

## Influenza D Virus Circulation in Cattle and Swine, Luxembourg, 2012–2016

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We detected antibodies against influenza D in 80.2% of the cattle sampled in Luxembourg in 2016, suggesting widespread virus circulation throughout the country. In swine, seroprevalence of influenza D was low but increased from 0% to 5.9% from 2012 to 2014–2015.

Influenza D virus (IDV), a new orthomyxovirus distantly related to influenza C virus, was described in pigs with respiratory symptoms in 2011 (1). Although mild symptoms only were reported in experimental pig and calf infections, the virus has been implicated in bovine respiratory disease complex (1–3). Cattle are currently considered the main host of the virus, but other livestock species are also susceptible (4). In Europe, IDV circulation has been reported in France (5), Italy (6,7), and Ireland (8). Recent serosurveys in Italy showed extremely high seroprevalence rates in cattle (92.4% seropositive) (9) and a low but increasing seroprevalence in swine, from 0.6% in 2009 to 11.7% in 2015 (7). We investigated the presence of IDV in cattle and swine farms in Luxembourg.

In 2016, we collected serum samples from 450 asymptomatic cattle from 44 farms throughout Luxembourg (Figure, panel A; online Technical Appendix, <https://wwwnc.cdc.gov/EID/article/24/7/17-1937-Techapp1.pdf>). We screened the samples for IDV antibodies by using hemagglutination inhibition (HI) assays. We also screened serum samples collected from pigs at 2 slaughterhouses in 2012 (n = 258, 27 farms) and 2014–2015 (n = 287, 29 farms). Because HI titers as low as 20 were measured in farms with demonstrated influenza D circulation (7), we considered HI titers  $\geq 20$  positive. In addition, we screened nasal swab specimens from asymptomatic pigs sampled at slaughter in 2009 (n = 232, 56 farms) and 2014–2015 (n = 427, 36 farms) by

real-time reverse transcription PCR (1). No cattle samples were available for molecular screening.

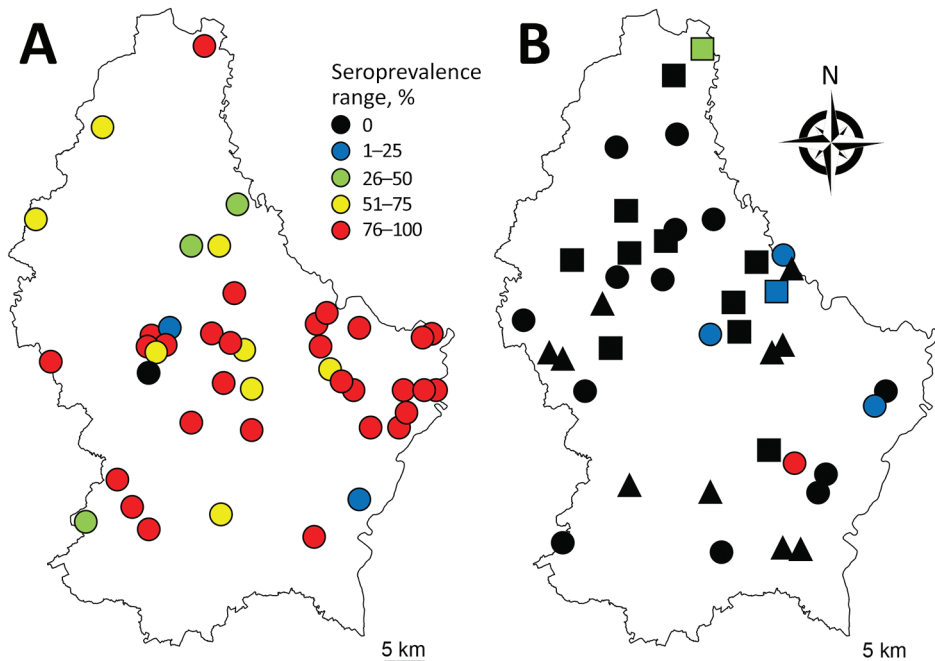
We found an overall seroprevalence of 80.2% in cattle (361/450; HI titer range 20–1,280) (online Technical Appendix Figure); 97.7% of herds (43/44) had  $\geq 1$  seropositive animal. Average within-farm seroprevalence was 83.0% (range 20%–100%; Figure, panel A). These results suggested that IDV affects most animals in nearly all farms (Figure, panel A). All animals were much older than 6 months (average 70.5 mo, range 23–209 mo), so it is unlikely that the antibodies were maternally derived (10). The median age of seropositive animals (61 months) was significantly higher than the median age of seronegative animals (41 months;  $p < 0.001$ ). Seroprevalence was higher in beef cattle (88.0%, 95/108) than in dairy cattle (75.6%, 133/176; meat or dairy production type was not known for 166 animals), but beef cattle were also on average older than dairy cattle. A binary logistic regression model including herd as a random effect and age and production type as fixed effects revealed that only age substantially affected IDV seropositivity.

