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Genomic Investigation of Bacterial Co-Infection in Southern Pudu (*Pudu puda*) with Fatal Outcome: Application of Forensic Microbiology in Wildlife Impacted by Anthropogenic Disasters

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Simple Summary

The southern pudu is a vulnerable deer species impacted by anthropogenic threats and infectious pathogens. In this context, we investigated bacterial infections affecting a southern pudu admitted to a wildlife rehabilitation center after suffering dog bites and limb burns caused by wildfires. Utilizing genomic tools, we characterized a triple bacterial infection comprising *Escherichia coli* ST224, *Klebsiella oxytoca* ST145, and *Acinetobacter baumannii* ST1365. Due to its broad resistome and virulome, a clone of *E. coli* ST224 progressed from a soft tissue infection to fatal sepsis. This work highlights the significant utility and accuracy of genomic research and forensic microbiology in understanding the infectious disease process that threatens wild animals.

Abstract

The southern pudu (*Pudu puda*) faces significant threats from anthropogenic activities and infectious diseases. Using whole-genome sequencing (WGS) and forensic microbiology research, we describe a triple bacterial co-infection in a southern pudu impacted by wildfire disasters. The deer presented infected burn wounds on the extremities and dog bite wounds in the lumbosacral region, from which a multidrug-resistant CTX-M-1-producing *Escherichia coli* sequence type (ST) ST224 and a *Klebsiella oxytoca* ST145 were isolated, respectively. The patient died 13 days after admission in a Wildlife Rehabilitation Center. During the necropsy, a sample from intracardiac blood was collected, and WGS analyses confirmed systemic dissemination of *E. coli* ST224 clone. The broad virulome (adhesins, invasins, toxins, and immune evasion genes) and resistome against beta-lactams (*bla*$_{\text{CTX-M-1}}$), aminoglycosides [*aac(3)-IId*, *aph(3')-Ia*, *aph(3'')-Ib*, *aph(6)-Id*], macrolides [*mph(A)*], sulfonamides (*sul2*), trimethoprim (*dfrA17*) and fluoroquinolones (*gyrA* and *parC* mutations) of *E. coli* ST224, contributed to treatment failure and death of the wild animal. Additionally, an oval nodule was identified in the abdominal cavity caused by *Acinetobacter baumannii* ST1365, the first WGS-confirmed report in wildlife. This study highlights the value of applying forensic microbiology and WGS to investigate and understand One Health pathogens threatening wildlife impacted by natural and anthropogenic disasters.

Keywords: ESBL; wildlife conservation; *Escherichia coli* ST224

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1. Introduction

Rehabilitating wildlife species and understanding the effects of pathogens and infectious diseases are crucial aspects of veterinary medicine. These efforts improve the health and survival of wildlife and safeguard human populations from zoonotic threats [1]. Infectious diseases pose a significant threat to the conservation of wild species, such as the southern pudu (*Pudu puda*), one of the smallest deer species in the world. Their conservation status is vulnerable, with declining populations mainly due to anthropogenic effects, including roadkill, forest fires, and free-roaming dog attacks [2]. There is molecular and serological evidence of the presence of potential pathogens in the southern pudu [3–6]. Therefore, the only documented case linking specific infectious agents to disease in this species is the association of mycotic pneumonia caused by Aspergillus fumigatus or Mucor spp. and encephalitis caused by *Curvularia spicifera* in zoo-captive southern pudus [7].

Wildlife microbiology forensic science is currently an underexplored field; however, it can help to investigate the causes of unusual wildlife mortality or death in individuals in captivity in rehabilitation centers [8]. The whole genome sequencing (WGS) is a valuable and versatile tool, and its use in clinical settings has been proposed to investigate the genomics of infectious diseases [9]. Its application in the forensic field is noteworthy, as it allows genomic epidemiology and microbial genomics, identifying the origin and potential spread of infectious pathogens [10].

