

# Spatial distribution of antimicrobial resistance of extra-intestinal clinical *Escherichia coli* isolated from poultry farms in western provinces of Cuba



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## Distribución espacial de resistencia antimicrobiana en aislados clínicos extraintestinales de *Escherichia coli* procedentes de granjas comerciales de provincias occidentales de Cuba

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**ABSTRACT:** Antimicrobial resistance (AMR) is a worldwide concern and a threat to global public health. On the other hand, *Escherichia coli* has played a significant role in the evolution of AMR. The current study aimed to characterise the spatial pattern of AMR of extra-intestinal clinical *E. coli* isolated from commercial poultry in western provinces of Cuba. Data for the study covered January-2014 to December-2017. Trend analysis and exploratory description were carried out using *R environment 4.0.4*. *ArcMap 10.4* was used for the spatial analysis by the Kernel Density Estimation method and visualisation map. Incremental trends in the frequency of resistance were observed during the study period. Kernel Density indicated that AMR was spatially distributed across the whole geographical region under study, although the highest density (high values) of AMR was located mainly in municipalities of Artemisa province. Areas of significantly higher and lower risk of AMR were identified in the Southeast and North of the region, respectively. Finally, the identification of the spatial distribution and relative risk surface of *E. coli* antimicrobial resistance from poultry farms in Cuba is a major step that contributes to optimise antimicrobial stewardship practices across the western region. This allows for improved preventive health measures and control strategies to prevent diseases and increase epidemiological surveillance.

**Key words:** Antimicrobial resistance, *Escherichia coli*, Kernel Density, relative risk, poultry.

**RESUMEN:** La resistencia antimicrobiana (RAM) es una preocupación mundial y una amenaza para la salud pública global. Por otra parte, *Escherichia coli* ha marcado una forma significativa en la evolución de la RAM. El presente estudio tuvo como objetivo caracterizar el patrón espacial de la RAM en aislados extraintestinales de *E. coli* procedentes de aves comerciales de provincias occidentales de Cuba. Los datos del estudio abarcaron de enero del 2014 a diciembre del 2017. El análisis de tendencias y la descripción exploratoria se realizó con el lenguaje de programación de *R versión 4.0.4*. Para el análisis espacial se utilizó *ArcGIS 10.4* mediante el método de estimación de densidad de Kernel y salida cartográfica. Se observaron tendencias al incremento en la frecuencia de resistencia durante el periodo de estudio. La densidad de Kernel indicó que la RAM se distribuyó espacialmente en toda la región geográfica de estudio, aunque la mayor densidad (valores altos) de RAM se localizó mayoritariamente en municipios de la provincia Artemisa. Se identificaron áreas de riesgo significativamente mayor y menor de RAM en el Sudeste y Norte de la región, respectivamente. Por último, la identificación de la distribución espacial y la superficie de riesgo relativo de resistencia antimicrobiana de *E. coli* procedente de granjas avícolas en Cuba es un paso importante que contribuye a optimizar las prácticas de administración de antimicrobianos en la región occidental. Esto permite mejorar las medidas sanitarias preventivas y las estrategias de control para evitar enfermedades y aumentar la vigilancia epidemiológica.

**Palabras clave:** Resistencia antimicrobiana, *Escherichia coli*, densidad de Kernel, riesgo relativo, aves comerciales.

## INTRODUCTION

Antimicrobial resistance (AMR) is a complex problem that best illustrates the One Health approach and it is widely perceived as a threat to human, animal, and environmental

health (1). The spread of resistance from animal sources negatively affects human health, directly by the spread of the resistant bacteria typically through food or indirectly by the spread of resistance genes from animal bacteria to human bacteria (2).

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To address related threats to AMR, various efforts and initiatives are coordinated through alliances between international and national organisations including a global action plan to tackle AMR (3). A recent review recognizes the collective need for limiting the emergence and spread of resistant pathogens and highlights the importance of an integrated and holistic multisectoral One Health approach to face AMR (4).

