First detection of Hedgehog coronavirus 1 in Poland

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Hedgehogs are common in the majority of European countries and are known to host various pathogens, including viruses. The recent discovery of MERS-related coronaviruses (CoVs) in hedgehogs from Germany, France, the UK, China, and Italy suggests that hedgehogs may represent a wild reservoir of betacoronaviruses. This study reports the first detection and characterization of novel betacoronavirus, subgenus Merbecovirus in wild hedgehogs in Poland. The CoV RNA was detected in 10 out of 40 hedgehogs’ rectal swabs and in 1 out of 18 samples of the lung. No viral RNA was identified in the duodenum and kidney. There was no significant relationship between clinical status, gender, hedgehogs’ age, and coronaviral RNA detection. Phylogenetic analysis showed that CoVs detected in our study grouped together with other representatives of Hedgehog coronavirus 1 species identified in Western Europe. Our findings provide further evidence that hedgehogs are a natural reservoir of Merbecovirus. Considering the high mutation rate of CoVs and their potential for crossing interspecies barriers, the proper management of hedgehogs admitted to wildlife rehabilitation centres is needed. It cannot be excluded that merbecovirus strains detected in hedgehogs may recombine with other CoVs leading to new viruses with potential for interspecies transmission.

European hedgehogs (Erinaceus europaeus) and Northern white-breasted hedgehogs (Erinaceus roumanicus) are small nocturnal, insectivorous mammals that are active from April to September and hibernates from October to March, widely spread in Europe1. They feed primarily on invertebrates such as beetles, earthworms, and molluscs but also on pet food that is frequently found in gardens and shared with dogs and cats1,2. Initially, those species lived in semi-open rural areas or forests with many trees and lush vegetation to provide hiding places and nesting material1. With increasing urbanisation, hedgehogs are becoming more synanthropic and live in the majority of European villages and urban areas nowadays. Those habitats provide more food sources, daily nest sites, and protection from badgers (Meles meles), the most dangerous natural predators for hedgehogs4. Living in parks, backyard gardens, and other urban green areas exposes them to various human-related risks, such as traffic accidents, poisoning from multiple chemicals used in urban green spaces, and attacks from stray cats.

The increasing number of hedgehogs, especially in gardens and city parks, influence animal-human contact frequency. People trying to help hedgehogs may unwittingly expose themselves to various pathogens, including zoonotic ones. Hedgehogs are an essential vector of many zoonotic pathogens, including bacterial: Salmonella sp., Mycobacterium sp., Methicillin-Resistant Staphylococcus aureus, Leptospira spp5–11 and viral ones: tick-borne encephalitis virus (TBEV); Severe fever with thrombocytopenia syndrome virus (SFTSV)12,13. The recent discovery of MERS-related CoVs in hedgehogs from Germany14, France15, the UK16, China17, and Italy2 suggests that hedgehogs may represent a wild reservoir of CoVs.

Coronaviruses are enveloped positive-sense RNA viruses. They belong to the Coronaviridae family, order Nidovirales18,19. CoVs are pathogenic for mammals and birds, in which they cause infections manifested by a range of clinical signs (from respiratory, gastrointestinal, and nervous systems) and subclinical infections1,20. Their propensity to recombine allows them to easily transmit and adapt to new hosts16,19. Four genera of CoVs have been identified to date: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus21. The Betacoronavirus genus of mammal-infecting viruses includes three subgenera (Sarbecovirus, Embecovirus, and Merbecovirus)22. The discovery of Erinaceus CoVs (EriCoVs) belonging to Betacoronavirus (subgenus Merbecovirus) in hedgehogs from various parts of the world23,14–17 may indicate their potential role as reservoirs and/or vectors of these CoVs. The main representative of Merbecovirus subgenus, MERS-CoV, has been proven to be zoonotic and pathogenic to humans23. It has been shown that merbecoviruses undergo recombination and show
positive animals concerning all tested animals. France15, Germany14, Italy2, Great Britain16, but their prevalence were generally higher than in our study (Germany and kidney). The presence of bCoVs in hedgehogs has been reported previously in other European countries: confirmed in 1 out of 18 samples of the lung. No viral RNA was identified in other samples tested (duodenum while 25% of animals tested in our study were CoVs positive (95% CI: 14.2–40.2). The CoV RNA has also been identified in hedgehogs of subgroup 1 (GenBank numbers KC545384, KC545386). They also revealed high nt sequence similarity between 98.1 and 100% and possessed the highest similarity (96.3–97.0%) with the viruses from German hedgehogs of subgroup 1 (HK679660), China (MT002834-35, MK907286-87) were 92.8–93.3% and 83.5–84.7%, respectively. Hedgehog coronavirus 1