This study aims to analyze the genomic characteristics of infection-causing bacterial pathogens and investigate the cause of death of a specimen of *P. puda*, admitted to a Wildlife Rehabilitation Center in Chile.

2. Materials and Methods

2.1. Case Background and Sample Collection

In 2023, during the forest fires in the south-central zone of Chile, the Wildlife Rehabilitation Center at the University of Concepcion received an adult male southern pudu from Florida, Biobio region, Chile. The patient had wounds in the lumbosacral area attributed to dog bites and burned right hind limb, both of which showed signs of infection. The deer received empiric fluoroquinolone (i.e., enrofloxacin, 5 mg/kg, IM, q. 24 h), with unsuccessful results. Swabs from both injuries were taken for microbiological culture. The patient passed away thirteen days after being admitted. At necropsy, an intracardiac blood sample was collected. Additionally, an oval nodule measuring 70 x 100 mm was identified in the abdominal cavity. It had a smooth external surface, firm consistency, and calcified yellowish-white content, which was also sampled (Figure S1).

2.2. Isolation and Bacterial Identification

For samples from wounds and the internal abdominal nodule, microbiological cultures were performed using brain-heart agar, blood agar, and MacConkey agar, and incubated for 24 hours at 37°C. For an intracardiac blood sample, the culture was initially grown in brain-heart infusion broth for 24 hours at 37°C, and subsequently transferred to brain-heart agar, blood agar, and MacConkey agar for an additional 24-hour incubation at 37°C. Preliminary bacterial identification was performed through API® 20E™ or API® 20NE™ systems (BioMérieux, France). For the susceptibility testing, the selection of antibiotics varied according to the analyzed bacteria, including cefazolin, cefovecin, ceftriaxone, cefoperazone, ceftazidime, cefoxitin, cefoxitin, cefotaxime, cefepime, amoxicillin/clavulanate, piperacillin/tazobactam, imipenem, meropenem, tetracycline, gentamicin, amikacin, chloramphenicol, trimethoprim-sulfamethoxazole, enrofloxacin, and levofloxacin. Susceptibility interpretation followed the protocols and cutoff points established by the Clinical and Laboratory Standards Institute [11,12]. *Escherichia coli* ATCC 25922 was used as a control strain.

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2.3. Whole Genome Sequencing and Genomic Characterization

Total genomic DNA of the bacterial isolates was extracted using the InstaGene™ Matrix (Bio-Rad Laboratories) extraction method and subjected to WGS using the Illumina NextSeq 2000 platform. Genomic assembly was performed using the Shovill (Version 1.1.0) with the SKESA assembler, available in the Galaxy web-based platform (https://usegalaxy.org/). For *E. coli*, multilocus sequence type (MLST), resistome, serotype prediction, and plasmid replicons were identified using MLST 2.0, ResFinder 4.1.0, SerotypeFinder 2.0, and PlasmidFinder 2.1, respectively, available from the Center for Genomic Epidemiology (http://genomicepidemiology.org/). For the virulome analysis, the VFDB database (https://www.mgc.ac.cn/VFs/) was used. For *Klebsiella oxytoca*, the MLST, resistome, virulome, and plasmid replicons were analyzed using the same databases as those for *E. coli*. For *Acinetobacter baumannii*, the Pasteur scheme was used for MLST analysis available in the PubMLST database (https://pubmlst.org/organisms/acinetobacter-baumannii); while resistome and virulome were obtained from ResFinder 4.1.0 and VFDB, respectively. The *A. baumannii* capsule was also typed using the Kaptive online tool (https://kaptive-web.erc.monash.edu/) to predict serotypes (K-type and O-type). For all strains, mutations in quinolone resistance-determining regions (QRDR) were searched using the CARD database (https://card.mcmaster.ca/home). For all predicted resistance genes, a ≥ 97% identity/coverage threshold was used as a filter for identification. For virulome and plasmid replicons, the default results were considered for each database. The raw data is available at the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA1269607.

