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Characterization of *Escherichia coli* Strains Isolated from Foods of Animal Origin and the Hands of School Kitchen Operators in the Mono Department of Benin

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Abstract: Antibiotic resistance in pathogenic bacteria has become an issue of major global importance, which has hurt human and veterinary medicine, causing real public health problems. This study aimed to characterize *Escherichia coli* strains isolated from foods of animal origin sold in schools and the hands of school kitchen operators in the Mono department of Benin. *Escherichia coli* strains were isolated using Tryptone Bile X-Glucuronide (TBX BAC ISO-16649-2,3) and identified by using 16S rRNA sequencing. Antibiotic resistance was performed using a list of 10 antibiotics selected on 55 *Escherichia coli* strains. The disk diffusion method was used to perform the antibiogram. The virulence genes *stx1 stx2* and *eae were* then searched using their specific primers on 55 isolates by PCR. This study showed that *Escherichia coli* isolates were resistant to ampicillin, tetracycline, and amoxicillin-clavulanic acid (64 82 %). Thus, the phenotype AMP-AMC-CEP was present in most of the predominant multidrug resistance (MDR). Molecular characterization of virulence genes showed the presence of *stx1* in 16.4% ($n = 9$) of the isolates tested. Given that, problems of antibiotic resistance are increasingly being observed in most pathogenic bacteria, including *Escherichia coli*, it is essential to study the reservoir of germs in the commensal flora with potential transport and to prospect for alternative solutions to prevent the growth of pathogens after contamination of foodstuffs, for effective control of food-borne outbreaks caused by this pathogenic micro-organism.

Keywords: antibacterial resistance; *Escherichia coli*; foods of animal origin; virulence genes

1. Introduction

Foodborne outbreaks caused by *Escherichia coli are increasingly* common throughout the world today, with diarrhoeal forms representing the main cause of hospitalization in Africa [1]. In public health, food safety is an area of major importance and concern, especially when food of animal origin is handled in a highly contaminated environment [2,3]. In developing countries, food establishments such as collective restaurants or school canteens are characterized by a lack of hygiene and financial resources to invest in safer equipment, a lack of education for kitchen operators, and poor food handling practices that can contribute to a significant increase in the incidence of foodborne [4–6]. *Escherichia coli is* a bacterium found in the digestive tract of humans and animals, especially in the intestinal flora, which can survive for 4 to 12 weeks in water. It is therefore used as an indicator to determine fecal contamination in food and drinking water [7–9]. In foodborne infections caused by *Escherichia coli, the* most common pathogenic forms are enteropathogenic *E. coli* (EPEC), toxigenic *E.*

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coli Shiga (STEC), enterotoxigenic *E. coli* (ETEC), and diffuse adherence *E. coli* [10–12]. At the zoonotic level, one of the most important agents in terms of public health is *E. coli* EHEC, which can be transmitted to humans through the consumption of contaminated food, leading to bloody diarrhea and high mortality [13–15]. This bacteria is capable of producing a biofilm and is particularly resistant to antimicrobial agents [9]. Furthermore, the inappropriate and abusive use of antibiotics has increased bacterial resistance to antimicrobial agents [16,17]. Studies have shown the resistance and multi-resistance to antibiotics of *E. coli* EHEC isolated from food of animal origin in several collective restaurants in some African countries [18,19]. Food-borne diseases are often associated with antibiotic resistance in bacteria in developing countries [20,21]. This study aimed to characterize *Escherichia coli* strains isolated from food of animal origin sold in schools and palmar face of school kitchen operators in the Mono department of Benin. This will supplement the data already available, which is needed to raise awareness among livestock farmers, veterinary surgeons, and those involved in human medicine about the responsible use of antibiotics.

1.1. Food's Sample Collection

Samples of ready-to-eat food of animal origin presenting a risk of contamination by pathogenic bacteria and commonly consumed by schoolchildren were identified through a survey. They were aseptically collected in Stomacher bags and placed in a cooler containing ice packs. A total of 100 food samples, including 40 fried fish, 30 boullis eggs, and 30 fried sausages, were collected from 20 schools in the Mono department and transported over a two hours drive to the laboratory for analysis. A characteristic colony of each strain (*Escherichia coli*) from each sample was taken and cultured in an Eppendorf tube containing Brain Heart Infusion Broth (BHI OXOID CM1135), 10% glycerol and stored at -80° C for later analysis.

