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Urban Pigeons as Reservoirs of Critical Pathogens: Improved protocol for sequencing pigeon faeces in disease monitoring

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Abstract

The spread of pathogens by animals is a serious issue around the world that causes a severe threat to human health. Feral pigeons (*Columba livia forma domestica*) that live in urban areas are zoonotic carriers of various pathogens that can be transmitted to humans by faecal contamination. This study aimed to detect the presence of bacterial, viral, and specifically fungal pathogens in pigeon faeces based on the World Health Organization's priority pathogen list published in 2022. Fresh faecal samples were collected at Uppsala, Central Station, and Svandammen, the pigeons' most relevant gathering spots and feeding sites. Genomic DNA was directly extracted from these samples, and ScilifeLab performed High throughput sequencing through Oxford Nanopore Technologies (PromethION). Metagenomic analysis revealed that most of the critically prioritized viral and bacterial pathogens listed by World Health Organization were present in pigeon faeces. Regarding fungal pathogens, which were the main objective of this study, samples from both studied locations contained all critical, high and medium-important fungal pathogens published in the World Health Organization list, such as *Aspergillus fumigatus*, *Candida albicans*, *Candida auris*, *Cryptococcus neoformans*, *Nakaseomyces glabratus*, *Candida tropicalis*, and *Cryptococcus gattii*. These fungal pathogens pose the risk of invasive fungal diseases and severe infections in low-immunity individuals and vulnerable populations. The findings indicate the importance of conducting further research to comprehensively understand potential exposure to feral pigeons. Furthermore, keeping pigeons away from sensitive areas, such as hospitals, and implementing measures to control pigeon populations can significantly decrease the spread of pathogens.

Introduction

Microorganic spread is a public health issue as some microorganisms are health-hazardous to humans. Microorganisms transmitted by wild animals, *i.e.*, zoonotic spread, have caused pandemics such as the plague, where fleas associated with rodents spread bacteria, and the coronavirus pandemic that is assumed to have been spread by bats (Piret and Boivin, 2021). Zoonotic spread by birds is especially relevant as they can transport microorganisms between continents (Moschetti *et al.*, 2017; Briscoe *et al.*, 2021). Birds can live close to humans in cities, which can risk spreading pathogens through their littering (Glushakova *et al.*, 2021).

The feral pigeon (*Columba livia forma domestica*) is a bird species living close to humans and is globally common in urban areas (Haag-Wackernagel and Moch, 2004; Magnino *et al.*, 2009; de Vasconcellos *et al.*, 2022). In cities, feral pigeons have plenty of food, can build nests, have shelter and natural predators are absent. The pigeon is adapted to indoor nesting and can live in large colonies which can accumulate a lot of faeces (Moschetti *et al.*,2017; de Vasconcellos *et al.*, 2022). In Finland, it is reported that feral pigeon populations increase with human density, which is thought to be due to pigeons being omnivores (Jokimäki and Suhonen, 1998).

Pigeons in cities have a high pathogenic diversity. Transmission of microorganisms to birds as vectors is probable to occur through the environment and the food (Moschetti *et al.*, 2017). Feral pigeons can ingest trash, feed on landfills, and get fed by humans which could result in the spread of fungi, viruses, and bacteria, including antibiotic- and multidrug-resistant bacteria. Thus, human activities can promote the spread (Phan *et al.*, 2013; Moschetti *et al.*, 2017; Freire *et al.*, 2022). Pathogens can also colonize urban pigeons when drinking

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contaminated water *e.g.*, with human faeces (Freire *et al.*, 2022). This was reported in a Brazilian study where human multi-drug resistant diarrheagenic *Escherichia coli* strains were identified as present in urban pigeons' cloaca (Borges *et al.*, 2017). Several different bacterial, viral, and pathogenic fungal species have been reported present in pigeon faeces (Catroxo *et al.*, 2011; Phan *et al.*, 2013; Medina *et al.*, 2017; Naz *et al.*, 2017; de Vasconcellos *et al.*, 2022; Kowalczyk and Wójcik-Fatla, 2022; Nualmalang *et al.*, 2023). This indicates that urban pigeons have the potential to transmit pathogens to the environment (Borges *et al.*, 2017).