Table 1. Detection of CoV RNA among hedgehog split into risk factors. p value determined by two-sided Fisher’s exact test; p ≤ 0.05 considered significant. OR Odds ratio, 95% CI 95% confidence interval. *Number of positive animals concerning all tested animals.

significant genetic variation.22 The epidemiological, biological, and virological characteristics of CoVs, mainly based on Spike-protein plasticity, suggest species barriers to infection may be easily crossed.24,25 Thus, CoVs identified in hedgehogs may pose a potential risk to humans, especially as there is increasing contact between hedgehogs and humans.

This study aimed to assess the presence of coronaviruses in hedgehogs in an urban area in Poland.

Results
Twenty-six juvenile (65%) and 14 (35%) adult animals were included in the study. 67.5% were females (27/40), while 32.5% were males (13/40). The mean body weight of juvenile animals was 264.42 ± 104.81 (range 83–467 g), while in the adult group, it reaches 740.07 ± 200.12 (490–1150 g). The presence of coronavirus (CoV) RNA was detected in 10 out of 40 hedgehogs’ rectal swabs (25%). Additionally, when examining the organs of animals, coronaviral RNA was detected in 1 out of 18 samples of the lung (5.55%), and it was from a hedgehog previously identified as swab-positive. No viral RNA was identified in the duodenum and kidney collected from hedgehogs. In investigated group, the CoV presence was confirmed in 25% of samples (95% CI: 14.2–40.2). Four adult and two young hedgehogs were found to be underweight; the weight of the other animals was normal. One of ten CoV RNA positive hedgehogs was diagnosed with underweight, but the other animals were in normal condition. An equal number (n = 20) of clinically healthy and sick animals were used in this study (detailed characteristics are presented in Table 2). Most animals (70%) in which the CoV was detected were clinically healthy. The remaining positive hedgehogs were diagnosed with fractures (2/3) and severe ectoparasite infestation (1/3). Eighty % of positive hedgehogs were juveniles, while only 20% were adults, noting that the number of juveniles in our study was almost double that of adults. Females represented 90% (9/10) of positive animals. There was no significant relationship between clinical status, gender, age, and detection of hedgehog coronaviruses (Table 1).

The odds ratio calculated for gender indicates a 6 times higher (95% CI 0.671–53.685) likelihood of having positive results in females compared to the males. After controlling for age, the odds of being bCoV RNA positive was 2.67 times higher in juvenile animals (95% CI 0.481–14.789).

Phylogenetic analysis. Topology of the phylogenetic tree based on the obtained sequence fragments of the RdRp gene revealed that ten of the positive hedgehogs resulted in being infected with merbecoviruses (Fig. 1A). Phylogenetic analysis showed that CoVs from Polish hedgehogs grouped together with other representatives of Hedgehog coronavirus 1 (HedCoV1), species identified in Western Europe. All 10 Polish hedgehog CoV strains were grouped within the same subgroup (Fig. 1B). The alignment of sequences of detected CoV strains showed identity between 98.1 and 100% and possessed the highest similarity (96.3–97.0%) with the viruses from German hedgehogs of subgroup 1 (GenBank numbers KC545384, KC545386). They also revealed high nt sequence similarity with Italian (95.4–96.8%) and German strains of subgroup 2 (93.5–95.0%) (MW246795-802, MT024741, and NC039207, KC545385, respectively). The nt homology of Polish hedgehog CoVs to strains sampled in Great Britain (MK679660) and China (MT002834-35, MK907286-87) were 92.8–93.3% and 83.5–84.7%, respectively.

