

Bacteria Isolated from Free-range Chicken (*Gallus gallus domesticus*) Eggs Sold in Semiarid Conditions and its One Health Impact

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ABSTRACT

Background: Food contamination is an important and growing public health concern due to the risk of foodborne illnesses. In this context, the egg, consumed all over the world, stands out. This food has been pointed out as a carrier of several bacteria, causing outbreaks of food toxoinfection. The production of free-range chicken (*Gallus gallus domesticus*) eggs has been an alternative for generating income for producers in Brazil; however, there is no monitoring of the sanitary quality of this product. The objective of this study was to identify microorganisms in 128 free-range chicken eggs sold in open markets in the semiarid region of Northeastern Brazil.

Materials, Methods & Results: The study was carried out at commercial fairs in the state of Rio Grande do Norte, Brazilian semiarid region. The minimum number of eggs to be used was determined by the formula for simple random samples considering the following parameters: expected frequency of positive eggs of 50% (for sample maximization), 95% confidence level and sampling error of 10%. The minimum number of eggs to be sampled was 97; however, 128 eggs were obtained in the period from August 2018 to April 2019. The internal content of the eggs was subjected to bacteriological culture using an adapted methodology, and for the *in vitro* susceptibility assessment the disk diffusion method on Müller-Hinton agar was used. In addition to biochemical tests, Gram-negative bacteria were subjected to microbiological diagnosis using the MALDI-TOF (Matrix Associated Laser Desorption-Ionization - Time of Flight) technique. Bacterial growth was found in 40 (31.3%) eggs. The microorganisms most frequently isolated were *Staphylococcus* spp. (27.5%), *Bacillus* spp. (15%) and *Enterobacter cloacae* (25%). There was bacterial growth in albumens [n = 10; 7.8%] and in egg yolks [n=38; 29.7%] ($P < 0.001$). The antimicrobials that showed highest resistance rates were ampicillin (95.5%), amoxicillin + clavulanic acid (77.3%), cephalothin (72.7%), nalidixic acid (72.7%), cephalexin (68.2%), imipenem (63.3%) and ertapenem (59.1%).

Discussion: The frequency of bacterial isolation obtained in this study was high and reflects contamination of the eggs, which can occur through the shell, and factors such as air humidity, time and temperature favor the migration of bacteria from the shell to the internal content of egg. Food toxoinfection occurs due to improper conditions in food processing, and it is essential to guarantee correct handling, promoting health quality. Therefore, some care related to egg management is necessary, from primary production to commercialization, ensuring food safety for consumers. Another point to be highlighted concerns the way of marketing this product. Eggs from free-range chickens are usually sold in open markets, kept at room temperature from production to final distribution, mainly in the semiarid region of Northeastern Brazil. Temperature, storage time and chicken's characteristics (lineage, age, nutritional management and health status) are factors that have a direct influence on the quality of the food offered to the consumer. The high frequency of isolated bacteria warns of the implementation of control measures to avoid contamination of this product and the importance of the correct use of antimicrobials in poultry, in order to avoid the spread of resistance-carrying pathogens, minimizing economic, health and environmental impacts.

Keywords: *Enterobacteriales*, bacterial growth, eggs, free-range chickens, public health.

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INTRODUCTION

Food contamination throughout the production stages is an important and growing public health concern due to the risk of foodborne diseases [12]. In this way, the egg, a food consumed worldwide, stands out due to its high nutritional value. However, this food has been identified as a carrier of several bacteria, mainly of the *Salmonella* genus [8]. In addition to causing food deterioration, pathogenic microorganisms are capable of causing infectious diseases to consumers when introduced into the food chain [13]. These pathogenic bacteria resistant to antimicrobials have been a matter of concern for public health [10], especially those of the order *Enterobacterales*, which are agents that inhabit the intestinal tract of humans and animals [33].