Pigeons have, for a long time, been considered as pathogenic transmitters to humans. Haag-Wackernagel and Moch (2004) report almost 200 published papers between 1941-2003, which confirm cases of feral pigeon-human transmission of pathogens that resulted in disease. However, all cases are probably not documented. Pathogenic transmission to humans is common through inhalation (Haag-Wackernagel and Moch, 2004). Contaminated dust particles can get airborne and inhaled when pigeons beat their wings (Magnino *et al.*, 2009). Thus, there is a potential health hazard if pigeon nests are close to ventilation systems (Medina *et al.*, 2017). For instance, may the pathogenic fungi *Aspergillus fumigatus*, *Cryptococcus gattii*, *Mucorales*, *Cryptococcus neoformans*, and *Histoplasma spp.* be transmitted through inhalation of spores (World Health Organization, 2022). Airborne fungi spores have, for instance, been detected in urban pigeon-feeding areas in Karachi (Pakistan) which could result in these areas being health hazardous for humans (Naz *et al.*, 2017). In Baltimore (US), potentially viable spores of *Enterocytozoon bieneusi* were detected in both air and water samples. Therefore, it can also be a contamination risk after rainfall in water run-off systems (Graczyk *et al.*, 2007).

The fact that pigeons usually live near humans makes zoonotic disease transmission possible (Magnino *et al.*, 2009). It can result in unintentional and intentional encounters such as feeding pigeons or passing pigeon-dense areas (Haag-Wackernagel and Moch, 2004). Avoiding exposure to feral pigeons can be challenging as they can be close to hospitals and community transport systems (de Vasconcellos *et al.*, 2022). There is a potential health risk when pigeons live near hospitals, while immunocompromised people can be exposed to pigeon-spread pathogens. A study by de Vasconcellos and colleagues (2022) found pathogenic *Staphylococcus aureus* and *Enterococcus faecium* in pigeon faeces close to hospitals in Brazil. Moreover, transmission of pathogens to humans might occur when handling sick or dead pigeons (Magnino *et al.*, 2009).

Fungal pathogens may be an underestimated human health hazard (World Health Organization, 2022) since the risk of fungal infections is generally low. However, the risk of fungal infections is 1000 times higher for immunocompromised persons (Haag-Wackernagel and Moch, 2004). Further, fungi have the potential to cause even greater threats than viruses and bacteria since there are no vaccines and limited availability of antifungal medicine. In addition, there is an increase in invasive fungal diseases and fungal resistance is appearing. Invasive fungal diseases are mainly a threat to immunocompromised populations but can become a global health concern. For instance, comorbid invasive fungal infections with aspergillosis, mucormycosis, and candidaemia increased during the coronavirus pandemic, and infection range and dispersal of fungi could be increased with the climate change (Nnadi and Carter, 2021; World Health Organization, 2022). In 2022, World Health Organization (WHO) presented the first systematic global prioritization of 19 health-hazardous fungi to guide and encourage research and public health action. In that list, the fungi are ranked as critical, high, and medium risk.

To summarise, feral pigeons are a source of pathogenic spread to humans, which can cause disease and possibly even epidemic outbreaks (Haag-Wackernagel and Moch, 2004; Piret and Boivin, 2021). Thus, it is essential to monitor health-hazardous microorganisms to avoid outbreaks. Risk factors for pathogenic spread are increased human-animal interaction, urbanization, and globalization (Piret and Boivin, 2021). More specifically, fungal pathogens need to be more recognized (World Health Organization, 2022). Therefore, this study aimed to explore the prevalence of bacterial, viral, and especially fungal pathogens registered in the WHO's priority pathogen list (2022), in pigeon faeces in Uppsala.

Materials and methods

Sample collection

Four fresh stool samples from free-living feral pigeons were collected from two different urban locations of Uppsala, Central Station, and Svandammen, known to be the most relevant gathering spots and feeding sites for urban pigeons, respectively (Figure 1). Samplings were carried out in March 2023.