Discussion
Hedgehogs have been indicated as a possible wild reservoir of emerging CoVs with potential public health implications. This study confirmed the presence of betacoronavirus (bCoV), subgenus Merbecovirus in hedgehogs in Poland for the first time. Our preliminary results suggest that CoVs are quite common in hedgehogs, while 32.5% were males (13/40). The mean body weight of juvenile animals was 264.42 ± 104.81 (range 83–467 g), while in the adult group, it reaches 740.07 ± 200.12 (490–1150 g). The presence of coronavirus (CoV) RNA was detected in 10 out of 40 hedgehogs’ rectal swabs (25%). Additionally, when examining the organs of animals, coronaviral RNA was detected in 1 out of 18 samples of the lung (5.55%), and it was from a hedgehog previously identified as swab-positive. No viral RNA was identified in the duodenum and kidney collected from hedgehogs. In investigated group, the CoV presence was confirmed in 25% of samples (95% CI: 14.2–40.2). Four adult and two young hedgehogs were found to be underweight; the weight of the other animals was normal. One of ten CoV RNA positive hedgehogs was diagnosed with underweight, but the other animals were in normal condition. An equal number (n = 20) of clinically healthy and sick animals were used in this study (detailed characteristics are presented in Table 2). Most animals (70%) in which the CoV was detected were clinically healthy. The remaining positive hedgehogs were diagnosed with fractures (2/3) and severe ectoparasite infestation (1/3). Eighty % of positive hedgehogs were juveniles, while only 20% were adults, noting that the number of juveniles in our study was almost double that of adults. Females represented 90% (9/10) of positive animals. There was no significant relationship between clinical status, gender, age, and detection of hedgehog coronaviruses (Table 1).

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Figure 1. Phylogenetic analysis of betacoronaviruses based on the replicase gene fragment. (A) The tree constructed for 37 betacoronaviruses: 10 strains identified in hedgehogs in Poland (marked with a dot), 27 from GenBank including 5 reference strains (written in bold and underlined) representing ratified four species of the Merbecovirus subgenus, and fifth strain representing the only species of Sarbecovirus subgenus as the outgroup. (B) Separate subtree of betacoronaviruses of the Hedgehog coronavirus 1 species. The tree was constructed using MEGA 7 using the maximum likelihood method based on the T92 + G + I model and 1000 bootstrap replicates (bootstrap values shown on the tree).
a few months. After this period, their metabolism does not immediately reach the normative one. It could be expected that viruses in such hedgehog state replicate slowly and are not shed/shed in lower quantity than in the active state. However, the relatively high CoV RNA occurrence in hedgehogs included in our research may suggest that they play an important role as a wild reservoir of CoVs.

In accordance with previous reports, we did not observe any correlation between CoVs positivity and the health condition of the animals. CoVs positive hedgehogs did not reveal the clinical signs of CoV infection. One of ten CoV RNA positive hedgehogs was diagnosed with underweight, but the other animals were in good condition.

Coronaviridae family appear to include viruses, a few of which cause disease. The number of detected microorganisms that are not correlated with any disease has increased significantly with the widespread use of molecular diagnostic tools. This also applies to coronaviruses detected in, i.e. bats or wild birds.