Most of the cattle investigated were born in Luxembourg (90%, 405/450), but IDV antibodies were found regardless of country of birth (others were born in Germany, France, Belgium, and Italy). This information demonstrates that our results cannot be explained by importation of seropositive animals alone and that IDV transmission takes place in Luxembourg. Within-herd seroprevalence of cattle herds was similar for herds located near the borders as well as those further inland, suggesting that the virus could also spread to and from the neighboring countries (Belgium, France, and Germany), for example, through cross-border grazing.

In Luxembourg, IDV seroprevalence was low in swine compared with cattle but has increased during recent years (0% in 2012 to 5.9% [17/287] in 2014–2015), as it has in Italy (7). We detected seropositive animals in 6/29 (20.7%) swine herds (Figure, panel B). The low virus prevalence from nasal swabs (0% in 2009, 0.7% [3/427] in 2014–2015) and the low viral RNA concentration (9.7–94.5 copies/ $\mu$ L) were not conducive to amplification of genetic material for sequencing. The low levels of virus circulation in pigs shown by seroprevalence data, the absence of symptoms at the time of sampling (3), and the short shedding period under experimental infection (1) probably contributed to the low detection rates observed in swine nasal swab samples. The IDV RNA-positive nasal swab samples originated from 2 different herds, 1 of which was also seropositive (9/10 pigs with HI titer  $\geq 20$ ; no samples were available from the second herd). Although we collected all 3 PCR-positive samples on the same day at the same slaughterhouse, it is unlikely that the pigs were infected during their short stay there.

Taken together, our results suggested that IDV circulates widely throughout cattle farms in Luxembourg and can be considered hyperenzootic in the country. Once

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**Figure.** Within-farm seroprevalence range in A) cattle herds ( $n = 44$ ) sampled in 2016 and B) swine herds sampled in 2012 ( $n = 10$ , triangles), 2014–2015 ( $n = 12$ , squares) or both ( $n = 17$ , circles), Luxembourg.

introduced into a herd, IDV seems to spread very efficiently, given the high within-farm seroprevalence rates. In light of cross-border trade and grazing, the region beyond Luxembourg's borders may be also hyperenzootic for IDV. Although IDV mainly affects cattle, we detected IDV antibodies in pigs and an increased seroprevalence in pig herds. We are planning systematic serologic and virologic screenings along with epidemiologic surveys to investigate the genetic diversity of IDV strains in Luxembourg, to evaluate the effect of IDV infection on cattle and pig health and productivity, and to study IDV interaction with other pathogens.

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### About the Author

Dr. Snoeck is a researcher at the Luxembourg Institute of Health in Luxembourg. Her primary research interests include the epidemiology of livestock zoonotic viruses.