DNA extraction

Since DNA extraction from faecal samples were challenging, a combination of kit-based (specifically, the ZYMO RESEARCH Quick-DNA Faecal Microbe MiniPrep Kit) and non-kit-based protocols was employed to remove residual contaminations, proteins, and salts from the DNA samples, enhancing DNA concentration and minimizing fragmentation. DNA was extracted from 150 mg of faecal sample as recommended by the kit manufacturer. The extracted DNAs were eluted in 50 ul of elution buffer and assessed using Nanodrop, Qubit, and Tapestaion before being stored at -20°C. This resulted in four samples that were good enough for sequencing (Table 1).

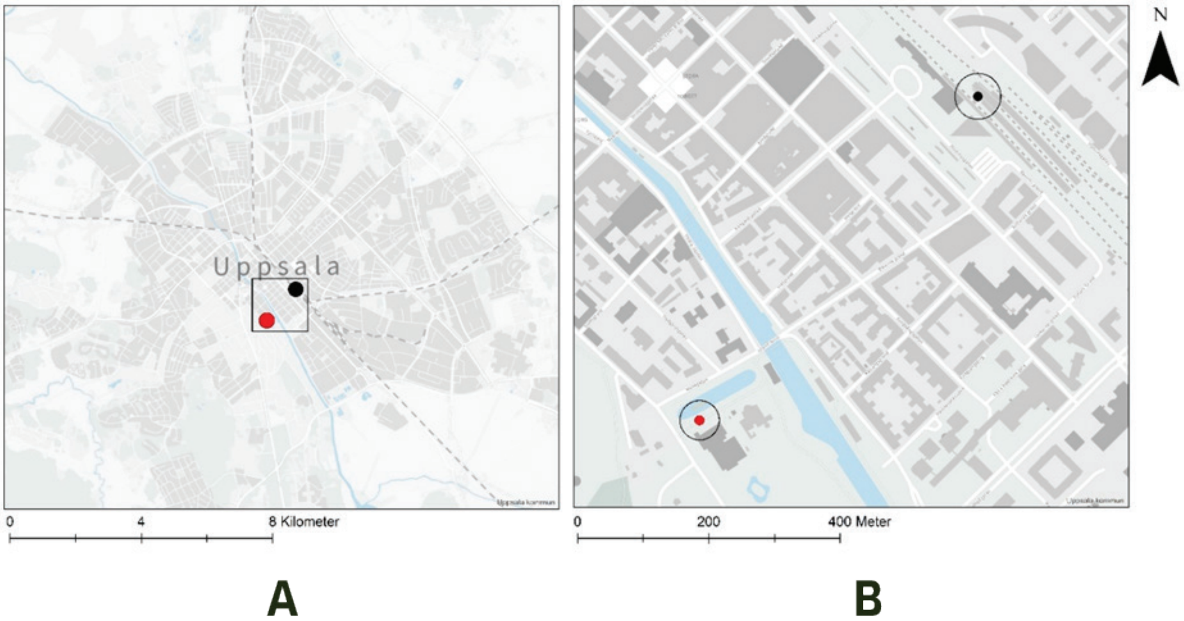

Figure 1. Collection sites of faecal samples, the train station (black bullet), and Svandammen (red bullet). In **A**, sites are presented in Uppsala where the square indicates **B**. In **B**, approximative collection ranges at each site are marked as circles. The figures are composed in ArcMap, the background map is from Uppsala Municipality. Each sample was collected using a wooden stick, placed into a 15ml falcon tube, and directly transported to the laboratory. Great care was taken to avoid the part of the droplet that had touched the ground.

Table 1. Sample ID, their location, the BioSample and Experiment Accession numbers.