### Table 2. Demographic information and health status of hedgehogs included in the study.

| Delivery date | Sex | Age | Body weight (g) | Clinical condition on admission, diagnosis | CoV status | GenBank number |
|---------------|-----|-----|----------------|-------------------------------------------|------------|----------------|
| 08.2020       | F   | juv | 335            | Weak, spinal fracture, euthanasia          |            |                |
| 08.2020       | F   | juv | 309            | Weak, jaw fracture, died in the WRC        |            |                |
| 08.2020       | M   | ad  | 781            | Weakness due to infestation of external parasites | +          | MZ605015      |
| 08.2020       | F   | ad  | 490            | Abscess (lungs), mucocele, weakness, uterus with ampullary changes | -          |                |
| 08.2020       | F   | juv | 225            | Mucocele, wounds on back, died in the WRC  | -          |                |
| 08.2020       | M   | ad  | 507            | Extensive back wound, extreme cachexia, mucocele, died in the WRC | -          |                |
| 08.2020       | M   | ad  | 780            | Extreme weakness, lots of ticks and fleas | -          |                |
| 08.2020       | F   | ad  | 574            | Fracture of the spine, pelvis and femur, euthanasia | -          |                |
| 08.2020       | M   | juv | 246            | Urinary tract haemorrhage, femur fracture, euthanasia | -          |                |
| 08.2020       | F   | ad  | 1150           | Clinically healthy, pregnant female        | -          |                |
| 08.2020       | M   | juv | 400            | Clinically healthy                         | -          |                |
| 08.2020       | M   | juv | 384            | Clinically healthy                         | -          |                |
| 08.2020       | M   | juv | 150            | Clinically healthy                         | -          |                |
| 08.2020       | F   | juv | 135            | Clinically healthy                         | -          |                |
| 08.2020       | M   | juv | 148            | Clinically healthy                         | -          |                |
| 08.2020       | M   | ad  | 540            | Extremely weak, died in the WRC            | -          |                |
| 09.2020       | F   | ad  | 777            | Spinal fracture, skinned left pelvic limb, euthanasia | +          | MZ605016      |
| 09.2020       | F   | juv | 136            | Dyspnoea                                   | -          |                |
| 09.2020       | F   | juv | 160            | Clinically healthy                         | -          |                |
| 09.2020       | F   | ad  | 1010           | Weak, died up to 24 h after admission; hepato- and splenomegaly | -          |                |
| 09.2020       | F   | juv | 289            | Clinically healthy                         | +          | MZ605017      |
| 09.2020       | F   | juv | 354            | Clinically healthy                         | -          |                |
| 09.2020       | F   | juv | 419            | Clinically healthy                         | +          | MZ605018      |
| 09.2020       | F   | juv | 292            | Clinically healthy                         | +          | MZ605019      |
| 09.2020       | F   | juv | 83             | Clinically healthy                         | +          | MZ605024      |
| 10.2020       | F   | juv | 197            | Clinically healthy                         | -          |                |
| 10.2020       | F   | juv | 103            | Clinically healthy                         | +          | MZ605020      |
| 10.2020       | F   | juv | 211            | Clinically healthy                         | -          |                |
| 10.2020       | F   | juv | 322            | Clinically healthy                         | -          |                |
| 10.2020       | F   | juv | 249            | Clinically healthy                         | +          | MZ605021      |
| 10.2020       | F   | ad  | 708            | Clinically healthy, slightly weakened      | -          |                |
| 10.2020       | F   | ad  | 817            | Weak                                       | -          |                |
| 10.2020       | F   | juv | 292            | Clinically healthy                         | +          | MZ605022      |
| 10.2020       | F   | juv | 277            | Weak, fracture of right femur, died after admission | +          | MZ605023      |
| 10.2020       | F   | juv | 302            | Weak, spinal fracture, euthanasia           | -          |                |
| 10.2020       | F   | ad  | 897            | Found dead                                 | -          |                |
| 10.2020       | M   | juv | 467            | Clinically healthy                         | -          |                |
| 10.2020       | M   | ad  | 510            | Dehydrated, diarrhoea, mucocele, severe supplicative arthritis | -          |                |
| 11.2020       | M   | juv | 390            | Trauma of the left pelvic limb, necrosis of the distal part of the limb | -          |                |
| 12.2020       | F   | ad  | 820            | Found dead                                 | -          |                |
hand, they could be opportunistic pathogens, the virulence of which is revealed under unfavourable conditions or in co-infections with other pathogens. In the present study, there was also no significant relationship between gender and age and detection of hedgehog CoVs, but juveniles and females had a higher likelihood of having positive results compared to the adult and males. It could be related to obtaining a more stronger amplification of CoV RNA observed in juvenile and lactating female bats of the species Myotis sp. Since hedgehogs are susceptible to all infections and only develop immunity over the years due to contact with different infectious agents. The CoVs detected in hedgehogs in our study were highly homologous to each other and similar to others from Europe in the compared fragment of the genome (93.5 to 97.0% homology). They differed most from the CoVs detected in Erinaceus amurensis in China (83.5 to 84.7% homology). It is unknown if these differences in the studied genome fragment sequence result from geographical segregation or reflect “species-dependent” grouping. However, it should be noted that in our study, hedgehogs came from a relatively small area. On the other hand, when the same genome fragment was compared, a “species-dependent” grouping of gamma- and delta-CoVs is observed30. Other reasons (spine, bone fractures) during their stay at WRC, were collected and stored at − 80 °C until analyses. The vaccine IBV 4/91 strain (Nobilis IB 4/91, MSD Animal Health, France) and stored at − 80 °C until laboratory examinations. In addition, samples of duodenum, lung, and kidney from 18 hedgehogs that were dead at admission died or were euthanised (with the use of xylazine and ketamine administered intramuscularly followed by intravenous pentobarbital administration) for ethical reasons (spine, bone fractures) during their stay at WRC, were collected and stored at − 80 °C until analyses. The following information for each hedgehog was recorded: delivery date, gender, body weight, age, health status. Detailed characteristic of animals used in the study is presented in Table 2.

Materials and methods

Sample collection. Since hedgehogs are protected by Polish law (Regulation of the Minister of the Environment of 16 December 2016, on the protection of animal species (Journal of Laws, item 2183) and (Journal of Laws 2020, item 26), all procedures were approved and carried out in accordance with the appropriate regulations and permits (Regional Directorate for Environmental Protection in Poznan (Poland): WPN-IL6401.366.2020.TE). We followed the ARRIVE guidelines during the study.