1.2. Hand's Sample Collection

Forty samples of the palmar surface of the hand before and after washing were collected from kitchen operators in the schools. Swabs were taken before and after cleaning and disinfecting the hands using two sterile cotton swabs, one wet with Maximum Recovery Diluent (MRD, Oxoid CM0733, Belgium) and the other dry, all inserted into a *Stomacher* bag containing 25 mL of MRD medium. Gloves were worn and changed between samples. The moistened cotton was placed in the *Stomacher* bag beforehand and taken out with sterile forceps for swabbing. Swabbing was carried out using the method described by [22]. Once swabbing was complete, the wet cotton was reintroduced into the *Stomacher* bag. The same surface was then swabbed again, this time with dry cotton, still using sterile forceps, and placed in the same *Stomacher* bag as the moistened cotton, and so on. After being transported to the laboratory, the samples were homogenized for 2 minutes to recover as much liquid as possible in a Falcon for analysis.

1.3. Escherichia coli Isolation

Of the tested food sample, 25 g was added to 225 mL buffered peptone water. Bacterial culture was performed with1 mL of initial suspension and successive decimal dilutions in-depth for food sample, and 1 mL of 25 mL initial suspension for hand swabs in depth using Tryptone Bile X-Glucuronide (TBX) BAC ISO-16649-2,3 agar. Petri dishes were incubated at 37 °C for 24h. Typical blue *E. coli* colonies with characteristic morphology were transferred in the Eppendorf tube containing Brain Heart Infusion Broth (BHI OXOID CM1135), glycerol at 10 %, and conserved at – 80 °C for future analysis.

1.4. DNA Extraction and 16S PCR

DNA extraction of *Escherichia coli* strains *was* using the NucleoSpin^{R} extraction kit obtained from Macherey Nagel. The manufacturer's protocol was followed. DNA samples were stored at -80 °C until use.

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16. PCR was performed under the conditions described below 20 µL reaction medium consisting of 2 µL of 10 x PCR buffer; 2 µL of dNTP (2 mM); 1.6 µL of MgCl2 (25 mM) ; 0.2 µL of Taq polymerase (5 U/µL) and 0.8 µL of each of the two (02) primers (forward primer 5'GAGTTTGATCMTGGCTCAG3' and reverse primer 5'TACGGTTACCTTGTTACGAC3'); 10.6 µl of molecular water and 2µl of DNA sample. Amplification was carried out using a thermal cycler under the following conditions: 94°C for 5 minutes for initial denaturation followed by 35 cycles of denaturation at 94°C for 30 seconds, then hybridization at 56°C for 30 seconds and elongation at 72°C for one minute with a final extension at 72°C for 5 minutes followed by a storage phase at 4°C. Migration was performed on a 0.6% agarose gel using a fluorescent intercalating agent with a non-specific affinity for DNA of 5 µg/ml and a molecular weight marker (DNA ladder small fragments - Eurogentec) at 100 Volts for 30 minutes, and the fragments obtained after migration were visualized using the Gel Doc EZ Imager BIO-RAD. The PCR products were subjected to sequencing after a purification step (Chong et al., 2017) using the Wizard Kit (genomic DNA purification Kit Wizard ®). An equimolar mixture of each product and the forward primer was used for sequencing using 16S rRNA sequencing.

1.5. Determination of stx1, stx2, and Eae Virulence Genes in Escherichia coli Strains

The virulence genes *stx1 stx2* and *eae* were determined in 55 *Escherichia coli* strains using specific primers (Table 1). A conventional PCR was performed under the conditions described below. The reaction mixture consisted of 2 µL of 10 x PCR buffer; 2 µL of dNTP (2 mM); 1.6 µL of $MgCl_2$ (25 mM); 0.25 µL of Taq polymerase (5 U/µL) and 0.75 µL of each of the two specific primer pairs. Amplification of the *stx1 stx2* and *eae* genes was achieved by initial denaturation at 94°C for 1 minute, followed by 30 cycles at 94°C for 30 seconds of denaturation, at 58°C for 30 seconds of hybridization, at 68°C of elongation for 30 seconds. The final extension was performed at 68°C for 5 min and maintained at 4°C. Amplification products were separated by 0.6% agarose gel electrophoresis using a fluorescent intercalating agent with a non-specific affinity for DNA of 5 µg/ml and a molecular weight marker (DNA ladder small fragments - eurogentec). Migration was performed at 100 V/cm for 30 minutes. The amplification bands were visualized using Gel Doc EZ Imager BIO-RAD.

