

1 **Screening Avian Pathogens in Eggs from Commercial Hatcheries in**
2 **Nepal- an Effective Poultry Disease Surveillance Tool**

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16

17 **Abstract**

18 **Background**

19 Commercial hatcheries play an important role in the overall poultry value chain- providing small to large
20 poultry farmers with day old chicks. Any outbreak in such hatcheries can spread diseases to other farms.
21 Regular screening of major avian pathogens, along with strict bio-security measures, can prevent spread of
22 diseases in hatcheries. Newcastle Disease Virus (NDV), Infectious Bronchitis Virus (IBV), *Mycoplasma*
23 *gallisepticum* (MG), *Mycoplasma synoviae* (MS), Infectious Bursal Disease Virus (IBDV) and Influenza A
24 Virus (IAV) are among the most prevalent poultry diseases which can be detected in egg albumin.

25 **Method**

26 We retrospectively (August 2020- August 2021, except October 2020) analyzed diagnostic results for six
27 selected avian pathogens (NDV, IBV, MS, MG, IBDV and IAV) on eggs (n=4343) received from eleven
28 major commercial poultry hatcheries located in the five adjoining districts of Kathmandu, Nepal. Albumin
29 from 10% randomly selected eggs from each hatchery were tested for the six avian pathogens using
30 multiplex PCR.

31 **Result**

32 Majority (7/11, 64%) of the poultry hatcheries had at least one of the six pathogens present. We detected at
33 least one avian pathogen in nine out of eleven months (82%) of screening. Except for IBDV, we found one
34 or more of the other major avian pathogens- Influenza A (IAV) (n=4 times) and *Mycoplasma gallisepticum*
35 (MG) (n=4 times) were detected the most, followed by Newcastle Virus (NDV) (n=3 times). Infectious
36 bronchitis virus (IBV) were detected twice, and *Mycoplasma synoviae* (MS) was detected once.

37 **Conclusion**

38 In a resource strapped country like Nepal, poultry disease outbreak investigation in particular and
39 surveillance in general are challenging. Meanwhile, poultry production is highly impacted by disease
40 outbreaks often triggered by poor bio-security and lack of pathogen screening practices. Our molecular
41 screening tests have picked up major poultry pathogens present throughout the year in eggs collected from
42 hatcheries. Influenza A was detected at 4 different incidences throughout the year, which is of concern to
43 both human and animal health. Quick systematic screening of eggs at key distribution points (hatcheries)
44 for major avian pathogens is an effective surveillance tool for early disease detection and containment of
45 outbreaks.

46 **Keywords:** eggs, avian pathogens, disease screening, surveillance, poultry health

47 **Introduction**

48 Globally, the poultry sector is a sizeable industry with a current market value of \$ 310.7 billion and is
49 expected to grow at a compound annual growth rate (CAGR) of 3.8%¹. Poultry is a rapidly growing
50 agricultural sub-sector in developing countries², however, product quality, safety, and avian diseases
51 continue to be a major challenge to this industry³.

52 Hatcheries occupy a focal position in the poultry production chain, connecting with multiple flocks⁴,
53 thereby acting as a reservoir, linkage and source of pathogenic microorganisms⁵. Nepal's \$240-million
54 poultry industry⁶ is buttressed by 21,956 poultry farms present in sixty four out of seventy five districts,
55 where 325 total commercial hatcheries represent this burgeoning industry⁷.

56 Animal trade related movement of poultry and poultry products from production sites, such as hatcheries,
57 can influence disease transmission dynamics into uncontaminated flocks⁸. For example, transmission of a
58 recent subtype of Avian Influenza virus in Bangladesh was associated with poultry movement⁹.

59 Several pathogens (both mono and multi-causal) have been implicated as probable causes of avian diseases.
60 Poultry can be infected or colonized with other potential organism via eggs³. Contaminated eggs can be a
61 source of infection and a vehicle for transmission of pathogens^{10, 11}. Contamination can occur horizontally
62 through egg shells¹² or vertically before oviposition stemming from infection of reproductive organs¹³. In
63 vertical/ trans-ovarian route, the disease is ascendingly transmitted from laying hen to its progenies- where
64 the yolk, albumen and membranes are contaminated via the reproductive organs¹⁴ before the eggs are
65 covered by shell in the uterus. Handling fecal material, dust, and dirt can contaminate eggs in hatcheries
66 through horizontal route. Extrinsic factors such as temperature, moisture, shell characteristics and
67 membrane properties are attributable to pathogen transmission¹⁵.