The World Health Organization report on global tricycle protocol (5) provides some specific and valuable approaches for integrated multisectoral surveillance to detect and estimate the prevalence of *Escherichia coli* as a key sentinel or indicator organism of AMR. Furthermore, *E. coli* strains are zoonotic pathogens, causing disease in animals and humans, respectively. Poultry meat is the food from animal origin most closely linked to human extraintestinal pathogenic *E. coli* (ExPEC) (6), requiring the proper use of antibiotics for its control based on susceptibility testing.

Current information about the geographical distribution of resistance is limited, laboratory capacity may be underdeveloped and the challenges of conducting comprehensive population-based surveillance are high (7). The demand for such data highlights the need for surveillance research and methods, given AMR surveillance is the core component of disease management and the foundation for a better understanding of the cause and spread of AMR (8). Calls for the monitoring, surveillance and prediction of AMR have prompted the design and implementation of surveillance systems at all geographic levels (9).

Some studies in Cuba address the environment, human patients, farm animals, and slaughterhouses as major components of the AMR problem (10-12). However, the identification of priority areas of intervention through trend analysis or AMR spatial distribution remains to be assessed. Consequently, there are knowledge gaps concerning the magnitude of AMR and intervention strategies in case of infections. Such information provides a meaningful basis for a more rational approach to the prescribing and use of antimicrobials, in agreement with regulations to prevent the development of AMR.

Retrospective studies can capitalise on AMR historical data aiming to identify trends, even spatially explicit. These approaches, besides identifying AMR spatial distribution, can anticipate opportunities for therapeutic success based on accumulated knowledge and categorise classes of antimicrobials or molecules whose use should also be reconsidered.

Kernel Density Estimation and Kernel smoothed relative risk surfaces to explore the spatial distribution of different events have been widely used (13,14). On this basis, Spatial-temporal models for the magnitude of AMR in food-producing animals could improve surveillance by targeting those areas and regions at

risk of higher AMR and help to anticipate when and where it may become a problem of greater concern.

This study, therefore, aimed to characterise the spatial pattern of antimicrobial resistance of extra-intestinal clinical *E. coli* isolated from commercial poultry in western provinces of Cuba.

## METHODS

### Study area

The study area included three western provinces (Artemisa, La Habana and Mayabeque), where high-density of commercial poultry farms are present. This area is about 8476,37 km<sup>2</sup> encompassing a Researching and Diagnosis Laboratory for avian diseases. The laboratory (LIDA, in Spanish) includes bacteriological diagnosis and AMR testing facilities (Figure 1).

