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Molecular analysis of zoonotic pathogens observed in *Thryonomys swinderianus* (Marsh cane rat) in the city of Daloa in Center Western Côte d'Ivoire

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Bush meat is a source of food and income for many people in Center Western Côte d'Ivoire. However, it can have adverse effects on the health of the population when food safety practices are not respected. The assessment of the health risk associated with the consumption of bush meat was carried out on 17 faeces samples of *Thryonomys swinderianus* (marsh cane rat) collected in the city of Daloa in Center Western Côte d'Ivoire. Coproscopy was performed on these faeces for the identification of intestinal parasites followed by DNA extraction from the isolated bacteria. This DNA was amplified by PCR using the 16S rDNA primer and sequenced. The resulting sequences were analysed using bio-informatics tools. This study revealed the presence of a diversity of parasites and bacteria pathogenic to humans in these animals. The *Trichuris trichiura* species is the most detected parasitic species in terms of intestinal parasites. The bacterial profile obtained is dominated by species belonging to the Enterobacteriaceae family, in particular *Klebsiella pneumoniae*, which is the most common species in the samples analysed. The presence of human pathogens in the faeces of wild animals demonstrates the zoonotic nature of parasitic and bacterial infections and the potential infectious risk of bush meat consumption.

Key words: *Thryonomys swinderianus*, Marsh cane rat, bush meat, zoonotic pathogens, health risk, molecular analysis, Center Western Côte d'Ivoire.

INTRODUCTION

Consumed wildlife or bush meat is a source of food and income for many populations in sub-Saharan Africa (Williamson and Backer, 2017; Chabi-Boni et al., 2019). Côte d'Ivoire, a country located in the West African tropical zone, is no exception.

Indeed, the consumption of bush meat is becoming increasingly noticeable in all regions of the country in general and in the Center Western region in particular (Gonédélé et al., 2017; Yéboué et al., 2020).

In the Center Western region of Côte d'Ivoire, which is

the subject of our study, it appears that the wildlife commonly poached and sold in restaurants is largely made up of small mammal species, mainly rodents. The most poached species and the one most prized by the population in this part of the country is the marsh cane rat *Thryonomys swinderianus* (Yéboué et al., 2020). However, the consumption of this bush meat can have adverse effects on the health of the population when hunting, transport, handling and cooking do not follow food safety practices (Van Vliet et al., 2017).

According to the World Health Organization, more than 50% of new infectious diseases in humans are caused by pathogens from animals or animal products, 70% of which are from wildlife (OIE, 2012).

These diseases, known as zoonosis, can spread in a variety of ways between animal hosts and humans. These include shared vectors, indirect contact through exposure to rodent faeces in a peri-domestic environment, but especially through direct contact with an animal through handling, consumption, bites, scratches, body fluids, tissues and excreta (Wolfe et al., 2005; Johnson et al., 2020).

Côte d'Ivoire is not on the fringe of risk nations with regard to the spread of zoonotic diseases (Liégeois et al., 2009). However, few studies have focused on health risks since the Ebola fever episode in West Africa and the Covid pandemic.

The objective of this study is to highlight the health risks associated with the consumption of bush-meat through the parasitological and cyto-bacteriological analysis of faeces samples from specimens of the marsh cane rat *T. swinderianus* found in the city of Daloa located in the Center Western Côte d'Ivoire.

MATERIALS AND METHODS

Collection of samples

Samples of faeces from seventeen fresh specimens of *Thryonomys swinderianus* (marsh cane rat) were collected from restaurants in the city of Daloa in the Center Western Côte d'Ivoire during March and April 2020.

Isolation of intestinal parasites from bush meat

Faeces samples were analysed by coproscopy after enrichment with Willis' liquid (33% saturated aqueous NaCl solution of specific gravity 1.2), using the flotation technique as described by Degbe et al. (2018). The purpose of this technique is to concentrate the principle is based on the density of the NaCl solution used and that of the parasites. It relies on the use of solutions with a density higher than that of most parasite eggs (Degbe et al., 2018).