68 Eggs form as great a proportion of the animal protein diet for Low and Middle Income Countries (LMICs),
69 overlooking a projected 76.6%¹⁶ growth in egg production. Egg production is imperative to the growing
70 population for providing an inexpensive source of protein¹⁷, thereby contributing to food security. Global
71 egg production continues to see substantial growth from 61.7 tons to 76.7 tons¹⁸, a 24% increase in the past
72 decade. Asia is the largest egg producing region, contributing to 60% of the total production volume¹⁶.

73 Avian pathogens can cause huge economic loss (>20%) in the overall poultry production, and three times
74 due to loss from mortality^{19, 20}. Egg and egg based product surveillance programs are highly effective in
75 controlling foodborne disease outbreaks- often providing information for timely intervention, control and
76 mitigation measures^{21, 22, 23}. Egg-based surveillance helped identify more than 895 foodborne disease
77 outbreaks in Spain (2000-2002), majority (85%) caused by *Salmonella*²⁴.

78 Most studies have focused on detecting of foodborne pathogens like *Salmonella* spp., *Camphylobacter* spp.
79 and *Escherichia coli* in eggshells^{26,27,28}, we posit that albumin-based screening is also a convenient tool and
80 useful in detecting other important avian pathogens such as- *Mycoplasma gallisepticum* (MG), *Mycoplasma*
81 *synoviae* (MS), Infectious Bronchitis Virus (IBV), Influenza A Virus (IAV), Newcastle Disease Virus
82 (NDV) and Infectious Bursal Disease Virus (IBDV) in hatcheries to minimize contamination through
83 horizontal and vertical transmission modes. Avian pathogens have been isolated from oral swabs, cloacal
84 swabs, serum samples, egg yolk, egg shells, and environmental swabs but albumin-based molecular
85 detection has not been intensively used till date. Due to the dearth of literature available on albumin
86 screening, we used Polymerase Chain Reaction (PCR) based tests to screen for six major avian pathogens
87 (IBD, IBDV, MS, MG, IAV and NDV) in egg albumin from eggs collected from eleven hatcheries, hence
88 devising cost-effective poultry pathogen surveillance tool in hatcheries.

89 **Selected Avian Diseases**

90 ***Mycoplasma synoviae* and *Mycoplasma gallisepticum***

91 *Mycoplasma* is a vertically transmitted disease²⁹ with pronounced effects in eggshell- altered surface,
92 thinning, translucency, consequently leading to a greater incidence of eggshell cracks and breaks³⁰. Though
93 it is a non-fatal disease³¹, it can significantly affect weight gain, feed conversion ratio, fertility, chronic
94 respiratory disease and hatchability^{32, 33} in birds. MG and MS are bacterial OIE-listed respiratory
95 pathogens³⁴ which often persist in sub-clinical level³⁵ and are a key cause for economic loss in the poultry
96 industry³⁴. *Mycoplasma* infections, especially in farms with weak biosecurity, are often the cause of
97 eggshell abnormalities and decrease in egg production³⁶.

98 **Newcastle Disease Virus (NDV)**

99 Newcastle disease (ND), an OIE-notifiable List A disease, is caused by avian paramyxovirus serotype 1
100 (APMV-1) virus³⁸ of Avulavirus genus. It is one of the highly pathogenic viral diseases of avian species,
101 and a major cause of morbidity and mortality in flocks³⁹. Affected birds develop respiratory, digestive and
102 neurologic symptoms with profound immunosuppression⁴⁰. In many countries throughout Asia and Africa,
103 ND remains endemic in commercial poultry despite intensive vaccination program that have been applied
104 for decades⁴¹. NDV can replicate in the reproductive tract of hens and contaminate internal components of
105 eggs and eggshell surface⁴².