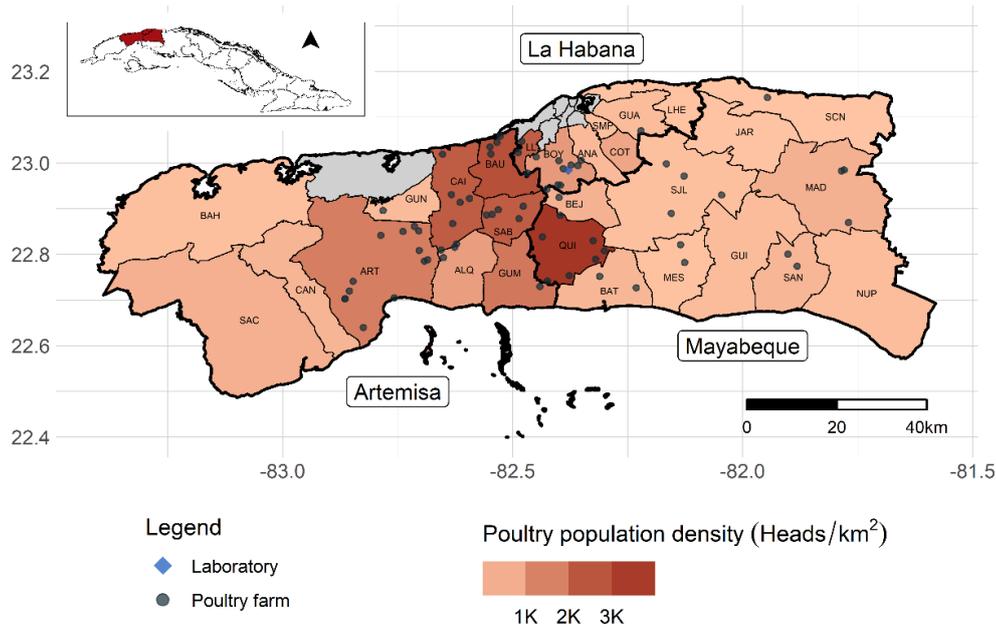
### Data sources

An analysis, derived from retrospective data on the susceptibility profile of extra-intestinal *E. coli* isolates from clinical cases associated with avian colibacillosis, was conducted. Data collection was carried out at the Poultry Research and Diagnosis Laboratory (LIDA) "Jesús Menéndez". Data correspond to isolates (n = 287) originating from 68 commercial poultry farms for four years (January-2014 to December-2017). Data were manually entered into a worksheet using *Microsoft Office Excel 2016* (15).

### Bacteria isolation/identification and antimicrobial susceptibility determination

Isolated samples were cultured in Tryptone Soya Broth and incubated aerobically overnight at 37°C. Subsequently, isolates were also streaked on MacConkey Agar. After 24 hours, plates were observed for examination of similar characteristic with *E. coli* (pink colony in MacConkey; Gram-negative coccobacilli). Samples were obtained from heart, kidney and liver of poultry suffering from septicaemia in the past 24 hours.

Antimicrobial susceptibility testing was performed by diffusion agar following the instruction of Clinical Laboratory Standard Institute guidelines (16). *E. coli* strains were evaluated against four veterinary important antibiotics from Oxoid disks (Oxoid™, Basings-toke, United Kingdom): enrofloxacin (ENR 5µg), norfloxacin (NOR 10µg), tetracycline (TE 30µg), and oxytetracycline (T 30µg). *E. coli* ATCC 25922 was used as a reference strain for the quality control of antibiotic sensitivity testing. Results were interpreted using CLSI clinical breakpoint for Enterobacteriaceae (16), as reference guide to classify in 3 categories as Susceptible (S), Intermediate (I) or Resistant (R).



**Figure 1.** Spatial location of commercial poultry farms and National Laboratory in the geographical studied area. Legend: Bahía Honda (BAH); San Cristóbal (SAC); Candelaria (CAN); Artemisa (ART); Guanajay (GUN); Caimito (CAI); Alquízar (ALQ); Güira de Melena (GUM); San Antonio de los Baños (SAB); Bauta (BAU); La Lisa (LL); Boyeros (BOY); Arroyo Naranjo (ANA); Cotorro (COT); San Miguel del Padrón (SMP); Guanabacoa (GUA); La Habana del Este (LHE); Santa Cruz del Norte (SCN); Jaruco (JAR), Madruga (MAD); San José de las Lajas (S JL); Bejucal (BEJ); Quivicán (QUI); Batabanó (BAT); Melena del Sur (MES); Güines (GUI); San Nicolás (SAN) and Nueva Paz (NUP). / Localización espacial de las granjas avícolas comerciales y del Laboratorio Nacional en la zona geográfica de estudio.

### Exploratory descriptive analysis

Resistance frequency was calculated as the proportion of isolates resistant to the antimicrobials researched in relation to the number of isolates tested. A linear regression analysis was made to determine the tendency of resistance in the study period. Time (years) was considered as an independent variable and antibiotic resistance per year as a dependent variable. The formula used was  $Y = \beta_0 + \beta_1 X$ , where  $Y$  is a dependent or outcome variable;  $X$  is an independent variable or so called predictor; and  $\beta$  model parameters (17). Determination coefficient  $R^2$  was used as a reference for measuring the goodness of fit of the models.

A descriptive analysis was performed to check the quality of the data, filter and describe the variables, and extract their general statistics (minimum value, maximum value, mean, median, 95% confidence interval, standard deviations, and standard error).