### References

- Hause BM, Ducatez M, Collin EA, Ran Z, Liu R, Sheng Z, et al. Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. *PLoS Pathog.* 2013;9:e1003176. <http://dx.doi.org/10.1371/journal.ppat.1003176>
- Ferguson L, Olivier AK, Genova S, Epperson WB, Smith DR, Schneider L, et al. Pathogenesis of influenza D virus in cattle. *J Virol.* 2016;90:5636–42. <http://dx.doi.org/10.1128/JVI.03122-15>
- Ng TF, Kondov NO, Deng X, Van Eenennaam A, Neiberghs HL, Delwart E. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. *J Virol.* 2015;89:5340–9. <http://dx.doi.org/10.1128/JVI.00064-15>
- Salem E, Cook EAJ, Lbacha HA, Oliva J, Awoume F, Aplogan GL, et al. Serologic evidence for influenza C and D virus among ruminants and camelids, Africa, 1991–2015. *Emerg Infect Dis.* 2017;23:1556–9. <http://dx.doi.org/10.3201/eid2309.170342>
- Ducatez MF, Pelletier C, Meyer G. Influenza D virus in cattle, France, 2011–2014. *Emerg Infect Dis.* 2015;21:368–71. <http://dx.doi.org/10.3201/eid2102.141449>
- Chiapponi C, Faccini S, De Mattia A, Baioni L, Barbieri I, Rosignoli C, et al. Detection of influenza D virus among swine and cattle, Italy. *Emerg Infect Dis.* 2016;22:352–4. <http://dx.doi.org/10.3201/eid2202.151439>
- Foni E, Chiapponi C, Baioni L, Zanni I, Merenda M, Rosignoli C, et al. Influenza D in Italy: towards a better understanding of an emerging viral infection in swine. *Sci Rep.* 2017;7:11660. <http://dx.doi.org/10.1038/s41598-017-12012-3>
- Flynn O, Gallagher C, Mooney J, Irvine C, Ducatez M, Hause B, et al. Influenza D virus in cattle, Ireland. *Emerg Infect Dis.* 2018;24:389–91. <http://dx.doi.org/10.3201/eid2402.170759>
- Rosignoli C, Faccini S, Merenda M, Chiapponi C, De Mattia A, Bufalo G, et al. Influenza D virus infection in cattle in Italy [in Italian]. *Large Animal Review.* 2017;23:123–8.
- Ferguson L, Eckard L, Epperson WB, Long LP, Smith D, Huston C, et al. Influenza D virus infection in Mississippi beef cattle. *Virology.* 2015;486:28–34. <http://dx.doi.org/10.1016/j.virol.2015.08.030>

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## Technical Appendix

### Materials and Methods

#### Sampling Design and Sample Collection

The 2012 annual census performed by the National Institute of Statistics and Economic Studies of the Grand Duchy of Luxembourg indicated there were 1,218 farms rearing  $\geq 20$  cattle and 86 farms rearing  $\geq 20$  swine in the country. Our study included cattle serum samples ( $n = 450$ ) from 44 herds collected in 2016 in the framework of infectious bovine rhinotracheitis serologic monitoring. On average, we screened 10.2 animals (range 9–20)/herd for the presence of influenza D antibodies. No information regarding past respiratory disease in the farms was available.

For pigs, we screened an average of 9.6 serum samples (range 1–19/herd, total = 258) from 27 pig farms sampled in 2012. In the 2014–2015 cohort, we collected an average of 9.9 serum samples/herd (range 9–10/herd, total = 287) from 29 pig farms. We collected all pig samples at the time of slaughter and, as with the cattle, no information regarding past respiratory disease in the herd was available.

Given the paucity of between- and within-herd seroprevalence data at the time of study design (1–3), and the complete lack of information from Europe, we arbitrarily and conservatively set values of between-herd seroprevalence to 10% and within-herd seroprevalence to 30%. According to the formula of Cannon and Roe (1982; cited in (1)), screening 10 animals/herd was sufficient to find  $\geq 1$  seropositive animal in a seropositive herd if the within-herd seroprevalence is  $\geq 30\%$ , irrespective of herd size (assuming a 90% test sensitivity and a desired certainty of detecting past infection in an infected herd of 95%). Similarly, sampling 44/1,218 cattle herds and 27/86 swine farms was sufficient to find  $\geq 1$  seropositive herd if between-herd seroprevalence was  $\geq 10\%$ .

We screened nasal swab samples from asymptomatic pigs at slaughter for the presence of influenza D virus. In 2009, we sampled 56 farms and screened 4.1 samples/herd on average (range 1–15/herd, n = 232). In 2014–2015, we collected 11.9 nasal swabs/herd on average from 36 pig farms (range 5–20/herd, n = 427).

### **Hemagglutination Inhibition (HI) Assays**

We screened all samples for the presence of IDV antibodies by HI tests performed as previously described (2) using D/bovine/France/5920/2014 viral strain (D/swine/Oklahoma/2011 lineage). We pretreated all serum samples with receptor-destroying enzyme (RDE, DebenDiagnostics, Germany) and hemadsorbed them on horse red blood cells before testing in 2-fold serial dilutions from 1:10 until 1:1280. We expressed antibody titers as the reciprocal of the highest serum dilution that inhibited hemagglutination.

### **Real-time PCR**

Pig nasal swabs were screened by a real-time RT-PCR (3) with Quantitect Probe RT-PCR kit (QIAGEN, Venlo, The Netherlands). No fluorescence and no probe degradation was visible in any negative controls. We retested positive samples in triplicate to confirm their positive status and quantified them in a qRT-PCR reaction. We generated a standard curve from 10-fold dilutions of a plasmid quantified by absorbance measurement with NanoDrop ND-1000 (Isogen Life Science, De Meern, The Netherlands).