Sample ID	Location	BioSample Accession	Experiment Accession
Sample1	Central station	SAMEA115741869	ERX12635354
Sample2	Central station	SAMEA115741870	ERX12635601
Sample3	Svandammen	SAMEA115741871	ERX12635579
Sample4	Svandammen	SAMEA115741872	ERX12635531

Sequencing

High throughput sequencing was performed by ScilifeLab through the Oxford Nanopore Technologies (ONT) method (PromethION) using SQK-NBD114-24 protocol. Reads were Quality controlled with third-party software pycoQC. The trimming of the adapter after sequencing was also done by the ScilifeLab.

High-Throughput Sequencing Analysis

Raw sequenced reads were quality-controlled with fastQC.Taxonomic classification of metagenomic reads at the species level was performed by aligning metagenomic reads against the “nr” database updated on 10-Mar-2022. This database is one of the databases provided by Kaiju[1] and contains all the sequences from the NCBI BLAST nr database belonging to Archaea, bacteria and viruses. For fungi detection, metagenome reads were aligned against the Fungi Database provided by Kaiju and updated on 29-Mar-2022 This database contains all the fungi sequences coming from the NBCI Refseq database.

Krona[2] was used to enhance the interpretability of the complex taxonomic data generated by Kaiju, global results of fungi, bacteria and viruses are presented in (Figure 2) (see all Krona results and the Kaiju read count for the 4 samples using NR and Fungi databases in Supplementary files[3] 1 to 40).

All the tools, their version and the parameters used are available in Supplementary file[3] 41.

Results

1. Detection of Viral Pathogens

Analysis showed that specific viruses that pose significant public health risks were discovered in both Uppsala Central Station and Svandammen. These viruses have

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[1] https://github.com/bioinformatics-centre/kaiju

[2] https://docs.csc.fi/apps/krona/

[3] http://journal.embnet.org/index.php/embnetjournal/article/downloadSuppFile/1059/1059_supp_1