The spread of bacterial resistance among different ecosystems has been studied as a broad concept within the context of One Health, emerging as a consequence of human, animal and environmental convergence, and has been growing as a strategy for better understanding and resolution of contemporary health problems [28]. In this context, considering the factors related to the importance of microbiological quality of eggs for human consumption, as well as the need to consume a healthy product free of pathogens and the scarcity of studies with free-range chicken eggs, the objective of this study was

to identify the presence of microorganisms in free-range chicken (*Gallus gallus domesticus*) eggs marketed in open markets in the semiarid region of Northeastern Brazil and their implication in One Health.

MATERIALS AND METHODS

Study area and sampling

The study was carried out at commercial fairs in the state of Rio Grande do Norte, Brazilian semiarid region. The counties included were Acari, Caicó, Carnaúba dos Dantas, Currais Novos, Jardim do Seridó, Jucurutu, Lagoa Nova, São José do Seridó, São Paulo do Potengi and Serra Negra do Norte (Figure 1). The eggs were transported at room temperature to the Microbiology Laboratory of the Veterinary Hospital Dr. Ivon Macedo Tabosa of the Federal University of Campina Grande (UFCG), Campus Patos - PB, where they were processed.

The minimum number of eggs to be used was determined by the formula for simple random samples [32] considering the following parameters: expected frequency of positive eggs of 50% (for sample maximization), 95% confidence level and sampling error of 10%, which resulted in a minimum sample number of 97 eggs. However, 128 eggs were obtained in the period from August 2018 to April 2019.

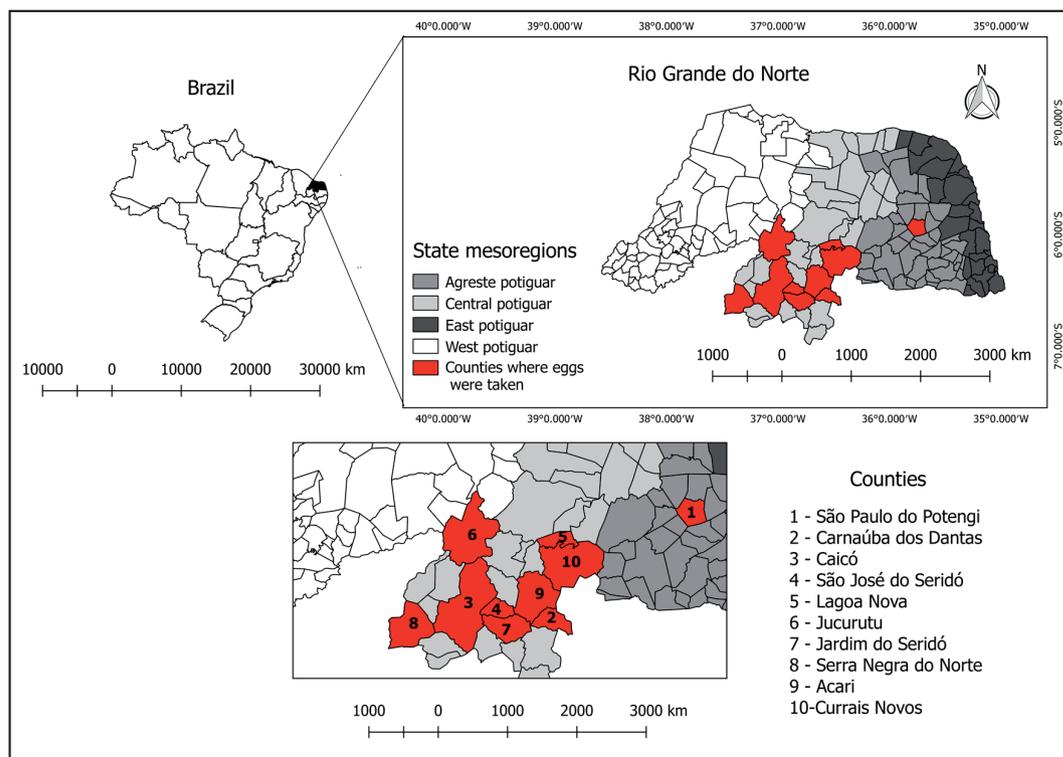


Figure 1. Counties from which the free-range chicken (*Gallus gallus domesticus*) eggs were collected from August 2018 to April 2019.