### Kernel Estimation Spatial Analysis

For spatial data representation, the Universal Transverse Mercator (UTM) coordinate system and Datum EPSG:4267- NAD27 were used as references. Data geo-processing was performed using the latitude and longitude (decimal degrees) of each poultry farm. Resistance of the Tetracycline and Fluoroquinolone classes were added in the context of point processes of

analysis (events), based on the geographical coordinates representing the exact location of poultry farms with the presence of *Escherichia coli* resistance.

A non-parametric Smoothing Method by Kernel Density Estimation (KDE) was carried out to analyse regions with high and low resistance to antibiotic classes. Therefore, Sheather & Jones plug-in (SJDPI) method was selected to calculate the bandwidth. According to those calculations, the spatial bandwidth was set to 2km and an output cell size of 1km<sup>2</sup>.

### Kernel Estimation of spatial relative risk

To study the relative abundance of AMR cases with respect to the at-risk population dispersion over a well-defined geographical region, the Kernel smoothing spatial relative risk surface method defined in Equation 4 was chosen.

$$\tilde{\rho}h_1, h_2(x|X, Y) = \frac{\log[\tilde{r}(x|X, Y)]}{\log[\tilde{f}h_1(x|X)] - \log[\tilde{g}h_2(x|Y)]}; x \in W \quad (4)$$

Where  $W$  : is the study window,  $X$  and  $Y$  the case and control data respectively, are two distinct samples of planar points assumed to originate from (unknown, possibly equivalent) density functions  $f$  (cases) and  $g$  (control),  $\tilde{f}h_1$  and  $\tilde{g}h_2$  the bandwidth kernel estimates of the case and control densities  $f$  and  $g$ . The ratio  $\tilde{r} = \tilde{f}h_1 | \tilde{g}h_2$  is an estimate of the relative risk

function  $r = f|g$ , more commonly expressed on the (natural) **log** scale as  $\rho = \log f|\log g$  (18).

A case-control data set was created for resistance to Tetracycline and Fluoroquinolone classes. AMR cases confirmed by LIDA were selected. As a control, the poultry farms studied without resistance signals but representative of the at-risk poultry population in the geographic area studied, were also selected. For all spatial risk surfaces, symmetric adaptive risk function estimates were calculated, using the pooled case/control data set to compute variable bandwidth factors (19). All estimates were edge-corrected to account for Kernel weight lost over the boundary of the study region and results were reported as log-relative risk surfaces **logf – logg** for symmetry around the ‘null’ log risk value of zero. Finally, the corresponding asymptotic p-value was estimated for each surface (18), and tolerance contours were superimposed at the 5 % significance level to delineate areas of significantly higher risk.

Descriptive statistical analysis and graphical visualisation were performed using the contributed package *ggplot2* v3.3.3 (20), bandwidth calculation was carried out using the package *EnvStats* v2.4.0 (21) and Kernel Estimation of spatial relative risk surface using the pa-

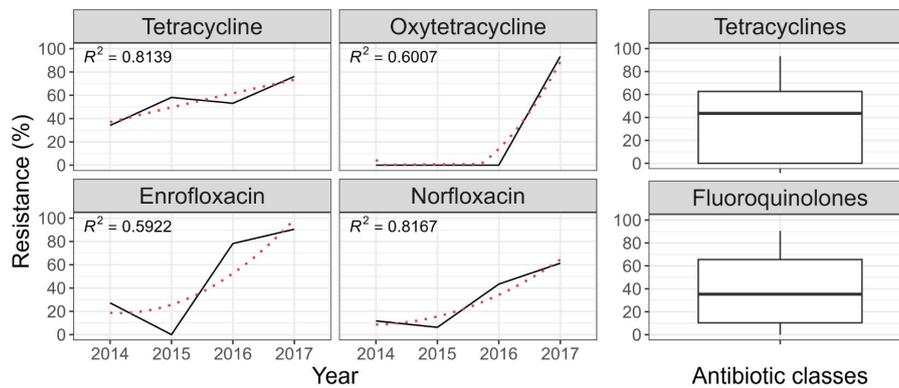
ckage *Sparr* v2.2.15 (18) in the *R environment* v4.0.4 (22). Spatial analysis, data preparation and mapping were performed using *ArcGIS* v10.4 (23).