106

107 **Infectious Bronchitis Virus (IBV)**

108 Infectious bronchitis in poultry is caused by IBV- an Avian Coronavirus (ACoV) of genus
109 Gammacoronavirus⁴³. IBV causes a fast-spreading respiratory disease in young chicks, with laying hens
110 experiencing reduced production, egg shell abnormalities, and decreased internal egg quality⁴⁴. Along with
111 commercial poultry, backyard poultry and free-ranging birds may serve as 'reservoir' for ACoV
112 transmission, and migratory birds often acting as an intermediary host spreading to wide and distant areas⁴⁵.

113 **Infectious Bursal Disease Virus (IBDV)**

114 Infectious Bursal Disease, commonly known as Gumboro, is an immunosuppressive disease transmitted
115 mainly horizontally through the feco-oral route⁴⁶. It is caused by a double stranded RNA virus- IBDV
116 (genus *Avibirnavirus* of family *Birnaviridae*)⁴⁷. There are two distinct serotypes of the virus, but only
117 serotype 1 viruses cause disease in poultry⁴⁸. Viruses belonging to one of these antigenic subtypes are
118 commonly known as variants, causing up to 60 to 100 percent mortality rates in chickens⁴⁹.

119 **Influenza A virus (IAV)**

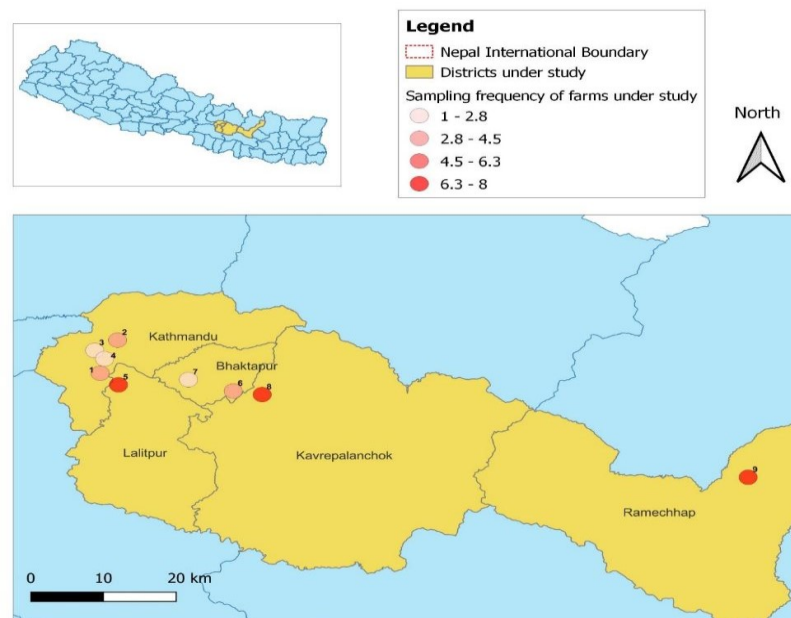
120 Avian Influenza (AI), caused by IAV, is a highly contagious viral infection which may cause up to 100%
121 mortality in domestic chickens or turkeys⁵⁰. The disease is caused by a highly mutable RNA virus that
122 belongs to the family *Orthomyxoviridae*⁵¹. Influenza viruses have two surface proteins, hemagglutinin (HA)
123 and neuraminidase (NA)⁵² that determine their subtype and the animal species they infect; there are 16 HA
124 and nine NA types⁵³. When AI viruses of two HA types, H5 and H7, infect domestic poultry (chickens and
125 turkeys) they often mutate and virulent disease arises in these birds which is called highly pathogenic avian
126 influenza (HPAI)⁵⁴. The initial infection that causes subclinical or mild disease is called low pathogenic
127 avian influenza (LPAI)⁵⁵. Wild water birds act as reservoir hosts of IAV, however these viruses generally
128 do not cause disease in these birds⁵⁶.