Bacterial isolation and identification

The outer part of the egg (shell) was cleaned with 70% alcohol and shortly afterwards, by viewing through an oviscope, a small part of this shell was broken with sterile forceps in the lower portion where the air chamber is located, forming an air hole of approximately 1 cm, without damaging the membrane between shell and albumen to separate the albumen and yolk using a syringe for aspiration. Then, the aspirated material (albumen and yolk separated) was enriched in Brain Heart Infusion (BHI)¹ broth in the ratio of 2 mL of egg content in 3 mL of BHI for 24 h at temperature of 37°C and later sown in Petri plates containing 5% defibrinated sheep blood agar¹, MacConkey agar¹, and agar agar¹ at a depth of 4 mm. After cultivation, the plates were incubated at 37°C in aerobiosis for 24-48 h to check for bacterial growth. The bacterial colonies were subjected to bacterioscopic examination using the Gram stain method and identified by biochemical tests: TSI, motility, malonate, indole production, urease production, gelatinase production, phenylalanine deaminase production, citrate use, methyl red reaction and Voges-Proskauer, lactose fermentation, esculin hydrolysis, nitrate reduction, catalase, oxidase and coagulase [22]. In addition to biochemical tests, Gram-negative bacteria were subjected to microbiological diagnosis using the MALDI-TOF (Matrix Associated Laser Desorption-Ionization - Time of Flight) technique [3].

In vitro antimicrobial susceptibility assessment

For the *in vitro* antimicrobial susceptibility assessment, the disk diffusion on Müller-Hinton¹ agar method was used [7,11]. The bacterial concentration to perform the antibiogram corresponded to the turbidity of the 0.5 McFarland scale and the thickness of the Müller-Hinton agar layer in a 4 mm plate. The antibiotic discs were kept at room temperature for 20-30 min before their use, with the placement of them on plates performed within 15 min. After sowing, the plates were kept at incubation temperature of 35-37°C. The results were interpreted by reading inhibition halos [7,8]. The antimicrobials² used were: aminoglycosides - amikacin (30 µg), gentamicin (10 µg) and neomycin (30 µg); beta-lactams - ampicillin (10 µg), cephalexin (30 µg), cephotaxin (30 µg), ceftazidime (30 µg), cephalotin (30 µg) and amoxicillin + clavulanic acid (30 µg); quinolones - piperimidic

acid (20 µg), nalidixic acid (30 µg), enrofloxacin (5 µg) and norfloxacin (10 µg); carbapenems - ertapenem (10 µg), imipenem (10 µg) and meropenem (10 µg); others - chloramphenicol (30 µg) and tetracycline (30 µg).

Statistical analysis

To compare the frequencies of bacterial agents isolated from eggs, the binomial test was used, and to compare the frequency of positivity between albumen and yolk the chi-squared test was used. The analyzes were performed in the R [27] environment and the R-Studio interface with 5% significance level.

RESULTS

Of the 128 eggs analyzed, bacterial growth was found in 40 (31.3%). Table 1 shows the frequencies of positivity according to the counties investigated. Only in 3 counties positive eggs were not obtained, and the frequencies of positivity ranged from 14.4% in Caicó to 72% in São José do Seridó.

Table 2 shows the frequencies of isolated microorganisms in the positive eggs (n = 40). Eighteen bacteria (45%) were Gram-positive and 22 (55%) Gram-negative. The microorganisms isolated were *Staphylococcus* spp. (27.5%), *Bacillus* spp. (15%), *Stenotrophomonas maltophilia* (7.5%), *Klebsiella oxytoca* (7.5%), *Cronobacter sakazakii* (2.5%), *Escherichia coli* (7.5%), *Corynebacterium* spp. (2.5%), *Enterobacter cloacae* (25%) and *Alcaligenes faecalis* (5%). There was significant difference in the proportions of isolated agents ($P = 0.004$).