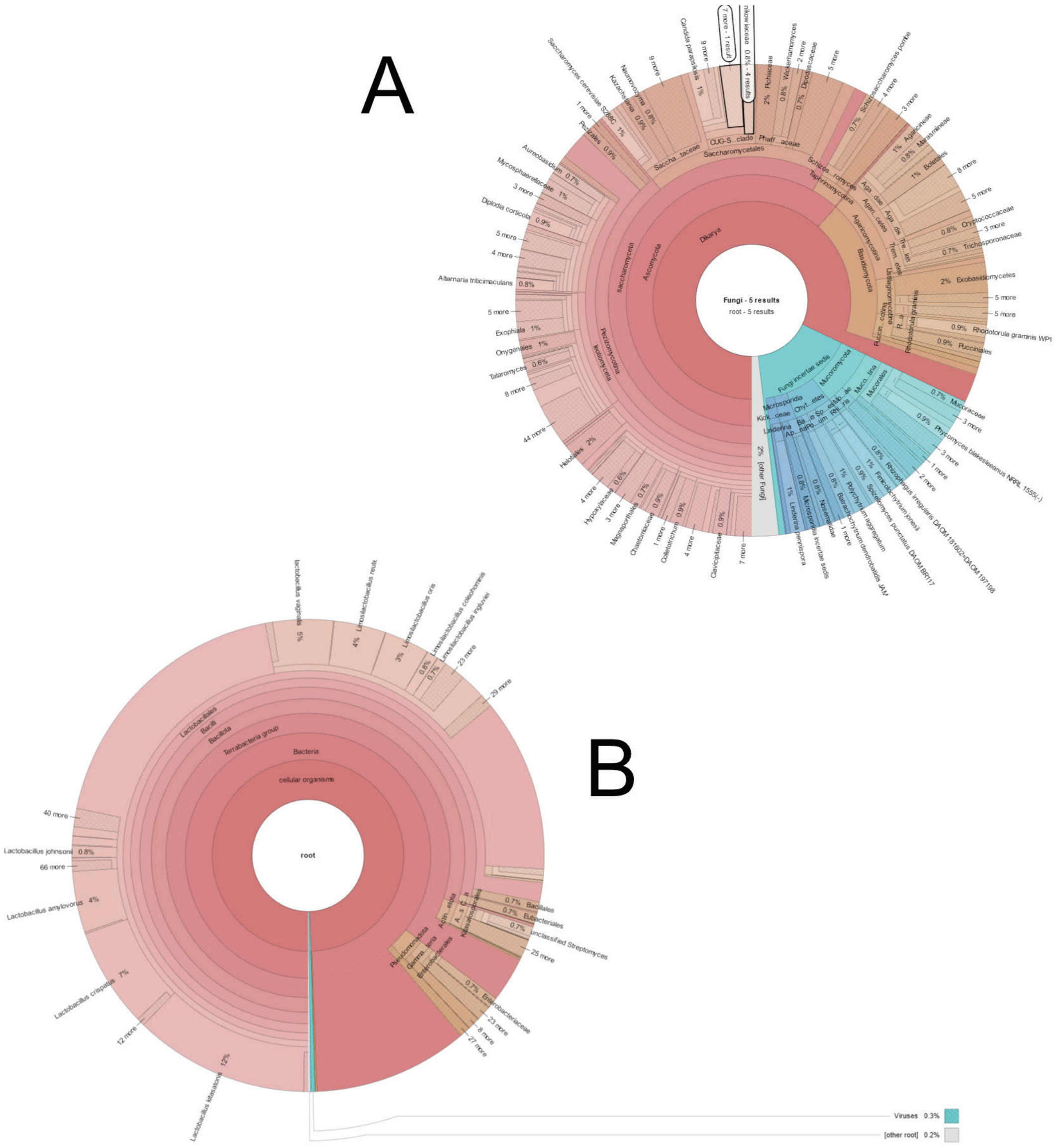

Figure 2. Krona representation of the global results using the Fungi (**A**) and NCBI nr (**B**) databases.

the potential to cause the diseases listed as priorities by the WHO because of their epidemic potential.

Identified Viral Pathogens

Several viruses were detected in both locations (Figure 3). All the samples from both locations contained the following viruses:

- *Crimean-Congo hemorrhagic fever virus* (CCHFV), responsible for causing Crimean-Congo hemorrhagic fever.
- *Lassa mammarenavirus*, which leads to Lassa fever.
- *SARS coronavirus* (SARS-CoV), causing Severe Acute Respiratory Syndrome (SARS) respiratory illness.
- *Monkeypox virus* (MPX), the causative agent of Monkeypox disease.
- *Alpha coronavirus*, associated with respiratory infections.
- *Beta coronavirus*, linked to respiratory infections.

2. Detection of Bacterial Pathogens

As bacteria develop resistance to antibiotics, they pose challenges in treating infections and increase the potential for diseases to spread. To address this, WHO has published a list of bacteria with critical, high and medium priorities based on the need for developing new antibiotics. The critical priority is linked to specifically threatening diseases within hospitals, while the high and medium priorities are associated with more common and severe illnesses.

Based on the Metagenomics analysis results, most of the bacteria mentioned in the WHO priority list were detected in fecal samples in both locations.

Identified Bacterial Pathogens

In the critical priority category, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and various enterobacteriaceae, including *Klebsiella*, *E. coli*, *Serratia*, and *Proteus*, were identified in all samples of both

