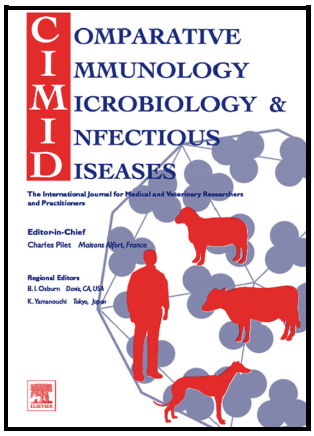


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PII: S0147-9571(23)00101-7

DOI: https://doi.org/10.1016/j.cimid.2023.102043

Reference: CIMID102043

To appear in: *Comparative Immunology, Microbiology and Infectious Diseases*

Received date: 5 April 2023
Revised date: 18 August 2023
Accepted date: 22 August 2023

Please cite this article as: C. Marin, M. Cerdà-Cuéllar, I. Rosario, L. Lorenzo-Rebenaque, S. Vega, A. Manzanares, D. Padilla, F. Real, E. Rodríguez-Ponce and B. Acosta-Hernández, Impact of genetic diversity and antibiotic-resistance of *Salmonella* isolated from feral cats: One Health approach., *Comparative Immunology, Microbiology and Infectious Diseases,* (2023) doi:https://doi.org/10.1016/j.cimid.2023.102043

# Impact of genetic diversity and antibiotic-resistance of *Salmonella* isolated from feral cats: One Health approach.

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## ABSTRACT

Free-living cats usually live in colonies in urban areas, especially close to parks and neighbourhoods where people feed them without any sanitary control. This can pose a human, animal and environmental health concern due to the close contact between uncontrolled colonies, the population and other domestic and/or wild animals. Thus, this study aimed to assess the genetic diversity and antimicrobial resistance (AMR) among *Salmonella enterica* subsp. *enterica* strains isolated from feral cats in a previous epidemiological study in the Gran Canaria island (Spain). A total of nineteen *Salmonella* isolates were obtained from November 2018 to January 2019 in a *Salmonella* epidemiological study in feral cats. All isolates obtained were genotyped by pulsed-field gel electrophoresis (PGFE) and were tested for antimicrobial susceptibility, in accordance with Decision 2013/652/EU. PFGE analysis revealed isolates clustering by serovar, with identical clones for serovars Bredeney and Grancanaria, while differing pulsotypes were

observed for serovars Florida (88.89% similarity) and Nima (83.23% similarity). All but two isolates were resistant to at least one antimicrobial. The results obtained demonstrate that feral cats in the region investigated are a reservoir of *Salmonella* strains resistant to gentamicin (94.1 %) and of the critically important antimicrobial tigecycline (23.5 %). Hence, they could excrete AMR strains through their faeces and contaminate the environment, favoring the spread of such bacteria to cohabiting pets. Moreover, this widespread presence of AMR *Salmonella* clones across various serovars highlights the urgent need to implement efficient antimicrobial stewardship and control programs by the local governments due to the ongoing need to protect human and animal health under a One Health concept.

**Key Words**: Zoonotic, Antibiotic, PFGE, Free-living cats

## INTRODUCTION

The phenomenon of urbanisation has a profound impact on ecological systems [1] and forces animals to change their feeding habits, breeding areas or even migration patterns, establishing stable populations of animals. A multitude of species coexist today in urban areas (pigeons, starlings, sparrows, geckos, lizards or even raccoons, wild boars and feral cats), using the resources that human activity provides them to live, feed and reproduce [2].

Free-living cats are also very numerous in urban areas. They usually live in colonies outdoors in public or private urban areas, especially close to parks and neighbourhoods where people feed them without any sanitary control. This can pose a human, animal and environmental health concern due to the close contact between uncontrolled colonies, the population and other domestic and/or wild animals. Several authors have shown that free-living cats could be an important factor in the transmission of zoonotic diseases [3], such as rabies, toxoplasmosis, tularaemia, murine typhus [4,5] and salmonellosis [6]. Consequently, different countries have established campaigns to control feral cat colonies. The most common control tool is based on trap-neuter-return campaigns that control the overpopulation of free-living cat colonies by trapping all or most of the cats

in a colony, sterilising them and returning them to their territory [7]. Otherwise, in several urban zones no control is carried out, increasing the danger of spreading pathogens.