Samples were also analyzed separately, with 128 albumens and 128 egg yolks. There was bacterial growth in 10 (7.8%) albumens and 38 (29.7%) egg yolks ($P < 0.001$). The frequencies of agents isolated in albumens (n = 10) and yolks (n = 38) are shown in Table 3. The most frequently isolated bacteria in albumens were *Enterobacter cloacae* (40%), *Staphylococcus* spp. (20%) and *Escherichia coli* (20%), and in yolks were *Staphylococcus* spp. (23.7%), *Bacillus* spp. (15.8%) and *Enterobacter cloacae* (12%).

The resistance patterns of 22 isolates of Gram-negative bacteria in relation to 18 antimicrobials are shown in Table 4. The antimicrobials that showed the highest resistance rates were ampicillin (95.5%), amoxicillin + clavulanic acid (77.3%), cephalothin (72.7%), nalidixic acid (72.7%), cephalexin (68.2%), imipenem (63.3%) and ertapenem (59.1%).

Table 1. Bacteriological isolation positivity in free-range chicken (*Gallus gallus domesticus*) eggs sold in commercial fairs in Brazilian semiarid from August 2018 to April 2019, according to county.

County	Eggs (N)	Positive eggs	
		Total	Frequency (%)
Acarí	5	2	40
Caicó	54	8	14.8
Carnaúba dos Dantas	3	2	66.
Currais Novos	5	0	0
Jardim do Seridó	7	0	0
Jucurutu	10	3	30
Lagoa Nova	5	0	0
São José do Seridó	25	18	72
São Paulo do Potengi	10	5	50
Serra Negra do Norte	4	2	50
Total	128	40	31.3

Table 2. Frequency of bacteria isolated (N = 40) in free-range chicken (*Gallus gallus domesticus*) eggs sold in commercial fairs in Brazilian semiarid from August 2018 to April 2019.

Bacteria	Positive (N)	Frequency (%)
<i>Staphylococcus</i> spp.	11	27.5
<i>Bacillus</i> spp.	6	15
<i>Stenotrophomonas maltophilia</i>	3	7.5
<i>Klebsiella oxytoca</i>	3	7.5
<i>Cronobacter sakazakii</i>	1	2.5
<i>Escherichia coli</i>	3	7.5
<i>Corynebacterium</i> spp.	1	2.5
<i>Enterobacter cloacae</i>	10	25
<i>Alcaligenes faecalis</i>	2	5
Total	40	100

Table 3. Bacteria isolated in albumen (N = 10) and yolk (N = 38) from free-range chicken (*Gallus gallus domesticus*) eggs sold in commercial fairs in Brazilian semiarid from August 2018 to April 2019.

Bacteria	Albumen		Yolk	
	Positive (N)	Frequency (%)	Positive (N)	Frequency (%)
<i>Staphylococcus</i> spp.	2	20	9	23.7
<i>Bacillus</i> spp.	0	0	6	15.8
<i>Enterobacter cloacae</i>	4	40	12	31.6
<i>Klebsiella oxytoca</i>	1	10	4	10.5
<i>Escherichia coli</i>	2	20	4	10.5
<i>Corynebacterium</i> spp.	0	0	1	2.6
<i>Alcaligenes faecalis</i>	1	10	2	5.3
Total de amostras	10	100	38	100

Table 4. Antimicrobial susceptibility profile of 22 Gram-negative bacteria isolated from free-range chicken (*Gallus gallus domesticus*) eggs sold in commercial fairs in Brazilian semiarid from August 2018 to April 2019.

Antimicrobial agent	Resistance profile (%)		
	Susceptible	Intermediate	Resistant
AMINOGLYCOSIDES			
Amikacin (30 µg)	77.3	4.5	18.2
Gentamicin (10 µg)	63.6	4.5	31.9
Neomycin (30 µg)	45.5	18.2	36.4
β-LACTAMS			
Amoxicillin + clavulanic acid (10 µg)	18.2	4.5	77.3
Ampicillin (10 µg)	4.5	0	95.5
Cephalexin (30 µg)	22.7	9.1	68.2
Cephotaxin (30 µg)	54.5	9.1	36.4
Ceftazidime (30 µg)	95.5	0	4.5
Cephalotin (30 µg)	22.7	4.5	72.7
QUINOLONES			
Pipemidic acid (20 µg)	31.8	18.2	45.5
Nalidixic acid (30 µg)	22.7	4.5	72.7
Enrofloxacin (5 µg)	68.2	4.5	27.3
Norfloxacin (10 µg)	59.1	4.5	36.4
CARBAPENEMS			
Ertapenem (10 µg)	41	0	59.1
Imipenem (10 µg)	31.9	4.5	63.3
Meropenem (10 µg)	40.9	13.6	45.5
OTHERS			
Chloramphenicol (30 µg)	68.2	4.5	27.3
Tetracycline (30 µg)	54.5	9.1	36.4