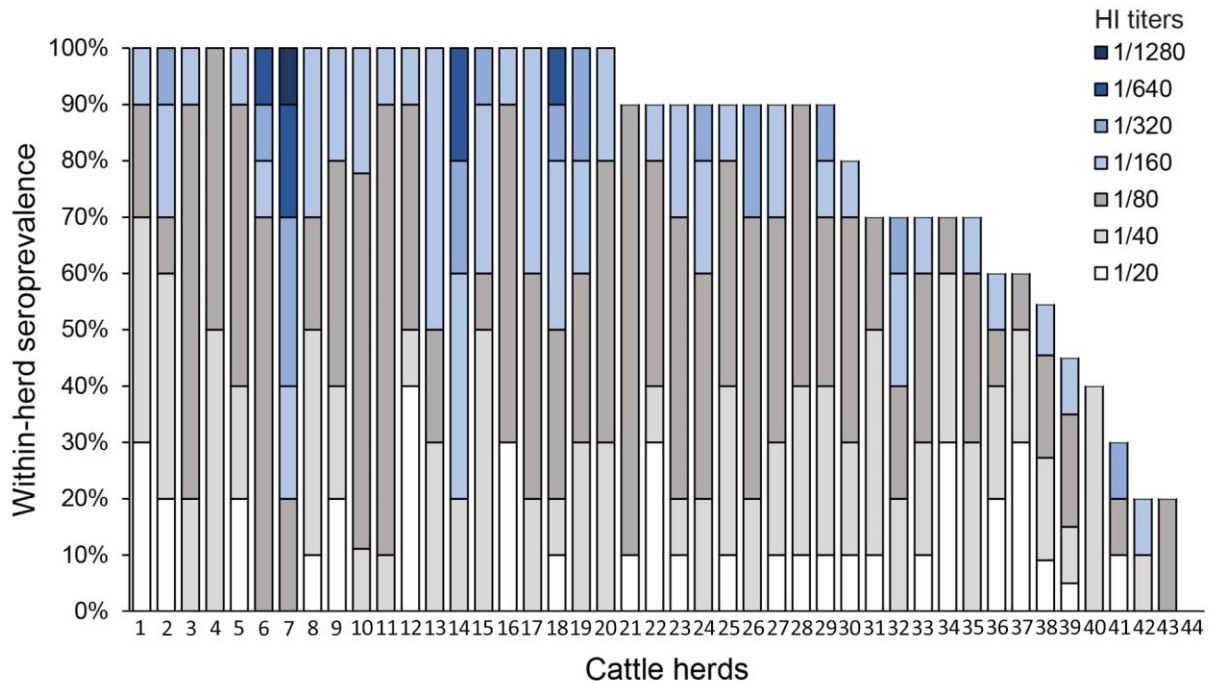
### **Statistical Analyses**

We performed statistical analyses (Mann-Whitney Rank Sum test,  $\chi^2$  test) in SigmaPlot version 12.0 (Systat Software Inc., San Jose, CA, USA). We tested a generalized linear mixed-effect model using binary outcome including herd as random effect and age and production type as fixed effects using the R package “lme4” (4) in R Studio.

### **References**

1. De Wit JJ. Detection of infectious bronchitis virus. *Avian Pathol.* 2000;29:71–93. [PubMed](https://pubmed.ncbi.nlm.nih.gov/11511111/)  
<http://dx.doi.org/10.1080/03079450094108>
2. Salem E, Cook EAJ, Lbacha HA, Oliva J, Awoume F, Aplogan GL, et al. Serologic evidence for influenza C and D virus among ruminants and camelids, Africa, 1991–2015. *Emerg Infect Dis.* 2017;23:1556–9. [PubMed](https://pubmed.ncbi.nlm.nih.gov/28111111/) <http://dx.doi.org/10.3201/eid2309.170342>

3. Hause BM, Ducatez M, Collin EA, Ran Z, Liu R, Sheng Z, et al. Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. PLoS Pathog. 2013;9:e1003176. [PubMed http://dx.doi.org/10.1371/journal.ppat.1003176](http://dx.doi.org/10.1371/journal.ppat.1003176)
4. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Softw. 2015;67:1–48. <http://dx.doi.org/10.18637/jss.v067.i01>



**Technical Appendix Figure.** Within-herd influenza D seroprevalence of each cattle herd (n = 44) tested and distribution of HI titers of individual animals within each herd (10 animals tested/herd; except for herd #10, n = 9; herd #38, n = 11; herd #39, n = 20).