2.4. Phylogenetic and Clonality Analysis

To elucidate the phylogenetic relationship of the two *E. coli* ST224, the genomic sequences of our isolates were deposited in the Enterobase database (https://enterobase.warwick.ac.uk/). Subsequently, a phylogenomic analysis was conducted using the cgMLST V1 + HierCC V1 scheme and MSTree V2 algorithms. This analysis included 737 available genome sequences of *E. coli* ST224, containing information regarding the sample origin, country, and collection year (Table S1). To determine clonality among strains, the clade including our *E. coli* ST224 strains was subjected to single-nucleotide polymorphism (SNP) analysis using the CSI Phylogeny 1.4 platform of the Center for Genomic Epidemiology, and clonality was interpreted according to Schürch et al. (2018). Closely related genome assemblies (less than 100 SNP differences) were selected to construct the final SNP-based phylogenetic tree (Table S2). The resistome of the closely related genome assemblies was obtained using the databases previously utilized with *E. coli* to compare genomic and epidemiological data.

3. Results

3.1. Bacterial Isolation and Antimicrobial Susceptibility

From infected wounds in the lumbosacral area attributed to dog bites, a *K. oxytoca* MVL-12-23 strain was isolated. From burn wounds on extremities and intracardiac blood samples, *E. coli* MVL-11-23 and MVL-123-23 strains were isolated, respectively. Finally, from the oval nodule detected at the necropsy, the *A. baumannii* MVL-13-23 strain was detected. *K. oxytoca* MVL-12-23 strain exhibits phenotypic resistance to cefazolin, tetracycline, gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole, and enrofloxacin. The two *E. coli*, MVL-11-23 and MVL-123-23 strains, displayed resistance to cefazolin, cefovecin, ceftriaxone, gentamicin, trimethoprim-sulfamethoxazole, enrofloxacin, and intermediate resistance to cefoperazone, amoxicillin/clavulanate, and amikacin. A. baumannii MVL-13-23 strain exhibits intermediate resistance to cefotaxime (Table 1).

Table 1. Information and antimicrobial susceptibility profile of infection-producing bacteria in southern pudu.

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Strain	Bacteria	Source	Antibiotic resistance profile[a]		
			R	I	S
MVL-012-23	*Klebsiella oxytoca*	Wounds in the lumbosacral area	KZ, TE, CN, AK, C, SXT, ENR	-	CRO, CFT, CAZ, FOX, AMC, IMP, MEM, AK
MVL-011-23	*Escherichia coli*	Infected burn wounds	CRO, CVN, KZ, CN, SXT, ENR	CFT, AMC, AK	CAZ, FOX, IMP, MEM, C
MVL-123-23	*Escherichia coli*	Intracardiac blood	CRO, CVN, KZ, CN, SXT, ENR	CFT, AMC, AK	CAZ, FOX, IMP, MEM, C
MVL-013-23	*Acinetobacter baumannii*	Internal abdominal nodule	-	CTX	CAZ, FEP, PTZ, IMP, MEM, TE, CN, AK, SXT, LEV

[a] KZ, cefazolin; CVN, cefovecin; CRO, ceftriaxone; CFT, cefoperazone; CAZ, ceftazidime; FOX, cefoxitin; CTX, cefotaxime; FEP, cefepime; AMC, amoxicillin/clavulanate; PTZ, Piperacillin/tazobactam; IMP, imipenem; MEM, meropenem; TE, tetracycline; CN, Gentamicin; AK, amikacin; C, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; ENR, enrofloxacin; LEV, levofloxacin.
R, resistant; I, intermediate; S, susceptible.