DISCUSSION

A limitation of the survey was the availability of eggs at the time of collection, which could justify the amount (n = 128) of eggs used, however, this number was deemed by sample calculation. Furthermore, the adapted methodology used to bacterial isolation is very laborious and didn't allow the processing of a larger amount of eggs at the same time.

The frequency of bacterial isolations obtained in this study was high and reflects high contamination of eggs, which can occur through the shell, and factors such as air humidity, time and temperature favor the migration of bacteria from the shell to the internal content of egg [2]. It is known that most foodborne infections occur due to improper conditions in food

processing, so that it is essential to guarantee correct handling to promoting sanitary quality. Therefore some precautions related to egg handling are necessary to preserve their original quality as much as possible, from primary production to commercialization, ensuring food safety for consumers.

Another point to be highlighted concerns the way of marketing this product. Eggs from free-range chickens are usually sold in open markets, kept at room temperature from production to final distribution, mainly in the semiarid region of Northeastern Brazil. Temperature, storage time and chicken's characteristics (lineage, age, nutritional management and health status) are also factors that have a direct influence on the quality of the egg offered to the consumer [25]. A wide variety of isolated bacteria was verified,

especially the *Staphylococcus* spp. (27.5%), *Bacillus* spp. (15%) and *Enterobacter cloacae* (25%). This high frequency can result from improper handling, storage and transport, causing the deterioration of this food. *Staphylococcus aureus*, for example, multiplies in food and produces toxins leading to staphylococcal poisoning [21], being responsible for foodborne diseases and is considered the third most prevalent etiological agent in the development of these diseases, with about 5.7% of cases [4].

Of the 6 Gram-negative bacterial genera found, some enterobacteria responsible for egg deterioration, such as *Alcaligenes faecalis*, stood out. It is not known for certain whether this high contamination comes from the chicken itself, environment or egg handling, but it is known that the egg can be contaminated after laying, when it comes into contact with feces through the microorganism's penetration and formation of the follicle of the yolk and/or formation of the albumen in the oviduct, before the formation of the shell, allowing the production of contaminated eggs [17]. However, microbiological studies reveal that contamination occurs, for most bacteria, mainly after laying. This horizontal spread is characterized by bacterial contamination in the passage through the cloaca or in contact with the environment and in its manipulation, with a direct correlation between egg and environment contamination [19].

The presence of *Enterobacter cloacae* is a worrying factor for the integrity of the eggs, as this agent is one of the main microorganisms that have a great capacity to cross the egg shell and its membranes. In research carried out in Australia, it was observed that after the evaluation of the shell surface, shell pore and internal content of 153 eggs, *Enterobacter cloacae* was the most found. *Citrobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Leclercia* spp., *Proteus* spp., *Salmonella* spp., *Serratia* and *Yersinia* spp. were also found [14].

In Brazil, there is a concern in the inspection and control of food quality, however, the greater focus of research on eggs is related to the presence of *Salmonella* spp., and according to Normative Instruction No. 62 of the Ministry of Agriculture, Livestock and Supply (MAPA) of Brazil, the methodology recommended for the evaluation of hygienic-sanitary quality and inspection, as well as the Technical Regulation on microbiological standards for food (RDC01/2001),

which deals with microbiological standards related to this agent, is directed for eggs in laying hens systems, unlike the system considered free-range, in which there is no such control [5,6].