## RESULTS

### Exploratory descriptive analysis

Two hundred eighty-seven extra-intestinal clinical *E. coli* isolates originating from 68 commercial poultry farms were assessed during the study period (2014-2017). The overall frequency of resistance in *E. coli* showed a wide variation (0-93%) against the antimicrobial tested, although an incremental trend of resistance was observed. The highest levels of resistance were found to Oxytetracycline (93 %), Enrofloxacin (90 %), Tetracycline (70 %), and Norfloxacin (61 %), in the last year (2017). Only low levels of resistance were observed between 2014 and 2015 (Figure 2).

Most isolates were highly resistant to Tetracycline (41.36 %) and Fluoroquinolone (39.83 %) classes, with smaller variations (Figure 2). Correspondingly, a distribution of resistance to Tetracycline (0 %, 43.60 %, 62.68 %) and Fluoroquinolone (10.38 %, 35.32 %, 65.62 %) classes, were observed in quartiles (Table 1).



**Figure 2.** Time trends and boxplot graphs of the frequency of *E. coli* antimicrobial resistance from commercial poultry farms in western provinces of Cuba during 2014-2017. Dashed lines show the linear trends. No association was found between resistance and year ( $p > 0.05$ ). / *Tendencias temporal y caja de bigotes de la frecuencia de resistencia antimicrobiana de E. coli procedentes de granjas avícolas comerciales de provinciales occidentales de Cuba durante 2014-2017. Las líneas discontinuas muestran las tendencias lineares. No se encontró asociación entre la resistencia y el año ( $p > 0.05$ ).*

**Table 1.** Descriptive statistics for boxplot of *E. coli* antimicrobial resistance from commercial poultry farms during 2014-2017. / *Estadística descriptiva de caja de bigotes de resistencia antimicrobiana de E. coli procedentes de granjas avícolas comerciales durante 2014-2017*

Antibiotic classes	Descriptive Statistics								
	Min. Value	Max. Value	1 <sup>st</sup> Quartile	2 <sup>nd</sup> Quartile (median)	3 <sup>rd</sup> Quartile	Mean	CI (95%)	Standard deviations	Standard error
Tetracyclines	0	93.33	0	43.60	62.68	41.36	26.3471-52.3803	36.8164	13.0168
Fluoroquinolones	0	90.48	10.38	35.32	65.52	39.83	27.4792-51.9082	34.1661	12.0795

\*CI (95%) Confidence Interval

## Kernel Estimation Spatial Analysis

The AMR of Tetracycline and Fluoroquinolone classes was spatially distributed throughout the geographical region studied, although the density distribution indicated that resistance to Tetracyclines was more widespread in the West region, while to Fluoroquinolones, it was more widespread in the East and West regions of the study area (Figure 3). During the period 2014-2017, a higher density (high values) of AMR was mostly located in the municipalities of Artemisa province. Nevertheless, areas with medium densities occurred in all the provinces with variable extensions.

A higher density of *E. coli* resistance to the Tetracycline class was identified in six municipalities (Alquízar, Artemisa, Bauta, Caimito, Güira de Melena, and San Antonio de los Baños) in Artemisa province, and in two municipalities (Boyeros and La Lisa) in Havana province.

On the other hand, the highest density of *E. coli* resistance to the Fluoroquinolone class was located in five municipalities (Alquízar, Artemisa, Bauta, Caimito, and Güira de Melena) of Artemisa, and in two municipalities (Boyeros and La Lisa) of Havana province, respectively. It should be noted that the high density of Kernel was also evident in several municipalities of Mayabeque: Bejucal, Batabanó, Madruga, Quivicán, and San Nicolás.

## Kernel Estimation of spatial relative risk

The relative risk surfaces for *E. coli* resistance to Tetracycline and Fluoroquinolone classes are presented in Figure 4. Two main areas (in the South and East of the study area) were observed to be at higher risk with respect to the underlying risk population. Lower risk areas were limited to the North.