*Salmonella* is widely considered one of the most important zoonotic pathogens worldwide, and it has been considered one of the top ten multidrug-resistant (MDR) bacteria worldwide [8]. MDR *Salmonella* strains emerge as a potential concern for public health safety, with implications of increased disease severity, longer hospitalisations and higher cost rates [8,9]. In this context, the World Health Organisation (WHO) deemed antimicrobial resistance (AMR) one of the most important health threats, which could cause 10 million deaths a year by 2050, ahead of other diseases such as cancer [10,11]. In this sense, *Salmonella* has been included in the WHO priority list of twelve antibiotic-resistant bacteria [12].

In Europe, *Salmonella* is the first cause of human zoonotic outbreaks [13]. However, even though salmonellosis is mostly noticed as a foodborne disease, it has been estimated that about 11 % of the cases are due to direct contact with animals, including cats [6,14].

A previous study in the Canary Islands [7], where 100 feral cats were sampled for *Salmonella* detection, showed that 19 % of individuals sampled were *Salmonella* carriers. From a one health point of view, this is a critical result, as uncontrolled infected feral cats could provide favourable conditions for transmitting zoonotic pathogens to humans, domestic and wild animals and the environment. This poses a significant threat in cases of *Salmonella* infection among children, the elderly or immunocompromised individuals due to the severity of their symptoms [15]. However, there is a lack of knowledge concerning the genetic diversity and the AMR of *Salmonella* isolates that could be spreading among feral cat colonies, and increasing our understanding would significantly bolster our capacity to mitigate potential risks. Thus, this study aimed to assess the genetic diversity and AMR among *Salmonella* strains isolated from feral cats in previous studies in the Canary Islands.

## MATERIAL AND METHODS

### *Salmonella isolates origin and isolation*

Nineteen *Salmonella* isolates were obtained from November 2018 to January 2019 on the island of Gran Canaria in a *Salmonella* epidemiological study in feral cats [7]. The

*Salmonella* isolates were taken from rectal swab samples collected from 100 feral cats from Ingenio (n=35), Santa Brígida (n=30) and Las Palmas de Gran Canaria /Arucas (n=35) (Figure 1).

Samples taken were analysed for *Salmonella* presence and confirmed according to the ISO7679:2017 and serotyped at the Reference Laboratory for Animal Health (Algete, Madrid, Spain) following the White-Kauffmann-Le Minor scheme. All isolates were stored at -80 ºC until use. For this study, fresh cultures of the isolates were obtained onto Xylose Lysine Deoxycholate agar (Scharlab, Madrid, Spain). Plates were incubated at 37 ºC during 24 h. Then, the Analytical Profile Index (API) 20E (BioMérieux, Madrid, Spain) was performed for the confirmation of *Salmonella* spp., following the manufacturer's indications.

***Molecular typing of Salmonella isolates***

Genotyping of *Salmonella* spp. isolates was performed by pulsed-field gel electrophoresis (PGFE) according to the PulseNet standardised protocol (www.pulsenetinternational.org/protocols/pfge/) to assess the genetic diversity and relatedness among isolates belonging to different serovars. Genomic DNA of the isolates was digested with Xbal restriction enzyme (Roche Applied Science). We analysed the resulting PFGE band patterns using Fingerprinting II v3.0 software (Bio-Rad) and isolates were compared with those present in our own PFGE database, from other regions and hosts (mostly of avian origin, wild and domestic). Similarity matrices were calculated using the Dice coefficient with a band position tolerance of 1.5 %, and cluster analysis was performed by the unweighted-pair group method with arithmetic mean (UPGMA). A cut-off of 90 % was used for determination of the different profiles (PFGE type or pulsotype).

***Antimicrobial susceptibility testing***

*Salmonella* spp. strains were inoculated onto Müller-Hinton agar (Scharlab, Madrid, Spain) to form a bacterial lawn; antibiotic discs were then placed on the plates, which were incubated at 37 ºC for 24 h. Antimicrobial agents were selected following those set out in Decision 2013/652/EU [16], including two quinolones: ciprofloxacin (CIP; 5 µg) and nalidixic acid (NAL; 30 µg); one aminoglycoside: gentamicin (GEN; 10 µg); one potentiated sulphonamide: trimethoprim-sulfamethoxazole (TRS; 25 µg); one phenicol:

chloramphenicol (CHL; 30 µg); one pyrimidine: trimethoprim (TRI; 5 µg); three b-lactams: ampicillin (AMP; 10 µg), cefotaxime (CTA; 30 µg), ceftazidime (CTZ; 30 µg); one macrolide: azithromycin (AZI; 15 µg); one polymyxin: colistin (COL; 10 µg); and one glycylcycline: tigecycline (TIG; 15 µg). After the 24 h incubation period at 37 ºC, the inhibition zone around each disc was measured. These zones were interpreted as susceptible (S) or resistant (R) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) indications [17] (http://www.eucast.org/clinical_breakpoints/) for *Enterobacteriaceae*, and when not possible, Clinical and Laboratory Standards Institute (CLSI) indications were applied (https://clsi.org/media/2663/m100ed29_sample.pdf) [18]. Multidrug resistance (MDR) was defined as acquired resistance to at least one agent in three or more antimicrobial classes [19].