Although *Salmonella* spp. was not detected in the analyzed samples, it was found that all samples showed contamination by bacteria with greater prominence in the order *Enterobacteriales*. This is also consistent with the study by Gomes Filho *et al.* [15], who evaluated 180 eggs from backyard hens and 80 eggs purchased from local markets and did not detect *Salmonella* spp.; however, there was growth of other agents. In our work, there was a concern to detect pathogens, not only *Salmonella* spp., but others that can compromise the integrity of this food and, consequently, cause damage to public health. Thus, an adaptation of the methodology recommended by MAPA was made, demonstrating its effectiveness in isolating Gram-positive and Gram-negative bacteria without the need to establish different isolation methods for each pathogen.

Regarding the comparison of the isolation frequencies in albumen and yolk, a higher proportion of bacteria isolated from yolk (29.7%) was found in comparison with albumen (7.8%). Once contamination by bacteria in the internal content of eggs occurs, they can cause damage to the consumer [31], such as foodborne diseases. These diseases can lead the patient to septic shock and death, in addition to affecting the economy in general [23]. To reduce the damage, refrigeration of the product would be an alternative; however, it would increase the production cost, which makes most of the free-range eggs that are sold in the Brazilian market at room temperature [20].

The patterns of antimicrobial resistance obtained in this study may be associated with the frequent use or even underdoses of these antimicrobials in disease therapy, or even with the use in breeding and feeding management. The use of antimicrobials in poultry is a common practice in the semiarid, aiming at increasing animal productivity, or controlling pathogens. However, this use is often made in an empirical and indiscriminate manner, providing the increasing appearance of bacterial strains resistant to these drugs, causing economic and health impacts. Within this context, research demonstrates an increase in the resistance of Gram-negative bacteria that are isolated from farm animals, especially those belonging to the

order *Enterobacteriales* [1,18,26]. This scenario has culminated in the increasingly frequent appearance of multidrug-resistant species, requiring multidisciplinary efforts for prevention and control, in addition to laboratory detection.

Veterinary drugs frequently used are β -lactam antimicrobials, aminoglycosides and tetracyclines [30], the latter being used as feed additive for poultry and pigs [33]. Quinolones are extensively used in the treatment of chickens infected with *Salmonella* Enteritidis, however, in the present study, in addition to resistance to classes of β -lactams and quinolones, resistance to classes of antibiotics such as carbapenems was also found, which causes surprise due to its unusual use in the routine of the veterinary medical clinic and animal production. These antimicrobials have a greater spectrum of action, used in clinical practice, indicated for severe hospital infections or as a last resort when other antimicrobials fail [16].

The presence of resistant bacteria in food is a worrying factor and has a worldwide impact in view of the potential for transferring antimicrobial resistant pathogens to humans, animals and the environment [24], which implies risks associated with the incorrect use of antibiotics in animal production, which consequently reflects on humans and the environment [9]. Antimicrobial resistance in bacteria of the order *Enterobacteriales* is a worldwide public health problem, as it is important in both veterinary and human medicine [29], and even though it is widespread in Brazilian territory, there is no notification of such resistance in all federative units. Nevertheless, Brazil does not yet have a national microbial resistance surveillance program.

Although the phenomenon of resistance is undeniable, there is, to date, no risk analysis model proposed for the study of bacterial resistance at the field level, that is, one that assesses the real contribution of the use of antimicrobials in farm animals and products of animal origin, such as free-range chicken eggs, for situational diagnosis of the magnitude of the problem. Therefore, the present survey guides measures of hygienic-sanitary control, prevention and control of the spread of bacterial resistance, such as the implementation of precautions for the correct use of antibiotics.

CONCLUSION

The methodology employed in the present study allowed the isolation of Gram-positive and Gram-negative bacteria from the internal content (albumen and yolk) of eggs from free-range hens from the Brazilian semiarid, including pathogens with an impact on public health and with potential for antimicrobial resistance. In addition, the high frequency of isolated bacteria warns of the implementation of control measures to avoid contamination of this product and the importance of the correct use of antimicrobials in poultry, in order to avoid the spread of resistance-carrying pathogens, minimizing economic impacts, health and environmental issues.

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