Table 1. Primers for virulence genes searched for.

Virulence genes	Primer	Sequence (5' - 3')	Amplicon size (bp)	Sources
stx1	F	CGCTGAATGTCATTCGCTCTGC	302	[23]
	R	CGTGGTATAGCTACTGTCACC		
stx2	F	CCTCGGTATCCTATTCCCGG	516	[23]
	R	CTGCTGTGACAGTGACAAAACGC		
eae	F	ACCAGATCGTAACGGCTGCCT	499	[1]
	R	AGTTTGGGTTATAACGTCTTCATTG		

1.6. Antibiogram

Antibiotic susceptibility testing of *Escherichia coli* strains was performed to determine their resistance profile using the disk diffusion method (CLSI, 2022). A bacterial suspension of 10^8 cfu/g at 0.5 McFarland was prepared and incubated for 18 to 24 hours using fresh strains grown for 24 hours on blood agar the previous day. Next, 0.1 mL of the inoculum was taken and spread on the surface of MHA (Mueller Hinton agar) medium previously poured into Petri dishes. The antibiotic discs were applied to the surface of the Petri dishes using sterile forceps. The dishes were incubated for 18 to 24

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hours at 37° C. Antibiotic discs included ampicillin (10µg), amoxicillin-clavulamic acid (20/10µg), aztreonam (30µg), cephalotin (30µg), cefoxitin (30µg), cefotaxime (30µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg) and tetracycline (30 µg) were purchased from Becton, Dickinson and Company (USA) (Table 1). Inhibition diameters were measured using digital calipers. The results obtained were used to classify isolates as 'resistant', 'intermediate' or 'susceptible' to a particular antibiotic using the EUCAST reference values (Table 2) [24].

Table 2. Antibiotics tested and their classes.

Antibiotics	Classes
Ampicillin (AMP) 10 µg	Beta lactamine Penicillin group A
Amoxicillin-clavulamic acid (AMC) 20/10 µg	Beta lactamine + Clavulamic acid, Penicillin group A
Aztreonam (ATM) 30 µg	Beta lactamine, Monobactam
Cephalotin (CF) 30 µg	Beta lactamine, Cephalosporin 1ère generation
Cefoxitin (FOX) 30 µg	Beta lactamine, Cephalosporin 2ème generation
Cefotaxime (CTX) 30 µg	Beta lactamine, Cephalosporin 3ème generation
Chloramphenicol (CHL) 30 µg	Phenocolate
Ciprofloxacin (CIP) 5 µg	Quinolone
Gentamicin (GMN) 10 µg	Aminoside
Tetracycline (TET) 30 µg	Tetracycline

Source : [24].

Table 3. Reference inhibition diameters.

Antibiotics	Reference inhibition diameters in mm	
	S ≥	R <
Aztreonam	26	21
Ciprofloxacin	25	22
Gentamicin	17	17
Tetracycline	-	-
Chloramphenicol	17	17
Cefotaxime	20	17
Cefoxitin	19	19
Amoxicillin	19	19
Amplicillin	19	19

Source : [24].

1.7. Statistical Analysis

The inhibition diameters obtained after the antibiogram were recorded and encoded in Excel (Microsoft Corporation). Statistical analyses were performed using SAS software (SAS Institute Inc, Cary, NC, USA). In this software, descriptive statistical analyses such as the calculation of frequencies, percentages or proportions were carried out to evaluate the resistance of the strains of *Escherichia coli* isolated from foods of animal origin and from the palmar surfaces of the operators to the antibiotics tested.

2. Results

2.1. Antibiotic Resistance and Virulence of Escherichia coli Strains Isolated from Food of Animal Origin and Hands

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The results obtained for all the antibiotics tested on the microorganism strains are shown in Figure 2. It can be seen that three antibiotics were ineffective on the *Escherichia coli* strains isolated from food of animal origin and hands. These were ampicillin, amoxicillin-clavulanic acid, and tetracycline, with resistance rates of 81.82% ($n = 45$), 72,73 % ($n = 40$), and 63,64 % ($n = 35$). In contrast to these antibacterial agents, *E. coli* strains were sensitive to aztreonam, cefotaxime, cefoxitin, chloramphenicol, ciprofloxacin and gentamicin respectively in the following proportions: 78.18% ($n = 43$), 58,18 % ($n = 32$), 63,64 % ($n = 35$), 78,18 % ($n = 43$), 72,73 % ($n = 40$), 52,73 % ($n = 29$).