129

130 Methodology

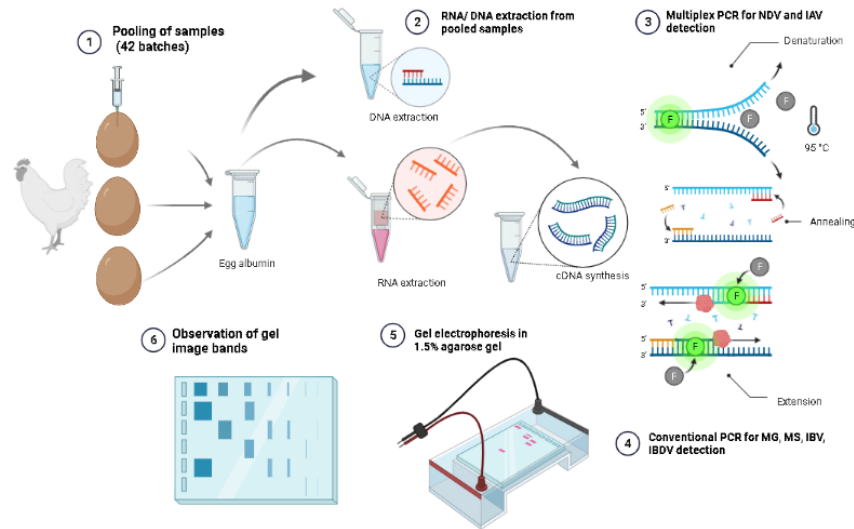
131 We retrospectively looked at the data on the presence of 6 major avian pathogens on eggs received
132 periodically every month (August 20- August 2021, except October 2020) from the eleven major hatcheries
133 in and around Kathmandu valley. These hatcheries participated in preventive pathogen screening services
134 provided by the BIOVAC Nepal's Poultry Diagnostic Laboratory (BNPDL). The sampling was performed
135 by trained personnel. No live animals were harmed and the study does not include handling of animal. No
136 embryonated eggs were killed during sampling process- qualifying this study to be exempted from any
137 ethical approval. To maintain anonymity of the hatcheries, they were coded with numeric digits on the basis
138 of time the samples were received. A total of 4343 eggs from eleven major hatcheries located in the five
139 surrounding districts (Kathmandu, Bhaktapur, Lalitpur, Kavrepalanchowk and Ramechhap) of Kathmandu,
140 Nepal were received and tested (Figure 1).

141 These eggs were brought to the BNPDL every month (except October 2020) in batches (133 ± 60 eggs per
142 batch) packaged in crates (30 eggs per crate). Albumin extracted from 10% random eggs from each batch
143 (3 eggs from each crate) were tested for six selected Avian pathogens (NDV, IAV, IBV, IBDV, MS and
144 MG) using PCR (Figure 2)



145

146 **Figure 1: Participating eleven major hatcheries located in Kathmandu and surrounding five districts**
147 *(Kathmandu, Bhaktapur, Lalitpur, Kavrepalanchowk and Ramechhap). As part of preventive disease*
148 *screening, eggs are routinely received by BIOVAC Nepal's Poultry diagnostic laboratory located in*
149 *Banepa (Nala), Nepal.*



150

151 **Figure 2: Laboratory testing for the detection of six avian pathogens:** *BIOVAC Nepal's Poultry*
152 *Diagnostic Laboratory (BNPDL) received samples from eleven participating hatcheries for preventive*
153 *diagnostic screening of avian pathogens.*

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156 **Molecular Detection of Six Avian Pathogens**

157 **Nucleic Acid Extraction and cDNA synthesis**

158 The nucleic acids (DNA/RNA) from pooled egg albumin samples were extracted using automated nucleic
159 acid extractor (abGenix™ AITbiotech, Singapore) following manufacturer's instructions. cDNA for the
160 extracted nucleic acids were synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories,
161 USA). For a single sample, 4 µL of 5X iScript reaction mix, 1 µL of iScript reverse transcriptase, 6 µL of
162 nuclease free water and 9 µL of the extracted nucleic acid was used to prepare 20 µL of cDNA. The cDNA
163 was synthesized in thermal cycler by incubating the mix at 25°C for 5 minutes followed by reverse
164 transcription at 46°C for 20 minutes and RT inactivation at 95°C for 1 minute. PCR for IBV, IBD, MG and
165 MS were performed using QIAGEN Multiplex PCR Kit (Qiagen, Catalog No. 206145). Multiplex PCR
166 was used to detect IAV and NDV simultaneously in the samples using QIAGEN Multiplex PCR kit.