In a graduated beaker, about ten (10) g of faeces were carefully triturated with a small amount of 33% saturated salt water until the mixture was homogeneous.

The level of the homogenate was reduced to 60 mL by adding the NaCl solution used. The suspension column was then sieved to remove any coarse material. Approximately 50 mL of homogenate was collected and transferred to a tube.

Subsequently, a slide was placed on the surface of the liquid without trapping air bubbles. After about 30 min, the slide was removed from the surface of the solution and placed on an object slide for observation of the parasite elements. After observation under a light microscope (x10, x40) (WHO, 1994) was used for the identification of the parasites.

Isolation of bacteria on CHROMAgar™ orientation medium

The presence of pathogenic bacteria was also investigated in the faeces of the Marsh cane rat *T. swinderianus*. Ten (10) microlitres of faeces samples, previously grown in enrichment medium, were inoculated onto CHROMAgar™ Orientation agar by tight and loose streaks, using single-use loops. The inoculated media were then incubated at 37°C for 24 h in an oven. CHROMAgar is a chromogenic medium that allows colorimetric differentiation of bacterial agents present in faecal samples. Thus, it allows a directed identification of the bacterial species.

Identification of isolated bacteria

Morphological identification: Gram stain test

A differential identification of the isolated strains was performed. Gram staining is the basic differential identification test in bacteriology (Delarras, 2007). It makes it possible to differentiate between gram-negative bacteria (coloured purple) and gram-positive bacteria (coloured pink) and to study their morphological character, in particular the form (cocci or bacillus). To do this, a pure bacterial colony is spread on an object slide. After drying at room temperature, this preparation is subjected to the action of four reagents allowing contrast effects: A first dye (0.5% crystal purple), a mordant (1/3 Gram iodine obtained from Lugol's iodine) which complexes the dye, a differentiator (Gram differentiator) which is a decolourising solution and finally, a second dye (1% safranin solution).

The reading is made by microscopic observation with an X100 objective. The enterobacteria appear as pink coloured bacilli.

Biochemical identification

Identification based on the biochemical characteristics of the bacteria was carried out using the classical gallery system described by Le Minor and Richard (1993). This is a system consisting of several enzymatic and metabolic tests.

DNA extraction from the faeces of the marsh cane rat *T. swinderianus*, polymerase chain reaction (PCR) amplification and sequencing

Bacterial cultures were made in Luria Bertani (LB) broth.

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Table 1. 16S rDNA amplification programme.

Step	Temperature (°C)	Time	Cycle
Initial denaturation	94	2 min	1
Denaturation	94	30 s	
Annealing	46	40 s	35
Extension	72	1:30 s	
Final extension	72	10 min	1
Conservation	4	∞	

Source: Authors

Subsequently, 1 mL aliquots of LB were made in cryotubes. They were sowed with a well-isolated colony on CHROMAgar agar medium and incubated for 24 h at 37°C.

Extraction of genomic DNA from the bacteria in the faeces was performed according to the protocol using phenol-chloroform described by Chan and Goodwin (1995) and checked on a 1% agarose gel for quality control. The quantities of reagents were slightly modified and adapted to the conditions of this work.

The reaction mixture for each simplex PCR was prepared to a final volume of 50 µL containing: 1µl of each primer (F: 5'-GCAAGTCGAGCGGTAGCACAG-3' and R: 5'-CAGTGTGGCTGGTCATCCTCTC-3') (260 bp), concentrated to 10 pmol/µL (Eurogentec), 5 µL of Mg^{2+} PCR buffer (10X), 1.5 µL of $MgCl_2$ (25 mM), 2.5 µL of dNTPs (200 µM), 0.1 µL of Taq polymerase (5U/µL), and 35.9 µL of ultra-pure water. Three (3) µL of genomic DNA extract to be amplified was added to this mixture for transfer to the thermal cycler.