2.2. Multidrug Resistance Profile of Escherichia coli Strains Isolated from Food of Animal Origin and Surfaces to Antibiotics

The multi-drug resistance of each isolate to antibiotics is described in Table 4. Of the 55 isolates tested, 35 were isolated from food and 20 from hands. It should be noted that several strains were multi-resistant to at least two antibiotics. It was found that approximately 11.43% ($n = 4$) of strains isolated from food were multi-resistant to 5 antibiotics compared with 10% ($n = 2$) of those isolated from hands. In addition, 35.14% ($n = 13$) *of Escherichia coli* strains isolated from foodstuffs were multi-resistant to 4 antibiotics, compared with 60% (of those isolated from hands. ($n = 12$) of those isolated from hands. Multi-resistance to 3 antibiotics was also observed in 20% of the strains isolated from foodstuffs and 20% of those isolated from hands. ($n = 7$) of strains isolated from food and 5% of those ($n = 1$) of those isolated from hands. Only 22.90% of ($n = 8$) of food isolates and 5% of hand isolates ($n = 1$) of those isolated from hands were multi-resistant to two antibiotics.

Table 4. Multi-resistance profile of *Escherichia coli* strains isolated from food and hands .

Type of resistance	Phenotypes	Isolates	
		Food	Hands
Multidrug resistance	AMP, AMC, CEP, GNM, TET	2 (5,7 %)	-
	AMP, AMC, CEP, CTX, TET	1 (2,9 %)	1 (5 %)
	AMP, AMC, CEP, FOX, TET	-	1 (5 %)
	AMP, AMC, CEP, CIP, TET	1 (2,9 %)	-
	AMP, AMC, CEP, TET	9 (25,7 %)	10 (50 %)
	AMP, AMC, FOX, TET	1 (2,9 %)	-
	AMP, CEP, GNM, TET	1 (2,9 %)	-
	AMP, AMC, CEP, CIP	-	1 (5 %)
	AMP, CEP, FOX, TET	1 (2,9 %)	1 (5 %)
	AMP, AMC, CEP, GNM	1 (2,9 %)	-
	AMP, AMC, CEP	3 (8,6 %)	4 (20 %)
	AMC, CEP, TET	6 (17,1 %)	1 (5 %)
	AMP, CEP, GNM	1 (2,9 %)	-
	AMC, CEP	8 (22,9 %)	1 (5 %)

With AMP: Ampicillin; ATM: Aztreonam; CTX: Cefotaxime; FOX: Cefoxitin; AMC: Amoxicillinclavulamic acid; CHL: Chloramphenicol; CIP: Ciprofloxacin; GMN: Gentamicin; TET: Tetracycline.

2.3. Virulence Gene Profile of Escherichia coli Strains Isolated from Foods of Animal Origin and Hands

The virulence genes detected in *Escherichia coli* strains were as follows. The search for virulence genes in a total of 55 *Escherichia coli* strains analyzed revealed that stx1 was present in 16.40% (n = 9) of the isolates, including 20% (n = 7) of food isolates and 10% (n = 2) of those isolated from surfaces. The virulence genes *stx2* and *eae* were not present in all the isolates analyzed.