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168

169 **Multiplex PCR for IAV and NDV**

170 We have developed and optimized a multiplex PCR that detects both IAV and NDV simultaneously in one
171 single test. A 291 bp fragment of Matrix protein gene of NDV and 156 bp fragment of Matrix protein gene
172 of IAV was amplified in 25 µL of the reaction mixture containing: 3 µL of cDNA, 5 µL QIAGEN®
173 nuclease-free water, 2.5 µL of 5X Q Solution, 0.5 µL NDV primer (forward), 0.5 µL NDV primer (reverse),
174 0.5 µL IAV primer (forward), 0.5 µL IAV primer (reverse) and 12.5 µL of 2X of QIAGEN® Multiplex
175 PCR Master Mix (HotStarTaq DNA Polymerase, MgCl₂, dNTPs and PCR buffer). PCR condition: 1 cycle
176 of initial denaturation at 95°C for 15 minutes, 45 cycles of denaturation at 95°C for 20 seconds, annealing
177 at 60°C for 20 second and extension at 72°C for 30 second. The PCR ended with a final elongation at 72°C
178 for 5 minutes. PCR products were visualized in Gel electrophoresis (1.5%) (Figure 3).

179 **PCR Primers- multiplex IAV and NDV**

180 For NDV, 10 pico-molar concentration each of forward primer (5'-GCTCAATGTCACTATTGATGTGG-
181 3') and reverse primer (5'-TAGCAGGCTGTCCCACTGC-3') were used and for IAV, 10 pico-molar
182 concentration each of forward (5'-CTTCTAACCGAGGTTCGAAACG-3') and reverse
183 (5'GGTGACAGGATTGGTCTTGTC-3') were designed using NCBI PrimerBlast®.

184 **PCR detection of IBV**

185 A 433 bp fragment of 3' UTR of IBV was amplified in 25 µL of the reaction mixture containing: 2 µL of
186 template cDNA, 8.5 µL QIAGEN® nuclease-free water, 1 µL All 1-F primer (forward), 1 µL Dell-R primer
187 (reverse) and 12.5 µL of 2X of QIAGEN® PCR Master Mix. PCR condition: 1 cycle of initial denaturation
188 at 95°C for 15 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 second
189 and extension at 72°C for 40 second. The PCR ended with a final elongation at 72°C for 5 minutes.

190 **PCR Primers- IBV**

191 10 pico-molar concentration of each All 1-F forward primer (5'-CAGCGCCAAAACAACAGCG-3) and
192 Dell-R reverse primer (5'-CATTTCCCTGGCGATAGAC-3') were used for detection of IBV as per Saba
193 et al. (2014)⁵⁷.

194 **PCR detection of IBDV**

195 A 643 bp fragment of complete hyper variable region of VP2 gene of IBDV was amplified in 25 µL of the
196 reaction mixture containing 2 µL of template cDNA, 8.5 µL QIAGEN® nuclease-free water, 1 µL
197 Infectious Bursal Disease Forward Primer, 1µL Infectious Bursal Disease Forward Primer and 12.5 µL of
198 2X of QIAGEN® PCR Master Mix. PCR conditions: 1 cycle of initial denaturation at 95°C for 15 minutes,

199 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 20 second and extension at 72°C
200 for 45 second. The PCR ended with a final elongation at 72°C for 5 minutes.

201 **PCR Primers- IBDV**

202 10 pico-molar concentration of each forward primer (5'-TCACCGTCCTCAGCTTAC-3') and reverse
203 primer (5'-TCAGGATTTGGGATCAGC-3') were used for the detection of IBD as per Kataria et al.
204 (2007)⁵⁸.

205 **PCR detection of MS**

206 A 207 bp fragment of 16s rRNA gene of MS was amplified in 25 µL of the reaction mixture containing 2
207 µL of template cDNA, 8.5 µL QIAGEN® nuclease-free water, 1 µL *Mycoplasma synoviae* forward primer,
208 1 µL *Mycoplasma synoviae* reverse primer, and 12.5 µL of 2X of QIAGEN® PCR Master Mix. PCR
209 conditions: 1 cycle of initial denaturation at 95°C for 15 minutes, 35 cycles of denaturation at 95°C for 30
210 seconds, annealing at 53°C for 20 second and extension at 72°C for 15 second. The PCR ended with a final
211 elongation at 72°C for 5 minutes.