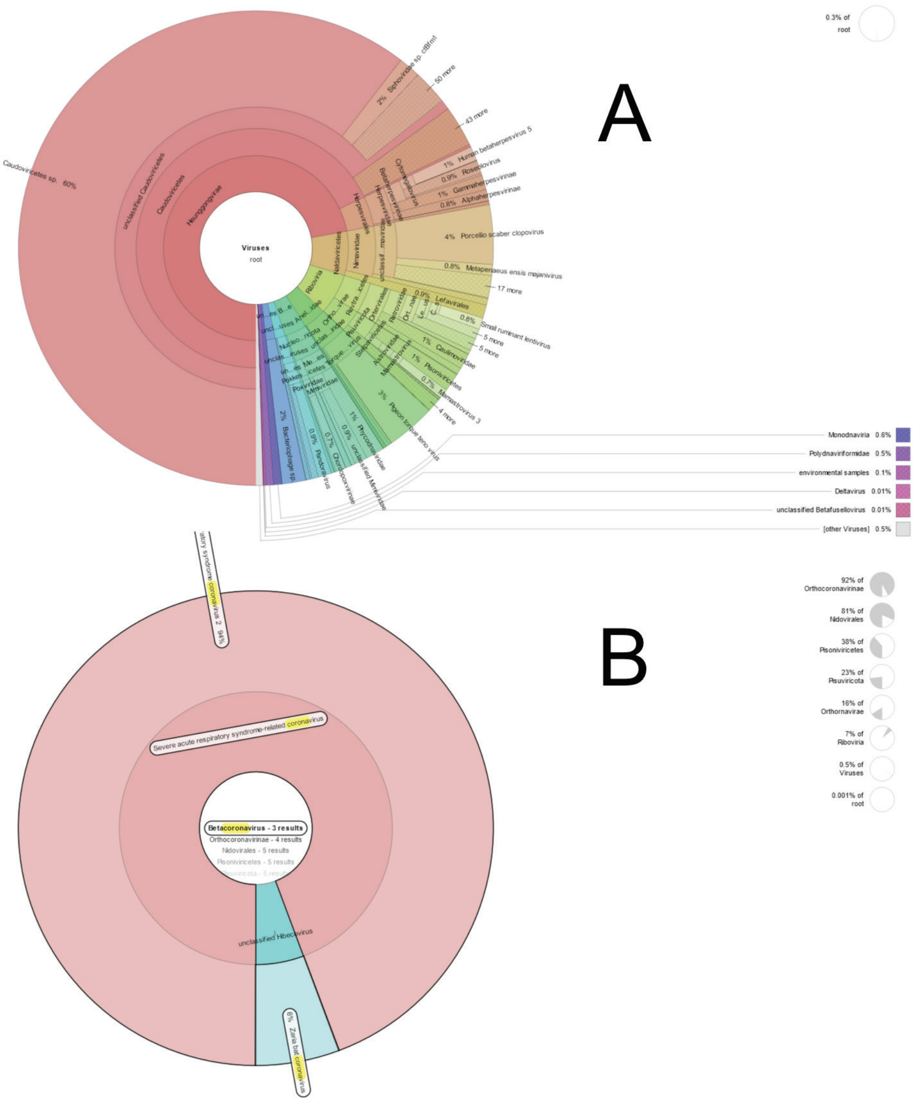

Figure 3. Krona representation of the Viral hit found in Kaiju with nr database. **A** represent all viral hits and **B** a magnification of the krona result to SARS-CoV.

locations. These bacteria are carbapenem-resistant and can lead to severe and potentially fatal infections.

Within the high-priority category, we observed the presence of *Enterococcus faecium* (vancomycin-resistant), *Staphylococcus aureus* (methicillin-resistant, vancomycin-intermediate resistant), and *Campylobacter spp.* (fluoroquinolone-resistant) in all samples.

Based on the third priority category, all samples contained *Streptococcus pneumoniae* and *Shigella spp.* These bacteria are penicillin-non-susceptible and fluoroquinolone-resistant respectively.

In addition to the priority list, our samples also included *Clostridium difficile* and *Mycobacterium tuberculosis*. These bacteria, as indicated by the WHO, have demonstrated a growing resistance to traditional treatments in recent years. *Clostridioides difficile* is acknowledged as an emerging pathogen with the potential to cause zoonotic diseases in humans (Mitchell *et al.*, 2022).

3. Detection of Fungal Pathogens

This study focused on the detection of fungal pathogens in the collected samples because the latest report from the WHO (2022) shows a high increase in cases of invasive fungal diseases (IFDs) and countries are advised to enhance their capacity to manage fungal infections.

WHO has classified fungal pathogens into three priority groups — critical, high, and medium — based on drug resistance and challenges in treatment and management.