For Tetracyclines, the greatest risk was observed in the South/South-East of Mayabeque province. Areas of lower risk were confined to the South-West, the North and a rural area in the North-East.

Compared to Tetracyclines, the highest risk surfaces for Fluoroquinolones were restricted to the extreme North and Northeast. Lower risk surfaces were more uniform throughout the study area, specifically in the West, South and a small portion in the Southeast.

## DISCUSSION

The current research provides the first insight about AMR trends and spatial risk in Cuba, specifically for AMR of extra-intestinal clinical *E. coli* from commercial poultry farms in western provinces. The followed approach offers alternatives for refining antibiotherapy interventions based on evidence from accumulated susceptibility data. The results evidenced the need to use other therapeutic options for the control of avian

colibacillosis in the farms where isolates with high resistance frequency to Tetracyclines and Fluoroquinolones were detected. This information is useful for overcoming the impossibility of defining antibiotherapy in near real-time based on susceptibility testing.

Existing studies have shown the limitations faced in the surveillance of AMR or have elucidated the difficulty in determining the antimicrobial susceptibility profile on a case by case before deciding on treatment in developing countries (7). As such, the approach based on AMR trends and spatial risk may represent a relevant solution, particularly in the cases of intensive commercial poultry, swine, or cattle, in which large populations at high rearing densities favour the rapidity of infectious processes and require timely control intervention.

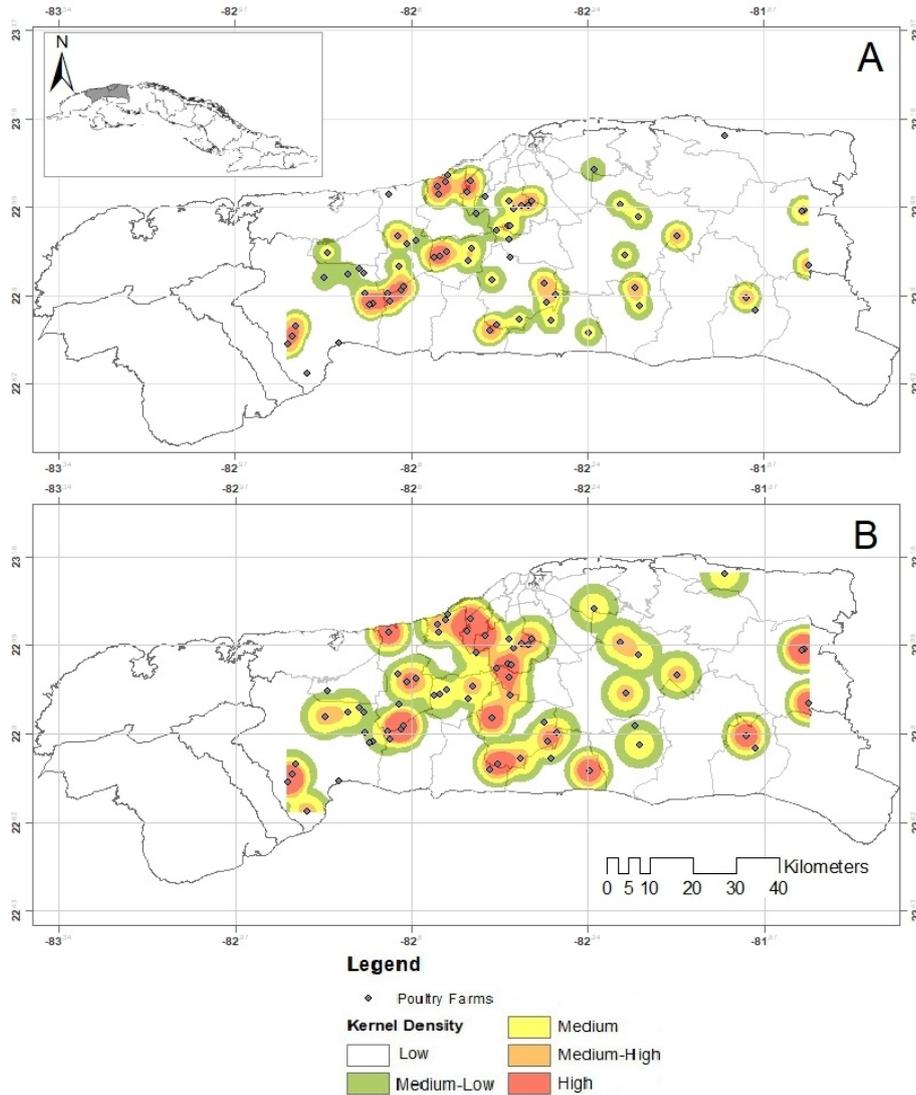
The present study focused on a region with a high density of poultry farms near a laboratory with AMR testing facilities. Such distinctiveness may offer a unique opportunity to understand AMR patterns and trends that may be representative and predominant among the poultry husbandry conditions. However, the evaluation of other areas in the country with high densities of food-producing animals where antimicrobial susceptibility testing may be insufficient is required.