212 **PCR Primers-MS**

213 10 pico-molar concentration of each forward primer (5'-GAGAAGCAAAATAGTGATATC-3') and
214 reverse primer (5'-TCGTCTCCGAAGTTAACAA-3') were used for detection of MS as per Kahya et al.
215 (2015)⁵⁹.

216 217 **PCR detection of MG**

218 A 185 bp fragment of 16s rRNA gene of MG was amplified in 25 µL of the reaction mixture containing 2
219 µL of template cDNA, 8.5 µL QIAGEN® nuclease-free water, 1 µL *Mycoplasma gallisepticum* forward
220 primer, 1 µL *Mycoplasma gallisepticum* reverse primer and 12.5 µL of 2X of QIAGEN® PCR Master Mix.
221 PCR conditions: 1 cycle of initial denaturation at 95°C for 15 minutes, 35 cycles of denaturation at 95°C
222 for 30 seconds, annealing at 53°C for 20 second and extension at 72°C for 15 second. The PCR ended with
223 a final elongation at 72°C for 5 minutes.

224 **PCR Primers- MG**

225 10 pico-molar concentration each of forward primer (5'-GAGCTAATCTGTAAAGTTGGTC-3') and
226 reverse primer (5'-GCTTCCTTGCGGTTAGCAAC-3') were used for detection of MG as per Kahya et. al.
227 (2015).

228 All PCR amplified products were visualized under 1.5% agarose gel electrophoresis.

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230 Results

231 We retrospectively looked at the data on the presence of 6 major avian pathogens on eggs received
232 periodically every month (August 20- August 2021, except October 2020) from the eleven major hatcheries
233 in and around Kathmandu valley. These hatcheries participated in preventive pathogen screening services
234 provided by BNPD. The hatcheries had experienced high morbidity and mortality in their birds; and had
235 seen decreased and defective egg production.

236 In an average we received 430 eggs every month from one or more of the eleven hatcheries, majority (7/11,
237 64%) had at least one of the six pathogens present. We detected at least one avian pathogen in nine out of
238 eleven months (82%) of screening. Except for IBDV, we found one or multiple occurrence of other major
239 avian pathogens- Influenza A (IAV) (n=4 times) and *Mycoplasma gallisepticum* (MG) (n=4 times) were
240 detected the most, followed by Newcastle Virus (NDV) (n=3 times). Infectious bronchitis virus (IBV) were
241 detected twice, and *Mycoplasma synoviae* (MS) was detected once (Table 1).

242 In hatchery 4, we detected IAV in samples received in two separate months (September and December
243 2020). Meanwhile, we received most consecutive samples from hatchery 9, where we detected MG 3
244 months in a row (April, May and June 2021), with multiple pathogens (MG, IAV and NDV) present in June
245 2021.

246 In the winter season (January- April 2021), four batches had four detectable pathogens (NDV, IBV, MS
247 and MG). In rainy or wet season (May- August 2021), three different pathogens (IAV, MG and NDV) were
248 found in the 9 batches; during this period MG was detected in three consecutive batches. During the fall
249 season (September-December), we detected only three pathogen (NDV, IBV and IAV) in four batches. We
250 detected more pathogens during rainy or wet season than in winter or fall season (Figure 4).



251
252 **Figure 3: Detection of Influenza A (IAV) and Newcastle Disease Virus (NDV) using Multiplex PCR:**
253 Newcastle Disease Virus (NDV) is detected as 300bp PCR amplicon, and Influenza A Virus (IAV) as
254 150bp. Lane 1: DNA ladder; Lanes 2-5: pooled albumin samples, Lane 6: negative control. Visualized
255 under 1.5% gel electrophoresis.