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3.2. Genomic Characterization of Infection-Causing Bacteria in Southern Pudu

The *K. oxytoca* MVL-12-23 strain belonged to the ST145 lineage, carrying resistance determinant genes against beta-lactams (*bla*$_{OXY-2-10}$), aminoglycosides [*aadA1*, *aadA5*, *aac(3)-Iia*], macrolides [*mph(A)*], phenicols (*catA1*), tetracyclines [*tet(B)*], sulfonamides (*sul1*), and trimethoprim (*dfrA17*). In addition, it has mutations in the *gyrA* (S83I) and *gyrB* (S463A) QRDR, associated with fluoroquinolone resistance (Table 2). The virulome comprised genes conferring bacterial adherence, iron uptake, secretion systems, efflux pumps, nutritional factor, virulence regulation, cell surface components, magnesium uptake, protease, and stress adaptation (Table S3). The plasmid replicons detected were *IncFIB(K)* and *IncM1* (Table 2).

Table 2. Multilocus sequence typing (MLST), resistome, serotype prediction, and plasmid replicons of *Klebsiella oxytoca*, *Escherichia coli*, and *Acinetobacter baumannii* strains detected in southern pudu.

Bacterial strain	MLST	Resistome[a]	Serotype prediction	Plasmid replicons
K. oxytoca MVL-12-23	ST145	*bla*$_{OXY-2-10}$[1], *aadA1*[2], *aadA5*[2], *aac(3)-IIa*[2], *mph(A)*[3], *tet(B)*[4], *sul1*[5], *dfrA17*[6], *catA1*[7], *gyrA (S83I)*[8], *gyrB (S463A)*[8]	ND	*IncFIB(K)*, *IncM1*
E. coli MVL-11-23	ST224	*bla*$_{CTX-M-1}$[1], *aac(3)-IId*[2], *aph(3')-Ia*[2], *aph(3'')-Ib*[2], *aph(6)-Id*[2], *mph(A)*[3], *sul2*[5], *dfrA17*[6], *gyrA* (D87N and S83L), *parC* (S80I)	O126:H23	*IncM1*, *IncQ1*, *p0111*
E. coli MVL-123-23	ST224	*bla*$_{CTX-M-1}$[1], *aac(3)-IId*[2], *aph(3')-Ia*[2], *aph(3'')-Ib*[2], *aph(6)-Id*[2], *mph(A)*[3], *sul2*[5], *dfrA17*[6], *gyrA* (D87N and S83L)[8], *parC* (S80I)[8]	O126:H23	*IncQ1*, *p0111*
A. baumannii MVL-13-23	ST1365	*bla*$_{OXA-413}$[1], *parC* (V104I, and D105E)[8]	KL138, OCL1	-

[a], genes encoding resistance to: [1], beta-lactams; [2], aminoglycosides; [3], macrolides; [4], tetracyclines; [5], sulfamethoxazole; [6], trimethoprim; [7], phenicols; [8], fluoroquinolones. ND, not determined.

The two *E. coli* strains belonged to the same ST224 lineage. The resistome was composed of genes conferring resistance to beta-lactams (*bla*$_{CTX-M-1}$), aminoglycosides [*aac(3)-IId*, *aph(3')-Ia*, *aph(3'')-Ib*, *aph(6)-Id*], macrolides [*mph(A)*], sulfonamides (*sul2*), and trimethoprim (*dfrA17*). In addition, *E. coli* strains displayed mutations in QRDR *gyrA* (D87N and S83L) and *parC* (S80I) genes, which confer resistance to fluoroquinolones. The serotype prediction of both *E. coli* strains was O126:H23 (Table 2).

For *E. coli* MVL-11-23 strain the virulome includes virulence factors associated to adherence, invasion, iron uptake, toxin, autotransporter, non-lee encoded ttss effectors, secretion system, and *Yersinia* O antigen (Table S3). On the other hand, *E. coli* MVL-123-23 strain carried a virulome composed by genes encoding for adherence, invasion, iron uptake, secretion systems, toxins, endotoxin, serum resistance, immune evasion, antiphagocytosis, magnesium uptake, quorum sensing, motility, autotransporter, non-lee encoded ttss effectors, virulence regulation, aminoacid and purine metabolism, anaerobic respiration, cell surface components, chemotaxis and motility, efflux pump, enolase enzyme, lipid and fatty acid metabolism, nutritional virulence, stress adaptation, acyltransferases and *Yersinia* O antigen (Table S3).