All fungi mentioned in the critical priority category, including *Cryptococcus neoformans*, *Candida auris*, *Aspergillus fumigatus*, and *Candida albicans*, were

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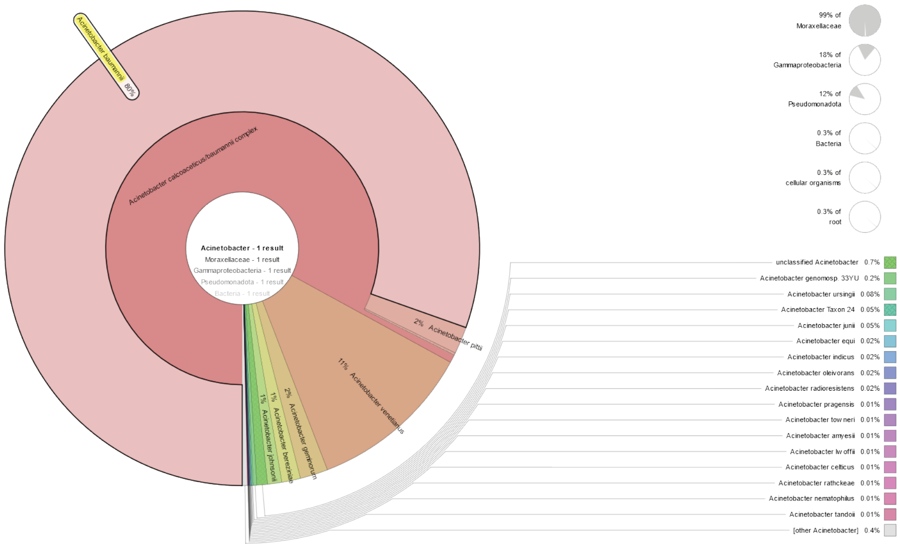

Figure 4. Krona representation of Acinetobacter baumannii. Other results are in Supplementary files[4].

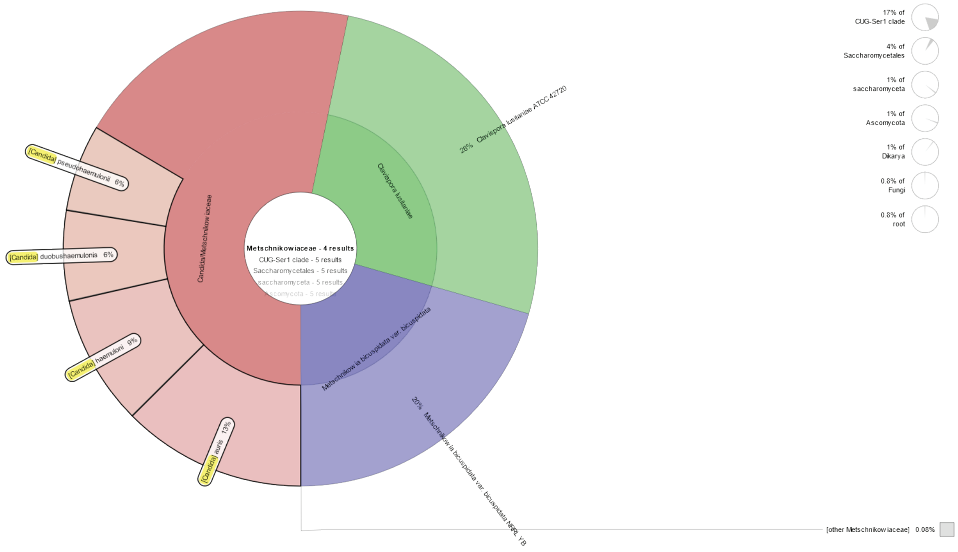

Figure 5. Krona representation of Candida auris.

present in samples from Uppsala Central Station and Svandammen.

Among seven fungal pathogens in the second priority group, six fungi, including *Nakaseomyces glabrata* (*Candida glabrata*), *Histoplasma spp.*, *Mucorales*, *Fusarium spp.*, *Candida tropicalis*, and *Candida parapsilosis*, were found in all samples.