DNA was amplified according to the protocol shown in Table 1. PCR products representative of all bacterial species isolated were coded and sent to BGI TECH SOLUTIONS (HONG-KONG) for sequencing.

DNA sequences assignment

Chromas Lite® 2.01 software was used to make the DNA sequences received in the ABI format files analyzable. The forward and reverse sequences of the genes were assembled and then corrected using DNA Baser Assembler 5.15.0 software and Chromas Lite® 2.01 software. *In silico* analysis of the sequences and phylogenetic characterization of bacterial species are made. The sequences obtained were compared with existing reference sequences in the public genomic database of the National Center for Biotechnology Information (NCBI) using the BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nih.gov) (Altschul et al., 1990).

Determination of phylogenetic relationships between bacterial species

The evolutionary history of the pathogens was deduced using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method on the 16S ribosomal DNA gene sequences. This method assumes an independent evolution of the different genetic profiles at a constant rate, with 1000 repetitions.

The optimal tree with the sum of the branch lengths = 2.77503562 is shown. The percentage of replicate trees in which the associated bacterial strains clustered in the bootstrap (1000 replicates) is indicated next to the branches.

The evolutionary distances were calculated using the Jukes-Cantor method and are in units of the number of base substitutions per site. This analysis was performed on 13 DNA sequences. Codon positions included were 1st, 2nd and 3rd. Positions observed in sequence pairs whose interpretation seemed uncertain were removed. There were a total of 269 positions in the final data set. These evolutionary analyses were performed in MEGA X software. Significance is observed for all statistical tests performed when the probability value (p) associated with the statistical tests is strictly less than 0.05.

Determination of the occurrence of microbial and parasitic agents

The occurrence of a micro-organism is the presence or absence in an analysed faeces sample. The percentage of occurrence was calculated in order to highlight the most represented microorganism in the intestinal microbiota of the animal under consideration.

Health risk analysis

The health risk associated with bush meat is highlighted by detecting and identifying the species of parasites and potentially pathogenic bacteria present in the faeces of *Thryonomis swinderianus* specimens and determining their occurrence.

RESULTS

Risk of parasitic infection

Analysis of the samples revealed the presence of four types of intestinal parasite eggs. These are: eggs of *Trichuris trichiura*, *Trichuris leporis, Trichostrongylus axei* and *Paraspidodera uncinata* (Figure 1).

Of the 17 samples of Marsh cane rat faeces analysed, eggs of *Trichuris trichiura* were detected in the majority (7; 41.2%), followed by eggs of *Trichuris leporis* (5; 29.4%), *Trichostrongylus axei* (3; 17.6%) and *Paraspidodera uncinata* (2; 11.8%).

Risks to bacterial infections

In addition to intestinal parasites, analysis of faecal

Figure 1. Parasite eggs isolated from the faeces of *Thryonomis swinderianus* (Marsh cane rat) collected in Center Western Côte d'Ivoire. A: Eggs of *Trichuris trichiura; B:* Eggs of *Trichuris leporis; C:* Eggs of *Trichostrongylus axei;* D: Eggs of *paraspidodera uncina.*
Source: Authors