The plasmid replicons detected were *IncM1*, *p0111*, and IncQ1 for *E. coli* MVL-11-23 strain and *p0111* and IncQ1 for *E. coli* MVL-123-23 strain.

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The *A. baumannii* MVL-13-23 strain belonged to ST1365 and serotype KL138, OCL1. It harbored a resistance gene *bla*$_{OXA-413}$ that confers resistance to beta-lactams, as well as mutations in *parC* (V104I and D105E) QRDR. The virulome comprises genes that confer characteristics such as bacterial adherence, biofilm formation, iron uptake, quorum sensing, phospholipases, immune evasion, serum resistance, sensor kinases, and catalase (Table S3).

3.3. Phylogenetic and Clonality Analysis of E. coli ST224

The initial phylogeny of *E. coli* ST224 was conducted with 737 genome assemblies from different countries around the world that met the established criteria (host, country, and year of collection data) (Table S2). The two *E. coli* strains isolated from *P. puda* were closely related (<100 SNPs of difference) to strains from Brazil (wild bird), Switzerland (human, cat, dog, and house environment), and the United States (human, pig, horse, and dog) (Table S2).

The collection years ranged from 2019 to 2024 (Figure 1). The resistome comparison of the strains includes resistance genes to beta-lactams (*bla*$_{NDM-5}$, *bla*$_{CTX-M-1}$), aminoglycosides [*aac(3)-IId*, *aph(3')-Ia*, *aph(3'')-Ib*, *aph(6'')-Ib3*, *aph(6)-Id*], macrolides [*mph(A), mph(E), msr(E)*], tetracyclines [*tet(A)*], sulfamethoxazole (*sul2)* trimethoprim *(dfrA17)*, phenicols (*catA2*) and rifampicin (*ARR-3*) antibiotics (Figure 1). The *E. coli* MVL-11-23 strain isolated from the infected wound differed by five SNPs from the *E. coli* MVL-123-23 strain isolated from the cardiac blood sample.