***Statistical analysis***

A Generalised Linear Model (GLM), which assumed a binomial distribution for *Salmonella* spp. shedding, was fitted to study the relationship between *Salmonella* spp. and their AMR. Analyses were carried out using a commercially available software application (SPSS 24.0 software package; SPSS Inc., Chicago, IL, 2002).

**RESULTS**

All isolates unfrozen (n=19) were confirmed as *Salmonella* spp. and belonged to the following five serovars: Bredeney, Florida, Grancanaria, Kottbus and Nima, with the latter being the most abundant (11 isolates). All the *Salmonella* isolates were from the Ingenio location.

PFGE analysis showed isolates clustering according to the serovar (Figure 2). The three isolates of ser. Bredeney showed the same pulsotype as did the two isolates of ser. Grancanaria. Two different pulsotypes were observed in ser. Florida (88,89% similarity), with one isolate each. Serovar Nima isolates revealed also two pulsotypes (83,23% similarity), one of them was a unique pattern (singleton) while the second one included the remaining 10 isolates. Isolates of serovars Bredeney, Grancanaria and Kottbus were compared with isolates present in our PFGE database, and no similarity was found (<60 %); this included one isolate of serovar Grancanaria recovered from a hoopoe in the same

region. Isolates of the remaining serovars are not available in our database, and therefore comparisons were not possible.

From all strains isolated, 89.5 % (17/19) were resistant to at least one of the 12 antimicrobials tested. The highest frequency of AMR was found to GEN (94.1 %, 16/17) and TIG (23.5 %, 4/17) ($P < 0.001$). No resistance was shown against CIP, AMP, NAL, CHL, TRS, TRI, COL, CTA, AZI and CTZ (Table 1). Furthermore, no isolate was MDR.

Overall, three different AMR patterns were found (Table 1, Figure 2). GEN alone (82.3 %, 14/17) was the most frequent pattern observed, followed by GEN-TIG (17.6 %, 3/17) and TIG alone (5.9 %, 1/17). Isolates with either of these AMR patterns showed the same (ser. Bredeney, Gran Canaria isolates) or different PFGE profiles (ser. Nima isolates) (Figure 2).

## DISCUSSION

Nowadays, urban areas constitute a shared environment between human and free-living animals, such as urban birds or mammals, which take advantage of the waste produced by human to feed [20,21]. The close association between free-living and domestic animals and human and urban environment facilitates the risks of pathogen dissemination of those persistent clones, able to survive long enough outside their hosts and so their AMR [22,23]. To the best of our knowledge, this is the first study in the literature assessing the genetic relationship and AMR of *Salmonella* spp strains isolated from feral cats.

The relationship between pets and their owners has evolved in recent years, becoming much closer. For this reason, concern about the transmission of zoonotic microorganisms between pets and their owners has increased the study of the epidemiology of zoonotic microorganisms in pets in recent years [24–26], especially *Salmonella* spp [24,27–29]. In Europe, the cat is the most common pet in households and in most cases they have access to the outside, where they can come into contact with free-living cats and other animals.

Free-living cats that inhabit urban areas live in colonies. In most cases, they are not subjected to sanitary control and can therefore present the risk of transmitting AMR *Salmonella* strains to other wild animals, domestic animals, humans and the environment, as demonstrated in other free-living urban animals [21]. Feral cats can freely roam and scavenge or hunt for food of unknown quality and are potential candidates as vectors for

*Salmonella* spp [24,30]. As this study showed, subclinical infections in carrier feral cats can lead to transmission of AMR *Salmonella,* which is a much more critical concern [31,32].

*Salmonella* ser. Nima is the serovar most frequently isolated from feral cats, one of the isolates with the highest incidence in humans [7]. Compared with other serovars analysed in this study, the same clone or closely related strains of ser. Nima, as revealed by PFGE, seems widespread among feral cats. Since all cats were sampled in the same area, this suggests a common source of infection, as all cats were sampled in the same area, which may have spread among the colony of cats in that area. The unrelatedness of genotypes of ser. Bredeney, Grancanaria, and Kottbus from cats with those from other origins (mostly of avian origin, wild and domestic) suggest particular host specificity.