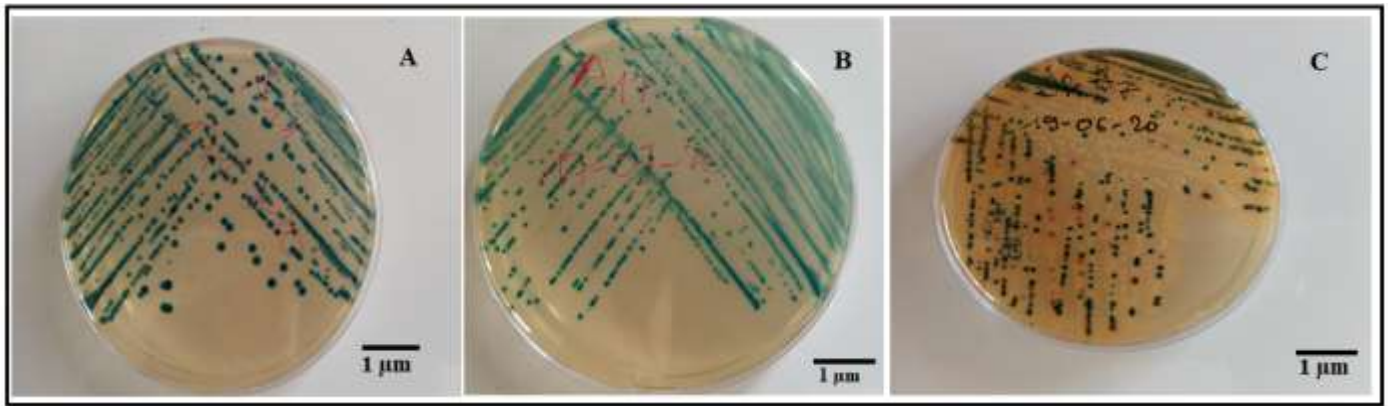

Figure 2. Gram-negative bacteria colony with metallic blue staining characterising the KESC group. KESC: *Klebsiella, Enterobacter, Serratia, Citrobacter; A and B: monobacterial infections; C: polybacterial infection.*
Source: Authors

samples revealed the presence of bacteria with infectious characteristics. On the basis of morphological and biochemical characteristics, the analyses described metallic blue staining Gram-negative bacteria characterising the KESC group (Figure 2). These bacteria belong to the Enterobacteriaceae family. Isolates of *Escherichia coli*, easily recognisable by their pink coloration, were also found in the intestinal bacterial flora of the Marsh cane rat. They are gram-negative. Also, polymicrobial infections are found (Figure 2).

In order to refine the characterisation of the gut microbiota of *Thryonomis swinderianus*, molecular typing of 16S rDNA was performed on 13 faeces samples. These Internal Transcribed Spacer (ITS) are characterised by DNA fragments of 260 base pairs.

Figure 3 shows the PCR amplified 16S rDNA

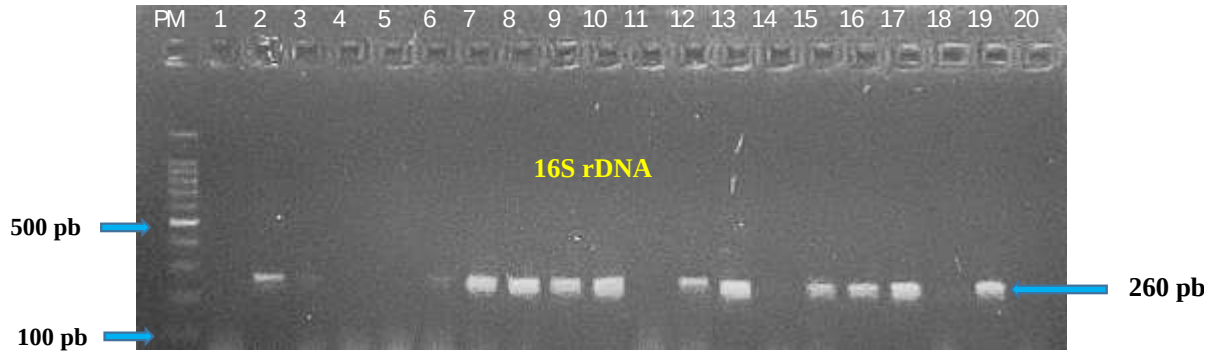

Figure 3. Electrophoresis gel showing PCR amplification products observed with the genetic markers 16S rDNA.
Source: Authors

fragments. The resulting PCR products were sequenced. Sequence analysis of the 16S rDNA gene, using the BLASTn programme, showed that the isolated bacterial strains are taxonically related to *Klebsiella* sp., *Enterobacter* sp., *Klebsiella pneumoniae, Burkholderia cepacia, Neisseria cineria, Erwinia endophytica, Serraria marcescens* and *Erwinia tasmaniensis* with percentages of identity ranging from 86.16 to 100%. These species are all enterobacteria.