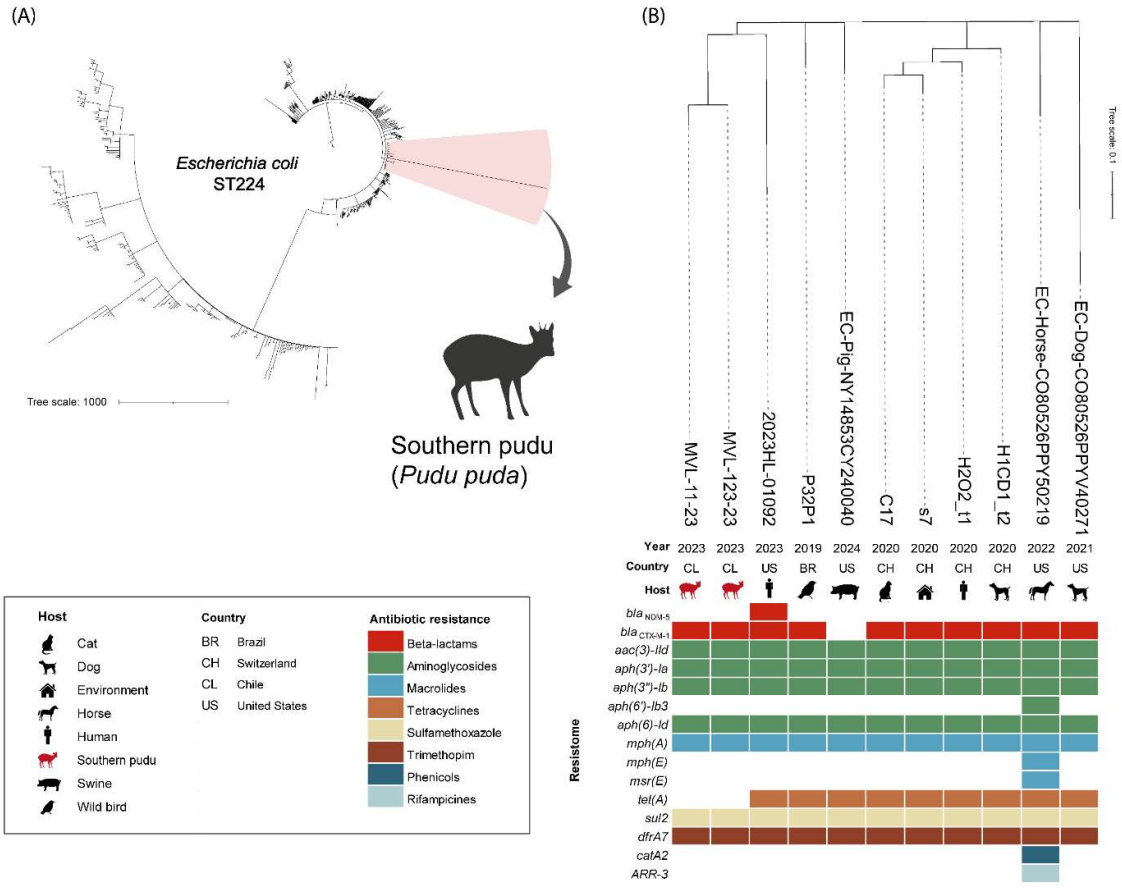

Figure 1. Phylogenomic analysis and comparison of *Escherichia coli* ST224 from infected wound and intracardiac blood samples isolated from southern pudu and closely related genomic sequences available in the Enterobase database. (A) Phylogenomic analysis of 737 genomes of *E. coli* ST224, highlighting the clade that includes strains isolated from the southern pudu. (B) Detailed view of the highlighted clade containing *E. coli* ST224 from

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southern pudu and closely related genomic sequences (less than 100 SNPs of difference) with comparison of epidemiological data and resistome.

4. Discussion

This study investigated the genomic characteristics of a triple bacterial co-infection in a vulnerable *P. puda* impacted by anthropogenic activities with a fatal outcome, using WGS as a relevant tool in the forensic field.

Wildfires result in the loss of millions of hectares annually due to uncontrolled blazes, severely impacting the environment, wildlife, and human life [14]. These disasters have a profound impact on biodiversity, leading to habitat loss, reductions in the population sizes of both flora and fauna, alterations in ecosystems, and environmental pollution [15–17]. While wildlife has evolved an escape response to fire, this does not guarantee survival in the face of such events [18–20]. As in the case described in this paper, disoriented escaped animals can be seen as prey of domestic or wild carnivores. On the other hand, wildlife that survives wildfires may be directly impacted by secondary infections due to wound contamination, which reduces the chances of survival for the burned animals [21].

E. coli is a diverse bacterial species comprising both commensal and pathogenic strains capable of causing intestinal and extraintestinal diseases in humans and animals. Advances in genomics have revealed that acquiring virulence-associated genes through horizontal gene transfer plays a key role in its pathogenic potential [22]. In this context, the southern pudu suffered from a secondary bacterial infection in its burned right hind limb caused by a multidrug-resistant CTX-M-1-producing *E. coli* ST224, progressing from local soft tissue infection to fatal sepsis. This was confirmed by SNP and virulome analyses, which verified that it was an *E. coli* ST224 clone identified in the bloodstream sample (with five SNPs of difference) [13]. However, the strain recovered from blood carried a more extensive virulome, which may have contributed to an increased pathogenic potential. Detected genes that encode for bacterial invasion, iron uptake, hemolysins, antiphagocytosis, chemotaxis and motility, endotoxin, immune evasion, quorum sensing, serum resistance, and stress adaptation could have facilitated the septicemia and fatal outcome of the case [23,24]. Unfortunately, we cannot confirm the specific mobile genetic elements (MGEs) carrying this extra virulome due to the short-read sequencing that does not allow a correct assembly of plasmids or other MGEs [25]. On the other hand, limited therapeutic options due to the antimicrobial resistance determinants, including the production of the extended-spectrum beta-lactamase (ESBL) CTX-M-1 by the strain, contributed to the death of the animal. ESBL-producing Enterobacterales are classified within the critical priority group of the WHO list, and the presence of this type of enzyme produced by *E. coli* complicates the treatment of patients [26]. Identifying *E. coli* ST224 lineage in the southern pudu adds to those reported worldwide in diverse hosts such as humans, pets, livestock, wildlife, and the environment [27]. This demonstrates that this One Health lineage adapts to different species and hospital and wild environments.