Rectal swabs were collected from 40 hedgehogs brought to the Wildlife Rehabilitation Centre (WRC) in Poznan after being found sick, injured, or too young to survive on their own for various diagnostic purposes. All hedgehogs were found on the urban area of the city of Poznan (16° 55′ E; 52° 25′ N), Wielkopolskie Voivodeship, Poland. When admitted to the WRC, hedgehogs were kept in isolation until sample collection (within 12 h of admission) to reduce the possibility of nosocomial infections. Between August 2020 and December 2020, the 40 individual rectal swabs were collected using swabs with transport medium (UTM: Viral Transport, COPAN Diagnostics, USA) and stored at − 80 °C until laboratory examinations. In addition, samples of duodenum, lung, and kidney from 18 hedgehogs that were dead at admission died or were euthanised (with the use of xylazine and ketamine administered intramuscularly followed by intravenous pentobarbital administration) for ethical reasons (spine, bone fractures) during their stay at WRC, were collected and stored at − 80 °C until analyses. The following information for each hedgehog was recorded: delivery date, gender, body weight, age, health status. Detailed characteristic of animals used in the study is presented in Table 2.

Molecular detection of coronavirus RNA. Hedgehogs’ tissues were homogenised in a sterile phosphate-buffered saline (Biomed, Lublin, Poland), obtained suspensions (10% w/v) were clarified by centrifugation (15 min at 3000g and 4 °C) and the supernatant used for RNA isolation. All swab transport media were also centrifuged before the process of RNA isolation. The RNA from 200 ul of the obtained fluids was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The samples were tested for CoV presence using an RT-PCR assay in a nested configuration using primers with a few degenerate nucleotides9. The assay amplifies a 555-nucleotide gene fragment of the non-structural protein 12 RNA dependent RNA polymerase (nsp12 RdRp), one of the components of viral replicate machinery present in all coronaviruses of mammalian and avian origin. All reactions were performed in a final volume of 25 µl. For RNA transcription and in the first step of DNA amplification, the One-Step RT-PCR kit (Qiagen, Hilden, Germany) was used. The Platinum Taq DNA Polymerase kit (Invitrogen, Carlsbad, USA) and the PCR product in dilution of 1:5 (v/v) were used in the second step. The vaccine IBV 4/91 strain (Nobilis IB 4/91, MSD Animal Health, USA) served as a positive control. The PCR-positive samples were identified using agarose gel electrophoresis.
Obtained amplicons were sequenced in both directions using Sanger sequencing technology by Genomed Sp. z o.o. (Warsaw, Poland).

**Sequence and phylogenetic analysis.** The forward and reverse nucleotide sequences were edited and aligned in the final consensus using Geneious v11.1.3 (Biomatters, Ltd., Auckland, New Zealand). They were then compared with sequences published in the GenBank database using the Basic Local Alignment Search Tool (BLAST) with the default parameters. Sequences with the highest homology were downloaded for further analysis. These sequences, together with 5 sequences of reference strains including 4 representing individual species from Merbecovirus subgenus (HedCoV1, Middle East respiratory syndrome-related coronavirus, Pipistrellus bat coronavirus HKU, Tylonycteris bat coronavirus HKU4), and the only one representing Sarbecovirus subgenus of the Betacoronavirus genus were then aligned using the MAFFT method in Geneious. The alignments were then exported to MEGA software, v7.0.2634. A maximum likelihood (ML) phylogenetic analysis was conducted using the best-fitting nucleotide substitution models. A bootstrap test including 1,000 replicates was performed for each resultant tree.

**Statistical analyses.** The associations between CoV RNA detection in samples, demographic features (species, gender, age), and health status variables were estimated using Fisher's exact test. The Wilson method for small n was used to calculate a 95% confidence interval (95% CI) for CoV RNA prevalence. All statistical analyses were performed in Statistica13.3 software (Tibco, USA).

**Ethics declarations.** All procedures were approved and carried out in accordance with the appropriate regulations and permits (Regional Directorate for Environmental Protection in Poznan (Poland): WPN-II.6401.366.2020.TE) and carried out according to the guidelines of the European Council Directive 86/609/EEC dated November 1986. The reported study complies with the ARRIVE guidelines.

**Data availability**
The viral sequences obtained in this study were deposited at NCBI Genbank, accession numbers: MZ605015-MZ605024. Other relevant data analysed during this study are included in this manuscript.

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