The phylogenetic relationship of the bacterial strains reveals that the gut microbiota of *Thryonomis swinderianus* is composed of two groups (group A and group B) of bacteria (Figure 4). Group A comprises 61.54% (n= 8 strains) of the strains analysed. This group is predominantly composed of human pathogenic strains (87.5%; in red), whose close relatives are *Klebsiella pneumoniae*, *Enterobacter* sp. and *Burkholderia cepacia*. One isolate from the group was identified as an uncultured bacterium (black). In group B, there were 5 strains representing 38.46% of all strains analysed. The strains in this group are genetically related to species such as *Neisseria cineria*, *Erwinia endophytica*, *Klebsiella* sp., *Serratia marcescens* and *Erwinia tasmaniensis*. Species of potential interest in human pathology for this group are *Klebsiella* sp. and *Serratia marcescens* (Figure 4).

DISCUSSION

The study of the health risk based on the analysis of marsh cane rat faeces samples revealed the presence of a diversity of microorganisms in these animals, notably parasites and bacteria. Four species of parasites were identified. These are *Trichuris trichiura*, *Trichuris leporis*, *Trichostrongylus axei* and *Paraspidodera uncinata*. Of these, *Trichuris trichiura* and *Trichostrongylus axei*, detected at rates of 41.2 and 17.6% respectively, are human pathogenic species.

Indeed, *T. trichiura* is a nematode responsible for trichocephalosis in humans. According to the WHO, this parasite infects more than one billion people, 220 million of whom are severely affected and one thousand of whom die each year (WHO, 2011). Trichocephalosis is a cosmopolitan parasite, most often without any symptoms. In tropical countries, poor rural areas with fecal peril, infestation can be massive and severe, especially in children (Sunkara et al., 2018). *Trichostrongylus axei* is an intestinal parasitic worm, generally found in ruminants, birds and primitive rodents, with a worldwide distribution (Anderson, 2000; Audebert and Durette-Desset, 2007).

In Africa (in the southern part of the continent), this species is one of the main parasites of its species found in ostriches (Smith, 2018). In these hosts, it causes decreased appetite and progressive weight loss. In addition, studies in Thailand have reported infection in humans, which appears to coincide with close contact with infected animals (Phosuk et al., 2013).

Infected humans present with stomach upset, abdominal bloating, diarrhoea and eosinophilia, which is a blood disorder due to increased eosinophils (Lattès et al., 2011; Wall et al., 2011). The presence and spread of *T. trichiura* and *T. axei* in the wild animal population in general and in marsh cane rat in particular, constitutes a health risk in case of contact with these animals. The zoonotic nature of these two pathogens therefore deserves special attention. Concerning the species *Trichuris leporis* and *Paraspidodera uncinata*, although human infection has never been demonstrated, they are responsible for serious infections in animals. Heavy infestation can cause anorexia, diarrhoea and weight loss associated with a rough coat, which could have serious consequences for the survival of the animals, and therefore for their health. Consequences for animal survival, and thus a threat to biodiversity (Schoeb et al., 2007; Bartholds et al., 2016).

Bacterial infections due to species such as *Klebsiella pneumoniae*, *Enterobacter* sp, *Serratia marcescens*, *Burkholderia cepacia*, *Neisseria cineria*, *Erwinia tasmaniensis* and *Erwinia endophytica* were detected.