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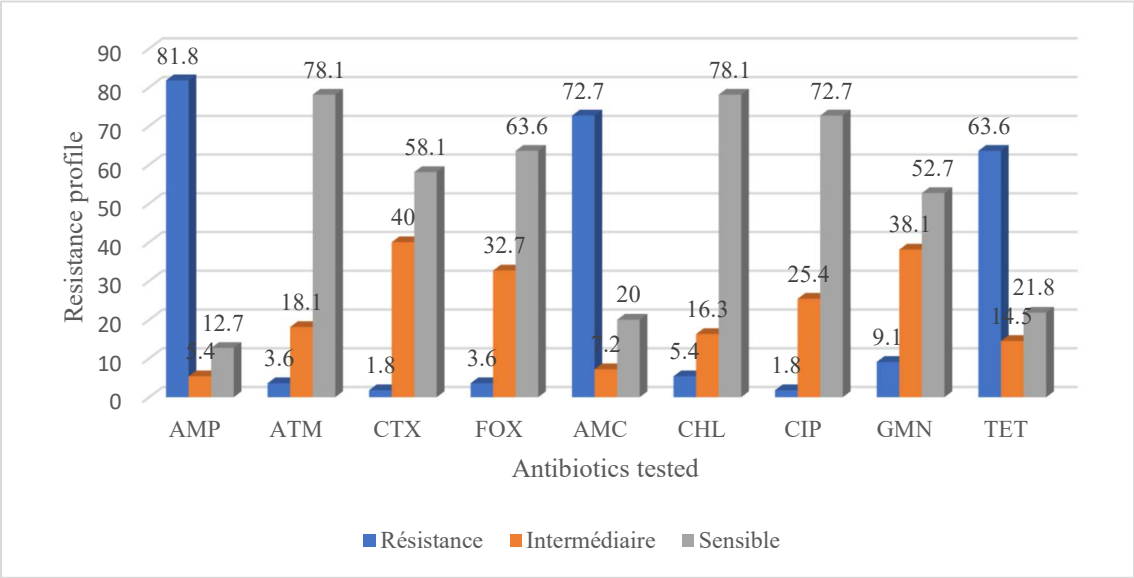

Figure 1. Antibiotic resistance of *Escherichia coli* strains isolated from food of animal origin and surfaces. AMP: Ampicillin; ATM: Aztreonam; CTX: Cefotaxime; FOX: Cefoxitin; AMC: Amoxicillin clavulamic acid; CHL: Chloramphenicol; CIP: Ciprofloxacin; GMN: Gentamicin; TET: Tetracycline.

4. Discussion

Foodborne outbreaks are of major importance in terms of food safety and public health. Most studies on the prevalence of food-borne illnesses have focused much more on the enumeration, research, and identification of the pathogenic microorganisms responsible for these illnesses in foods [25,26]. In Africa, several studies have been reported on the antibiotic resistance of *Escherichia coli* isolated from food, water, animal, and human samples [1,27–29]. In Benin, information on the antibiotic resistance of this bacterium isolated from food of animal origin and the hands is practically non-existent, and the few research studies that have been carried out are clinical studies [30].

This study on the antibiotic resistance of *Escherichia coli* strains isolated from food of animal origin and surfaces showed high frequencies of resistance of the bacteria to the three main antimicrobial agents tested, i.e. ampicillin, amoxicillin clavulanic acid and tetracycline, with frequencies of between 60 and 90%. These results are similar to those of [14] who, in their study of *Escherichia coli* O157:H7 strains isolated from foods of animal origin sold in institutional catering in Ethiopia, observed resistance of this bacterium to these three antibiotics in proportions ranging from 50 to 100%. However, our results are contrary to those of [31], who found resistance frequencies of between 16 and 40% in *Escherichia coli* strains isolated from foodstuffs on the campuses of the University of Abomey-Calavi in Benin, and those of [32], who found resistance frequencies of between 25 and 50% in strains isolated from drinking water and street foods in Maputo, Mozambique. [23] obtained an estimated frequency of resistance to amoxicillin-clavulanic acid of 57.14% for the same bacterium in their study of strains isolated from environmental samples and food products in Benin. This result differs from that obtained in this study for the same antibiotic, which was 72.73%. These different frequencies of resistance observed may be due either to spontaneous genetic mutation or to repeated exposure to antibiotics in animals and humans through their diet or during the treatment of bacterial diseases. However, among the *Escherichia coli* strains isolated from food and surfaces in the present study, others were sensitive to aztreonam, cefotaxime, cefoxitin, chloramphenicol, ciprofloxacin, and gentamicin in proportions ranging from 50 to 80%. These results are similar to those of [33], who observed frequency sensitivities of 100% for ciprofloxacin and cefoxitin for strains isolated from retail meat products in Ethiopia; and [14], for whom the bacterium's sensitivities to tetracycline and chloramphenicol were also 100% for strains isolated from poultry and beef collected in abattoirs and restaurants. In contrast to these results, other studies have shown that

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Escherichia coli is resistant to cefotaxime, aztreonam, gentamicin, ciprofloxacin, chloramphenicol, and other antibiotics [34–38].