In addition to the *E. coli* infection, the patient had an infection in a dog bite wound where a multidrug-resistant *K. oxytoca* ST145 was isolated, being the first report in wildlife. This lineage has been described in Poland, China, and Spain as an emerging pathogen primarily causing nosocomial post-surgical or wound infections in humans [28–31]. This increases the need to monitor the environment of wildlife rehabilitation centers and wild patients with secondary wound infections. Both *E. coli* ST224 and *K. oxytoca* ST145 were resistant to fluoroquinolones due to point mutations in quinolone resistance-determining regions, leading to the therapeutic failure with the empiric enrofloxacin administered.

Finally, *A. baumannii* ST1365 was a pathological finding in the southern pudu, establishing the first confirmed report of this bacterium by WGS in wildlife. While not characteristic, such presentations highlight the potential of *A. baumannii* to induce focal, nodular pathology under certain clinical conditions [32,33]. The duration and underlying causes of the lesion caused by *A. baumannii*

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in the southern pudu remain unknown. Although this pathogen is well studied in human medicine, its pathogenic potential in animals warrants further investigation [34,35].

5. Conclusions

This study highlights the utility of whole genome sequencing (WGS) as a powerful tool for forensic microbiology in wildlife, enabling the precise characterization of pathogens responsible for fatal infections. The investigation of a deceased southern pudu revealed a complex case of a triple bacterial infection involving *E. coli* ST224, *K. oxytoca* ST145, and *A. baumannii* ST1365, exhibiting significant antimicrobial resistance and extensive virulence gene repertoires. The detection of CTX-M-1-producing *E. coli* ST224 emphasizes the One Health implications of multidrug-resistant bacteria circulating across human, animal, and environmental reservoirs. This study demonstrates that the same *E. coli* ST224 clone, initially isolated from a wound infection, could cause fatal septicemia in the southern pudu, underscoring the pathogen's adaptability and virulent potential in wildlife hosts. The findings also underscore the underexplored role of opportunistic pathogens such as *A. baumannii* and *K. oxytoca* in wildlife health. This case study exemplifies the importance of implementing genomic epidemiology in wildlife rehabilitation settings to detect, monitor, and understand the emergence and spread of infectious diseases, ultimately contributing to wildlife conservation efforts and safeguarding public health.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Photography of lesions in the southern pudu during the necropsy. A, wounds in the lumbosacral area; B, burn wounds; C, internal abdominal nodule detected at the necropsy; Table S1: Epidemiological data of genomic assemblies of *Escherichia coli* ST224 available in the Enterobase database; Table S2: Matrix of SNP-based phylogeny analysis of closely related genome assemblies of *Escherichia coli* ST224; Table S3: Virulence genes (virulome) according to the virulence factor class of *Klebsiella oxytoca*, *Escherichia coli,* and *Acinetobacter baumannii* strains isolated from southern pudu.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

WGS	Whole-genome sequencing
MLST	Multilocus sequence type

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ST	Sequence type
ESBL	Extended-spectrum beta-lactamase

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