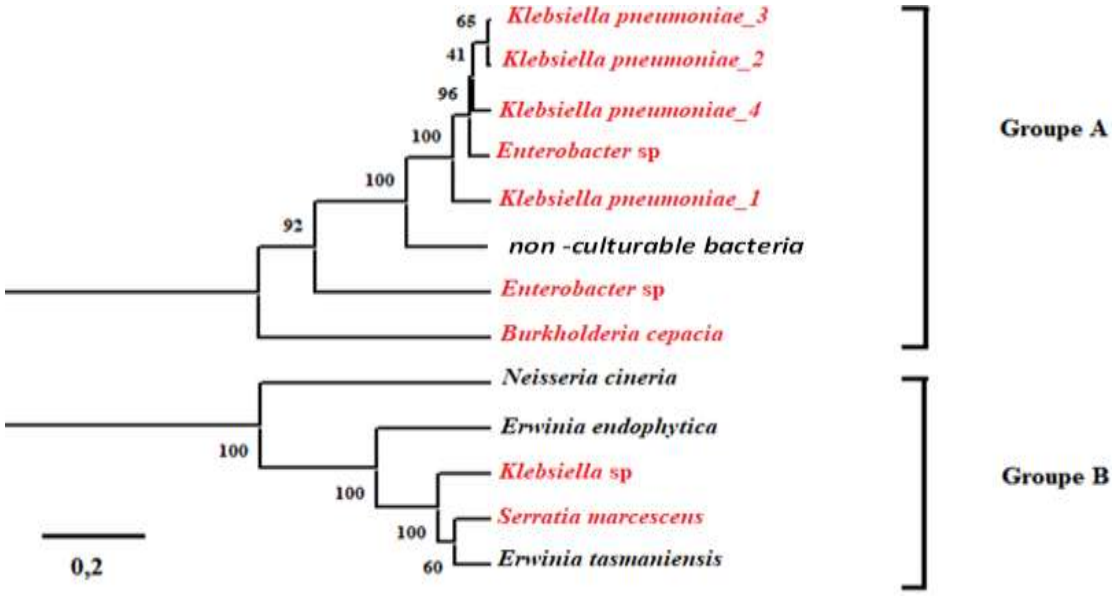

Figure 4. Dendrogram showing the phylogenetic relationship between the bacterial species identified from the 16S rDNA gene sequences. In red: Species accepted in human pathology; In black: Non-pathogenic species for humans or undefined.
Source: Authors

The bacterial profile obtained is dominated by species belonging to the Enterobacteriaceae family and known for their pathogenicity in humans. These are species of the genus *Klebsiella*, *Enterobacter* sp., *Burkholderia cepacia* and *Neisseria cineria*. *Klebsiella pneumoniae* is the most represented species of this family with an occurrence of 38.46% (in 5 samples out of 13 sequences analysed). This species has been medically recognised as one of the most important opportunistic pathogens, causing infections of the pulmonary system, urinary tract, circulatory system and soft tissues, acquired in the environment. Soft tissue infections acquired in hospitals or associated with healthcare worldwide (Hou et al., 2015). In addition, Vincent et al. (2010) and Jakobsen et al. (2012) have identified *Klebsiella* strains in pet feces. This study appears to be the first to demonstrate the presence of *K. pneumoniae* in wild animals in Côte d'Ivoire. The presence of human pathogenic bacteria in the faeces of wild animals shows a zoonotic character of bacterial infections.

The presence of "uncultured bacteria" in the faeces revealed by this study could be explained by the absence of genomic data, targeting the coding region of the ribosomal DNA gene sequence.

Conclusion

This study highlighted the health risks associated with the consumption of bush meat through the analysis of faecal samples of marsh cane rat *T. swinderianus* specimens. It revealed the presence of a variety of microorganisms in Marsh cane rat, including parasites and bacteria pathogenic to humans. The *Trichuris trichiura* species, responsible for trichocephalosis, is the most detected intestinal parasite. The bacterial profile obtained is dominated by species belonging to the Enterobacteriaceae family and known for their pathogenicity in humans. *K. pneumoniae* is the most represented species of this family. Although some authors have shown in their studies the presence of Klebsiella strains in the faeces of domestic animals. This study shows, for the first time, the presence of *K. pneumoniae* in wild animals in Côte d'Ivoire. The presence of human pathogens in the faeces of wild animals shows the zoonotic nature of these parasites and bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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