The fact that the study encompassed a major and densely populated poultry region reinforces its significance to prevent AMR impact by giving a more appropriate vision of *E. coli* drug resistance patterns. The research highlights *E. coli* which is considered an extremely relevant and representative indicator of an antibiotic resistance issue of global clinical importance because it is part of the normal microbiota of humans and animals and it is also present in the environment (24).

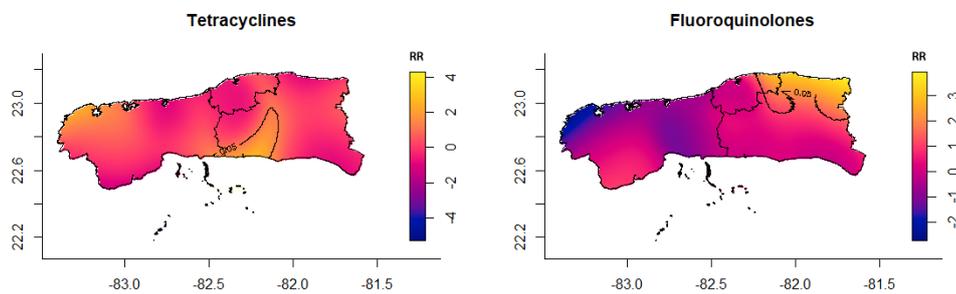
The spatial transmission of antimicrobial-resistant bacteria has been understudied in comparison to pathogens (25). Additionally, there is no consensus on AMR surveillance methodology, particularly on systematic data collection in Cuba. These further limits several studies.

Various drivers are contributing factors to the occurrence of AMR in farms and the environment (26). The identification of several patterns by KDE in Artemisa, La Habana and Mayabeque might suggest the implication of different AMR contributors such as antimicrobial usage, hygiene sanitation practices, horizontal transmission among animals, and feed strategies, as observed in other studies (25,27).

The increasing trend observed in resistance rates with broader spatial and temporal consideration is of concern, and closer monitoring of this problem is needed. A previous smaller-scale local and cross-sectional resistance study also revealed high rates of AMR (28), but lacked a geographically explicit consideration of AMR risk.



**Figure 3.** Kernel Density of antimicrobial resistance of extra-intestinal clinical *E. coli* isolated from commercial poultry farms in western provinces of Cuba (2014-2017). (A) Tetracycline class and (B) Fluoroquinolone class. / Densidad de Kernel de resistencia antimicrobiana en aislados clínicos extra-intestinales procedentes de granjas avícolas comerciales de provincias occidentales de Cuba (2014-2017). (A) Tetraciclinas y (B) Fluoroquinolonas.



**Figure 4.** Estimated log relative risk for antimicrobial resistance of extra-intestinal clinical *E. coli* isolated from commercial poultry farms in western provinces of Cuba. Tolerance contours are superimposed as solid lines at the 95 % confidence level. Solid lines indicate higher risk areas. / Riesgo relativo logarítmico estimado de resistencia antimicrobiana en aislados clínicos extra-intestinales procedentes de granjas comerciales de provincias occidentales de Cuba. Los contornos de tolerancia se superponen como líneas sólidas con un nivel de confianza del 95 %. Las líneas continuas indican zonas de mayor riesgo.

