	Swabs (tracheal/oropharyngeal) from 5 dead birds	Every 30 days : Swabs (tracheal/oropharyngeal) from 60 birds; At batch completion: blood samples from 20 birds
Testing?	Virological using M gene RT-PCR (If the result is positive, screening for H5/H7)	Virological using M gene RT-PCR (If the result is positive, screening for H5/H7) and NP ELISA serology
Type of laboratory?	A recognised laboratory	An approved laboratory

Thank you for your Attention!