During the last decade, and under the guidance of the One Health approach, significant progress has been made in controlling the occurrence rate and antimicrobial susceptibility rates of *Salmonella* spp. in livestock within the food chain [33–36]. However, there is a lack of information concerning other domestic animals [24]. The results obtained in this study aimed to gain insight into *Salmonella* strains' occurrence and their antimicrobial susceptibility profiles in feral cats. They showed that around 90 % of *Salmonella* isolates analysed were resistant to at least one antibiotic, and of these, 94.1 % and 23.5 % showed resistance to GEN and TIG, respectively. It should be noted that TGC is considered a critically important antibiotic for human medicine according to WHO. Notably, the *Salmonella* AMR results are in line with previous studies involving other urban species, such as reptiles sampled in the Canary Islands [7], urban birds from central Spain [21], and wildlife from other countries [37,38].

Bacterial resistance to GEN and TIG has been widely documented due to their extensive use in veterinary and human medicine [37,39–42]. Hence, the close contact between feral cats and other domestic animals could explain this study's high percentage of GEN and TIG resistant isolates. These and other authors' results suggest that AMR concern is not limited to its initial niches, potentially livestock and its derived products, but that free-living animals could also be essential in spreading these strains in the urban environment [43]. It is essential to highlight that *Salmonella* spp. often encode resistant genes to critically important antibiotics, and uncontrolled urban free-living animals could elevate the risk of shedding such resistant bacteria in the environment [44].

In conclusion, the present study demonstrates that, in the region investigated, feral cats may be considered a critical reservoir of AMR *Salmonella* strains, including to an antibiotic categorized as critically important for human medicine according to WHO. They may be excreting the resistant pathogen through their faeces and contaminate the environment, favouring dissemination of the bacterium to cohabiting pets. Moreover, the concern of resistance clones of *Salmonella* isolates analysed in this study highlights the urgent need for local governments to implement efficient antimicrobial stewardship and control programmes, due to the ongoing need to protect human and animal health under the One Health concept.

**ACKNOWLEDGEMENTS**

The authors wish to thank the "Improvement of Production System-related Food Safety and End Products" research group (Veterinary Faculty, University Cardenal Herrera-CEU) for the technical support. Authors would like to thank Teresa Ayats for her technical support. CERCA Programme from the Generalitat de Catalunya is also acknowledged. The English text version was revised by N. Macowan English Language Services.

**FUNDING**

This study was financed by Universidad Cardenal Herrera-CEU (IDOC 21/10, and INDI 20-21, 22/34).

**CONTRIBUTION**

For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, BAH, SV and IR.; methodology, CM, MCC, IR, LLR, AM, DP, FR,ERP, and BAH.; software, CM and LLR; validation, CM, MCC, IR, LLR, SV, AM, DP, FR,ERP, and BAH.; formal analysis, CM and LLR.; investigation, CM, MCC, IR, LLR, SV, AM, DP, FR,ERP, and BAH; resources, CM, MCC, IR, LLR, SV, AM, DP, FR,ERP, and BAH; data curation, CM and LLR; writing—original draft preparation, CM and LLR.; writing—review and editing, CM, MCC, IR, LLR, SV, TA, DP, FR,ERP, and BAH; visualization, CM, MCC, IR, LLR, SV, AM, DP, FR,ERP, and BAH; supervision, CM, MCC, IR, LLR, SV, AM, DP, FR,ERP, and BAH.; project administration, CM,

MCC, IR, LLR, SV, AM, DP, FR,ERP, and BAH.; funding acquisition, CM, MCC, IR, LLR, SV, AM, DP, FR,ERP, and BAH. All authors have read and agreed to the published version of the manuscript.

## TABLES

**Table 1**. AMR pattern of *Salmonella enterica* subsp. *enterica* strains isolated from feral cats.

| Serovar | n | CIP | NAL | GEN | TRS | CHL | TRI | AMP | CTA | CTZ | AZI | COL | TIG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bredeney | 3 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Grancanaria | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Florida | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Kottbus | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nima | 11 | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |

n: Number of *Salmonella* strains. CIP: Ciprofloxacin, NAL: Nalidixic acid, GEN: Gentamicin, TRS: Trimethoprim-sulphamethoxazole, CHL: Chloramphenicol, TRI: Trimethoprim, AMP: Ampicillin, CTA: Ceftazidime, CTZ: Cefotaxime, AZI: Azithromycin, COL: Colistin, TIG: Tigecycline.