256 **Table 1: Eggs received and avian pathogen detected**
 257 **from each hatchery:** *Between Aug 2020 through*
 258 *August 2021 (except October 2020), we received eggs*
 259 *from participating 11 major poultry hatcheries. Eggs*
 260 *received from some hatcheries (1, 2, 3 and 7) were free*
 261 *of screened avian pathogens. Influenza A (IAV) (n=4*
 262 *times) and Mycoplasma gallisepticum (MG) (n=4*
 263 *times) were detected the most, followed by Newcastle*
 264 *Virus (NDV) (n=3 times). Infectious bronchitis virus*
 265 *(IBV) were detected twice, and Mycoplasma synoviae*
 266 *(MS) was detected once.*

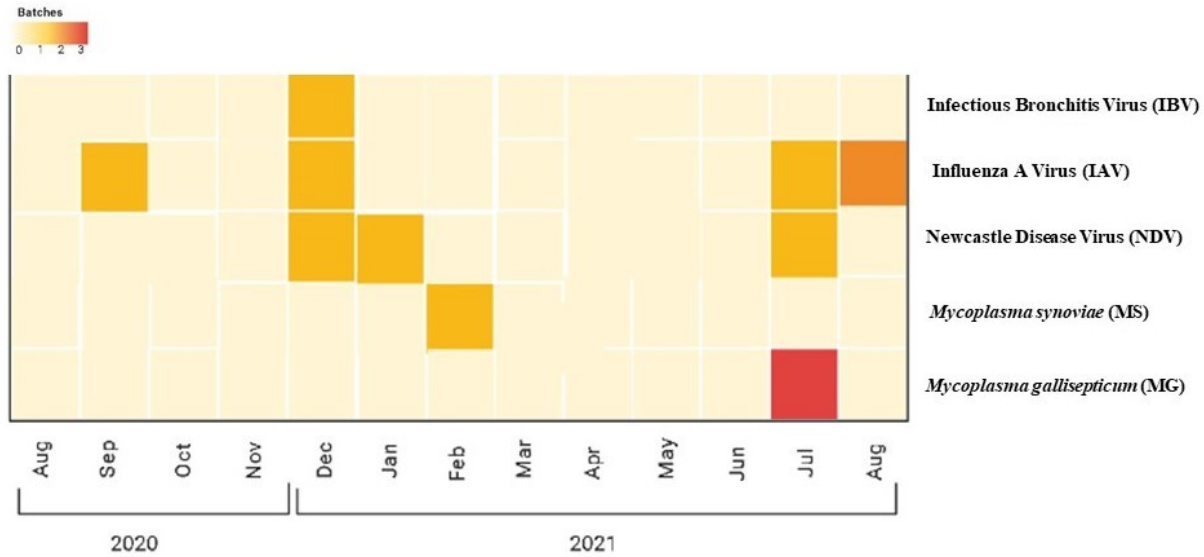
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HATCHERY	Months											
	Aug 20	Sep 20	Nov 20	Dec 20	Jan 21	Feb 21	Mar 21	Apr 21	May 21	Jun 21	Jul 21	Aug 21
1	N=260											
2	N=120											
3		N= 450										
4		N=150 IAV		N=120 IAV/IBV								
5			N= 60	N=240 NDV								
6					N=420 NDV							
7						N=60	N=210					
8						N=27 MS IBV						
9							N=65	N=30 MG	N=180 MG	N=300 MG, NDV, IAV	N=418	
10									N=10	N=270	N=870 MG	
11											N=90	N=1050 IAV

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272 **Figure 4: Detected avian pathogens in received batches from the eleven different hatcheries:** *In the*
273 *winter season (January- April 2021), four batches had four detectable pathogens (NDV, IBV, MS and MG).*
274 *In rainy or wet season (May- August 2021), three different pathogens (IAV, MG and NDV) were found in*
275 *the 9 batches; during this period MG was detected in three consecutive batches. During the fall season*
276 *(September-December), we detected only three pathogen (NDV, IBV and IAV) in four batches. We detected*
277 *more pathogens during rainy or wet season than in winter or fall season (Figure 3).*