Furthermore, high rates of multidrug resistance in *Escherichia coli* isolated from food and hands were observed in this study involving up to five antibiotics at a time. The frequencies of multidrug resistance varied between 17 and 26% for strains isolated from food of animal origin and between 20 and 50% for those isolated from hands. These results are in line with several studies carried out in Africa and Benin, in which the frequencies of multidrug resistance in this bacterium involved at least four antibiotics in roughly the same proportions [38,39]. The variation in the frequencies of multidrug resistance of the bacteria in the different studies may be linked to the uncontrolled use of antibiotics to treat foodborne diseases, the dose, and route of administration of the antibiotic, the presence of antibiotics in food additives, the geographical location of the studies, the immune response of the patient treated and the difference between the antibiotics tested [40–42]. All these forms of *Escherichia coli* resistance observed are the real causes of the therapeutic failures encountered when treating food-borne illnesses caused by this bacterium. It is therefore essential to accurately identify the pathogens involved in food-borne illnesses before prescribing antibiotics for their treatment. Any haphazard use of antibiotics should be avoided.

In addition to this bacterium's resistance to antibiotics, studies to determine virulence factors have shown the presence of *stx1* in 16.40% of isolates. Isolates possessing this virulence factor may therefore contribute to the production of the verocytotoxin characteristic of the bacterium's pathogenicity. This toxin is the basis of the hemolytic uraemic syndrome (HUS) caused by VTEC. These results are not consistent with those of [43], who, in their work on *Escherichia coli* isolates from foods sold in community and environmental restaurants in southern Benin, did not find the virulence factor *stx1* in their isolates. However, the results obtained for the search for this virulence factor in this study are similar to those of several authors who have also worked on *Escherichia coli* strains isolated from foods of animal origin, animal and human samples [44,45]. However, virulence factors such as *stx2* and *eae were* not found in the isolates tested in the present study. These results are similar to those of several authors who have worked on the same bacterium isolated from foods of animal origin [46–49].

Therapeutic failures in the treatment of food-borne outbreaks and food-borne illnesses caused by bacteria with virulence factors that are resistant to antibiotics in general, and *Escherichia coli* in particular, are becoming more and more frequent [50–52]. The epidemiological profile of each foodborne outbreak depends on several factors, including the infection rate and the mortality rate. The mortality rate depends essentially on the conditions in which patients are treated and, above all, on the virulence of the pathogenic microorganism in question [53,54]. The virulence of pathogens is therefore a very important parameter to take into consideration for the effective and efficient management of cases of foodborne outbreaks or food-borne illnesses [55]. It should also be remembered that compliance with good hygiene and manufacturing practices in mass catering is one of the measures to be taken upstream to prevent outbreaks of food-borne illnesses. In addition, alternative solutions to antibiotic treatment should be encouraged to further refine strategies for combating food-borne illnesses.

5. Conclusion

Antibiotic resistance and virulence in pathogenic bacteria are among the factors contributing to the high mortality rates observed in food-borne outbreaks worldwide and Africa in particular. In sub Saharan Africa, and especially in Benin, several upstream factors are at the root of food-borne outbreaks, including non-compliance with good hygiene and manufacturing practices, failure to regulate the food sector, including mass catering (in places such as nursing homes, prisons, public services, and schools), and a lack of quality control of the food sold. In addition, antibiotic resistance in bacteria means that treatments are no longer effective. The present study on antibiotic resistance in bacteria showed that *Escherichia coli* strains isolated from food of animal origin and hands in primary schools had developed multi-drug resistance involving at least two antibiotics. The study on

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the determination of virulence genes in the same isolates revealed the presence of the virulence factor *stx1* in 16.40% of the isolates tested. It can be concluded that the consumption of foods of animal origin sold in schools presents potential risks of food-borne outbreaks that could cause harm to public health.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: This study received approval from the ethics committee of the University of Abomey-Calavi of the Republic of Benin before the research was conducted. During the study, food operators voluntarily gave their informed consent before beginning the collection of samples. Participants were assured that the data would be collected anonymously and would only be used for the purposes of the study. They were under no obligation to participate in the study. Participation in the study was voluntary and free.

Data Availability Statement: Data will be made available by the authors upon request.

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

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