## FIGURES

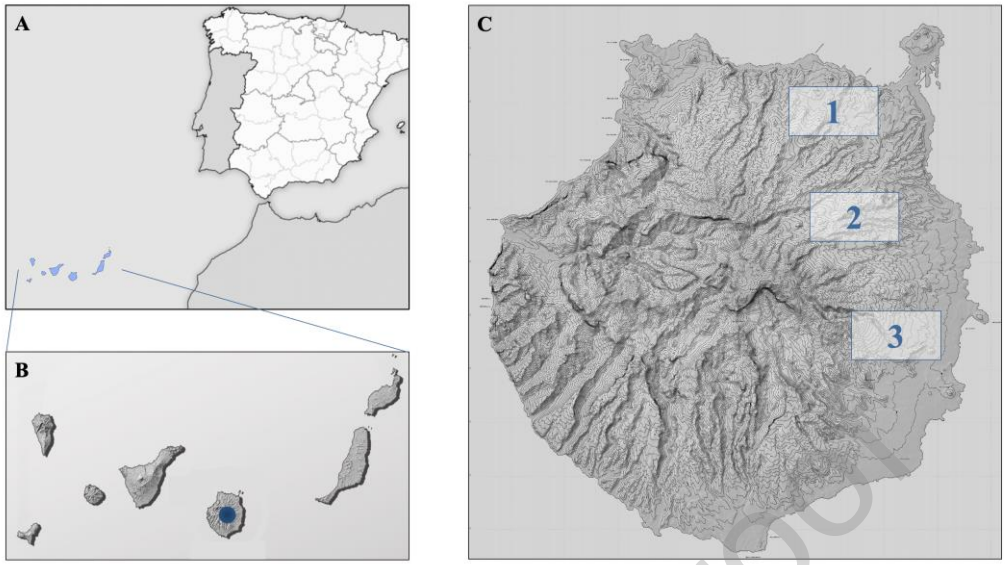


**Figure 1:** Distribution of cats sampled on the island of Gran Canaria. A. Location of the Canary Islands in the Atlantic Ocean; B. Location of the island of Gran Canaria in the Canary archipelago; C. Representation of the locations where samples were taken (1: Las Palmas de Gran Canaria/Arucas; 2: Santa Brígida; 3: Ingenio). Note: All the Salmonella positive samples were from the Ingenio (3) location.

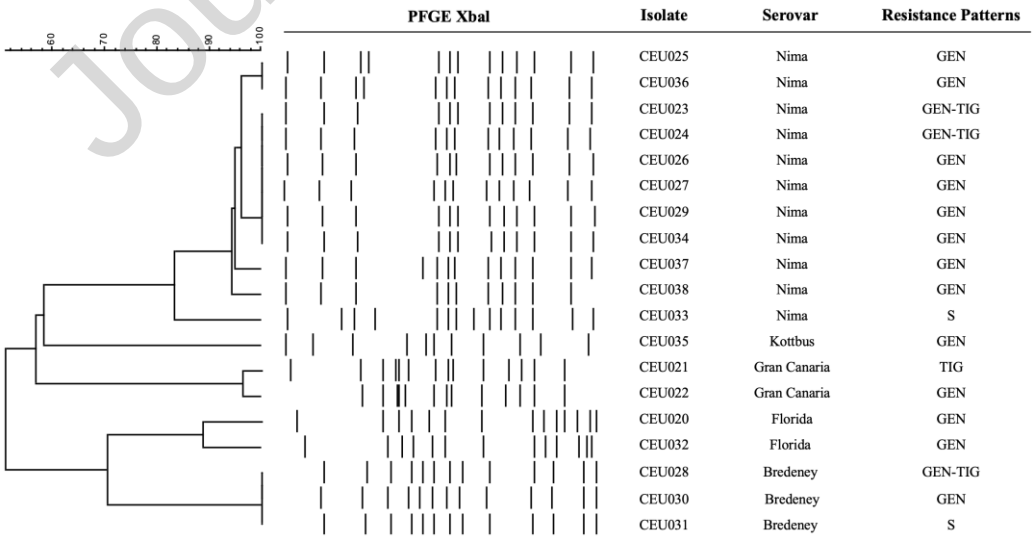

**Figure 2.** PFGE dendrogram of Xbal profiles of *Salmonella enterica* subsp. *enterica* isolates and their resistance patterns. GEN: Gentamicin, TIG: Tigecycline, S: Susceptible.

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**Conflicts of Interest**

The authors declare no conflict of interest.

# Highlights

- Free-living cats could be an important factor in the transmission of zoonotic diseases.
- Feral cats may be considered a reservoir of resistant *Salmonella* in Gran Canaria island (Spain).
- Same *Salmonella* clones suggest a common source of infection in feral cats colonies.