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281 **Discussion**

282 There is no active surveillance of most of the avian pathogens in Nepal, only outbreak related Avian
283 Influenza (Bird flu) is investigated^{60, 61} primarily due to human health concerns. Meanwhile, disease
284 outbreaks in poultry farms are often reported based on clinical symptoms and mortality. Lack of
285 comprehensive animal diagnostic facilities also limits the farmers' ability to have disease outbreaks
286 properly investigated. Preventive disease screening is still a novel concept in Nepal's poultry industry.
287 Against that backdrop, we have set up a poultry disease diagnostic laboratory (BNPDL) based in
288 Kathmandu and Nala, Nepal. We routinely receive requests to screen for avian pathogens from poultry
289 farmers, including hatcheries. Hatcheries can be a source of disease spread through contaminated eggs and
290 day old chicks. We have detected presence of avian pathogens in egg albumin using molecular (PCR)
291 detection. In a resource strapped country like Nepal, where disease surveillance is not well developed, a
292 routine egg screening based on accurate and relatively fast PCR screening, can offer an important insight
293 into floating avian pathogens in poultry population at any given time. This kind of information can be
294 helpful for poultry producers, including hatcheries, to prevent and mitigate their losses by adopting
295 appropriate interventions.

296 **Egg-shell based pathogen detection**

297 There are some egg-shell based poultry disease surveillance program being used in some countries,
298 however, they have only focused on food borne pathogens such as *Salmonella*, *Camphylobacter* and
299 *Escherichia coli*^{62,63,64,65}. Majority (41%) of all foodborne disease outbreaks in Spain were associated with
300 consumption of eggs and egg products⁶⁶, which explains a high level of contamination associated with eggs.
301 Similarly, quarterly surveillance measures have also been carried out under the Egg Product Inspection Act
302 (EPIA) as part of USDA's Shell Egg Surveillance (SES)⁶⁷. While successful in detecting these important
303 food safety related pathogens, such surveillance completely overlooks the poultry health related pathogens-
304 especially avian viral pathogens. We have demonstrated that egg (albumin) based screening and disease
305 surveillance can be pretty effective in picking floating pathogens, and help us understand the disease
306 burden, patterns and trends in general.

307 **Limitation of our study**

308 This study was based on and relied upon the eggs being provided by the participating hatcheries. Most of
309 these hatcheries only requested to have their eggs screened based on suspected clinical signs (and often
310 after some mortality). Hence, they did not provide eggs routinely and regularly. Because of this, we were
311 not able to establish the real disease occurrence trend across each hatchery nor were we able to tell whether
312 the de-contamination efforts they made actually worked. Furthermore, randomly selecting 10% of the eggs

313 might not have the sensitivity needed to pick all the pathogens in a given farm; we only screened a fraction
314 of eggs in each batch due to cost consideration. We could have integrated an environmental screening and
315 bio-security assessment as a part of a thorough disease surveillance system in poultry industry/farms,
316 however, we were not able to do that in this study. Interestingly, we did observe some seasonal variability
317 of disease occurrences- wet or rainy season harboring more pathogens than dry season. However, we need
318 more data points to look into this further.

319 **Implications and Utility of Egg based Pathogen screening**

320 Molecular detection of pathogens in egg albumin can provide important information to put together an early
321 containment strategies for poultry farmers in particular and animal health efforts in general. It can be
322 especially beneficial to hatcheries as they are often the contamination source- spreading disease from egg
323 to day-old chicks, and eventually to the whole poultry production value chain. Egg-based disease screening
324 can be an effective One Health surveillance tool as well, as it can pick up important Zoonotic pathogens
325 such as Influenza A, and help stem pathogen spill-overs, thereby safeguarding human health. Albumin
326 screening, as an early detection tool, can also assess biosecurity effectiveness in hatcheries and help curb
327 horizontal and vertical transmission of avian diseases. In a developing country like Nepal, where resources
328 are limited, easy to access pathogen screening samples like eggs and highly sensitive and accurate
329 molecular (PCR) tools can help in building important avian disease surveillance tool. With the advent of
330 next generation DNA sequencing and Genomics technology, we can even screen for a broader viral,
331 bacterial and other pathogens using same (single) sample source. High throughput in data acquisition made
332 possible by such new technologies certainly can make disease surveillance fast, easy and affordable. Our
333 study is an initial step towards that direction.

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346 **Acknowledgement**

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356 Conceptualization: Rajindra Napit

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358 Field support: Dhiraj Puri

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365 Writing – review & editing: Dibesh Bikram Karmacharya

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