From pathogens in the medium group, our samples contained nearly all fungi, including *Scedosporium spp.*, *Coccidioides spp.*, *Pichia kudriavzeveii* (*Candida krusei*), *Cryptococcus gattii*, *Talaromyces marneffei*, *Pneumocystis jirovecii* and *Paracoccidioides spp.*

Discussion

This study's extensive metagenomic analysis underscores the significant role that urban pigeons play in the epidemiology of critical pathogens. This includes the identification of viral, bacterial, and particularly fungal organisms, which have notable impact on public health, especially for immunocompromised populations. Our findings, which detail the detection of various pathogens in pigeon feces collected from Uppsala Central Station and Svandammen, reveal the diversity of the microbial

[4] http://journal.embnet.org/index.php/embnetjournal/article/downloadSuppFile/1059/1059_supp_1

community that urban pigeons can potentially harbor. This diversity is consistent with the WHO's priority pathogen list and suggests that pigeons could pose a risk as carriers of infectious diseases in areas where interactions between humans and pigeons are frequent. The identification of fungal pathogens such as *Aspergillus fumigatus*, *Candida auris* and *Cryptococcus neoformans* in all fecal samples from pigeons of both locations indicates an underestimated risk for public health. These fungi are known for their ability to cause invasive diseases, particularly in low immunity individuals, and could lead to outbreaks if not adequately monitored.

Similarly, the presence of bacterial pathogens, including those resistant to antibiotics, adds more concerns. The detection of carbapenem-resistant *Acinetobacter baumannii* among other bacterial species could indicate the threat of spreading antibiotic resistance which can significantly complicate both the treatment of the diseases caused by environmental exposure, and managing it in high populated areas.

Furthermore, presence of viral pathogens with outbreak potential, brings additional public health risk. The presence of viruses such as the *SARS coronavirus* and *Monkeypox virus* in pigeon feces suggests that pigeons could serve as carriers for viral diseases. This shows the requirement for more research on viral pathogen in urban regions.

The coexistence of these pathogens in urban pigeons faeces reveals the need for a multidimensional strategy. This strategy should aim not only to reduce human-pigeon interactions and manage pigeon populations in sensitive areas but also to increase monitoring of the presence of these pathogens in urban regions. Moreover, raising public awareness is important to inform urban residents about the potential health risks associated with pigeons and to reduce exposure to pathogens.

It is not entirely possible to discount the likelihood that some of the identified organisms might have originated from the soil surrounding the fecal samples. However, our testing approach aligns with standard procedures employed in collecting feral feces from various species. Therefore, we assert that our results are indicative of the microbial content typically present in feral pigeon feces.

Key Points

- Feral pigeons in urban areas can spread harmful pathogens to humans, primarily through fecal contamination.
- This study analyzed pigeon feces from Uppsala's central gathering spots to detect bacterial, viral, and fungal pathogens.
- Metagenomics analysis confirmed the presence of most high-priority viral and bacterial pathogens listed by the World Health Organization (WHO).
- All critical fungal pathogens identified by WHO, including *_Aspergillus_* and *_Candida_* species, were found in the samples, posing a risk to individuals with weakened immune system.
- Controlling pigeon populations and keeping them away from sensitive areas, like hospitals, could reduce the spread of these pathogens.

This study lays the foundation for future explorations into the transmission mechanisms and the formulation of strategies to hinder the spread of infections from pigeons to humans. These efforts are crucial in urban planning and public health policymaking, aimed at safeguarding community health, particularly in regions where human-pigeon interactions are commonplace.

Ethical consideration

Pigeons were not disturbed, captured, or harmed and fecal samples were collected after excretion. Therefore, ethical approval was not required.

Acknowledgments

We would like to express our deepest appreciation to the "Oxford Nanopore & NGI/SciLifeLab Project Competition" at the Long Read Conference in Uppsala (LRUA2022) for selecting us as one of the two winners, allowing us to have our samples sequenced for free at SciLife Lab. We also extend our sincere gratitude to the SLU Bioinformatics Infrastructure (SLUBI) for their invaluable support in the analysis of the data.

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