AMR is often inferred as dependent on antimicrobial usage (29,30). Accordingly, Tetracyclines, recognized as the most widely used antimicrobial in veterinary medicine (31), were the antibiotic class with the highest level of resistance, in the present study. The repeated exposure to therapeutic agents contributes to an increased selective pressure and, consequently, to a higher prevalence of antimicrobial resistance (32,33). Tetracyclines are a first-line antibiotic often used prior to determining the antibiotic resistance profile of a pathogen. As Tetracycline is a naturally derived compound, bacteria can be exposed to these agents in nature and outside of any human use for disease treatment, for livestock prophylaxis. In addition, Tetracycline resistance is plasmid-mediated, with a wide variety of genetic determinants (34).

On the other hand, the use of antimicrobials as growth promoters has been forbidden in Cuba (35). Therefore, the use of sub-therapeutic doses of antibiotics would be of minor importance as a driver of resistance. Nevertheless, there is still a need for global progress in recording and reporting antimicrobial use data (AMU) for more effective management of the associated risk. In fact, the maintenance of general health and hygiene practices in food-producing animals is paramount for improving animal welfare and production and, consequently, reducing AMU. In this sense, high-density poultry areas would be the first line to verify and promote good hygiene and husbandry practices, as well as future studies on AMU. Particular consideration is needed in the control of vectors (e.g., flies, beetles and cockroaches), which can play a potential role in the transfer of pathogens and resistance genes from different environmental levels (27).

In addition, intra-country trade and animal mobility may play a more important role than differences in antimicrobial exposure in explaining geographic differences in antimicrobial resistance levels (36). In Cuba, the commercial poultry farming at the national level is based on provincial enterprises that used to be self-sufficient for poultry replacements, which has given a certain level of regionalization. However, animal batches within the egg production system, as the most predominant in Cuba (37), usually change location two to three times from hatching to the final productive facilities within each province.

The feed composition and the poultry gut microbiome can lead to an increase in *E. coli* (38). However, the influence of this factor could not be considered in the present study. Such analyses could shape future research direction, given that *E. coli* is considered a major threat to the poultry industry and public health (39) and it is also likely the most actively transmitted microbe between humans and poultry (40).

### Limitations and strengths

Although the study was limited to one region, it included an important productive area totalling

152 poultry farms, with an animal population of over 5 109 542 individuals in 8476,37 km<sup>2</sup>. Through this study, the knowledge of AMR spatial patterns of *E. coli* from commercial poultry farms is enhanced. This research provides information on AMR risk areas to the most used antibiotic classes in poultry production areas in Cuba. Such territories must be prioritized in the intensification of AMR surveillance or containment measures in anticipation of the potential evolution of transmission events.

The study emphasises the need for antimicrobial stewardship interventions, to limit antimicrobial usage and preserve the effectiveness of these molecules wherever possible. More importantly, the research offers new insights for stakeholders to make evidence-based decisions and policies to prevent and control the occurrence and dissemination of AMR.

The possibility of analyzing more recent data was hampered by declining evidence and other limitations during COVID-19 pandemic, which broke out in 2020 and lasted more than three years. Nevertheless, the continuous four-year study period may reflect the current trend, although COVID-19 also involved a significant reduction in AMU, the influence of which on AMR profiles has yet to be assessed. This should be considered for further studies.

### CONCLUSIONS

This research revealed, for the first time, the trends and spatial risk for AMR of extra-intestinal clinical *E. coli* from commercial poultry farms in Cuba. These findings characterise a reference point for improving antibiotherapy intervention on commercial poultry farms throughout the western geographic region of Cuba. The results underline the need to reinforce the implementation of preventive measures that contribute to reduce the frequency of bacterial infection and act as the main driver of antibiotic use.

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