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Risk Factors of Extended-Spectrum β-Lactamase Producing Enterobacteriaceae Occurrence in Farms in Reunion, Madagascar and Mayotte Islands, 2016–2017

Noellie Gay [1,*], Alexandre Leclaire [2], Morgane Laval [1], Guillaume Miltgen [2], Maël Jego [1], Stéphane Ramin [1], Julien Jaubert [2], Olivier Belmonte [2], Eric Cardinale [1]

[1] UMR AnimalS, health, Territories, Risks and Ecosystems Unit, Department of Animal Health, Univ Montpellier, INRA, Montpellier, France; noellie.gay@cirad.fr; eric.cardinale@cirad.fr; laval.morgane1@gmail.com

[2] Bacteriology laboratory, Félix Guyon Hospital, Saint-Denis, Reunion, France; olivier.belmonte@chu-reunion.fr; alexandre.leclaire@yahoo.fr; guillaume.miltgen@chu-reunion.fr

* Correspondence: noellie.gay@cirad.fr, Tel.: +262-693-828-223

Abstract: In South Western Indian ocean (IO), Extended-Spectrum β-Lactamase producing Enterobacteriaceae (ESBL) are a main public health issue. In livestock, ESBL burden was unknown. The aim of this study was estimating the prevalence of ESBL on commercial farms in Reunion, Mayotte and Madagascar and genes involved. Secondly, risk factors of ESBL occurrence in broiler, beef cattle and pig farms were explored. In 2016-2017, commercial farms were sampled using boot swabs and samples stored at 4°C before microbiological analysis for phenotypical ESBL and gene characterization. A semi-directive questionnaire was performed. Prevalences observed in all production types and territories were elevated, except for beef cattle in Reunion which differed significantly. The most common ESBL gene was the CTX-M-1 subtype. Generalized linear models explaining ESBL occurrence varied between livestock production sectors and allowed identifying main protective (e.g., water quality control and detergent use for cleaning) and risk factors (e.g., recent antibiotic use, other farmers visiting the exploitation, pet presence). This study is the first to explore tools for antibiotic resistance management in IO farms. It provides interesting hypothesis to explore about antibiotic use in IO and ESBL transmission between pig, beef cattle and humans in Madagascar.

Keywords: Indian ocean; livestock; Extended-Spectrum β-Lactamase producing Enterobacteriaceae; risk factors; CTX-M; enzymes

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1. Introduction

Extended-spectrum β-Lactamase producing Enterobacteriaceae (ESBL) is a public and veterinary health burden worldwide and particularly in West Indian ocean (1). These multi-resistant bacteria have been identified as a priority in terms of epidemiological surveillance in humans and animals from the Indian Ocean Commission (IOC) state members (i.e. Comoros, Madagascar, Mauritius, Reunion, and Seychelles) and Mayotte (French oversea territory)(1).

ESBL are resistant to almost all beta-lactam antibiotic drugs including third generation cephalosporin (3GC), as well as other classes of antibiotics such as fluoroquionolones, aminoglycosides, sulfonamides, and cyclins, leading to the use of last-resort antibiotics (i.e. carbapenems) in ESBL infections in humans (2).

The occurrence of ESBL has been identified in broiler and swine farms in Europe (3-5) and the CTX-M β-lactamases enzymes group is the most frequently detected ESBL in livestock, especially CTX-M-1 subtype (4).

Selection pressure exerted by antibiotic drugs on microbiota favors carriage and persistence of ESBL in humans (hospital and community) (6, 7), livestock and pets (7-9); thus, all could act as potential reservoirs of ESBL.

The main known risk factor identified in ESBL occurrence in livestock was "use of 3GC or 4GC (ceftiofur, cefoperazone and cefquinome) in the last 12 months" in dairy and pig farms (10, 11).

Other risk factors such as storage of slurry in a pit, operating an open herd policy and infrequent cleaning of calf feeding equipment were also identified in dairy farms (4), and fish ponds in poultry farms of Vietnam (12).

In IOC no estimate of ESBL prevalence in livestock was available. Thus, the aim of this study was first estimating the prevalence of ESBL on beef cattle, broiler and pig commercial farms in Reunion, Mayotte and Madagascar Islands, and identify ESBL enzymes occurrence in each production type and territory. Secondly, potential risk factors of ESBLE occurrence in poultry and pig farms were explored.

2. Materials and Methods

2.1. Study Population

Reunion and Mayotte are French overseas territories located in South Western Indian Ocean. Reunion with an area of 2512 km2 is home for around 850 996 people (13). In Reunion, 156 poultry producers, 340 pig producers and 331 beef cattle producers are structured in breeding organization and could be considered as intensive or partially free ranging (Eric Cardinale, Personal Communication).

Mayotte with an area of 374 km2 is home for around 235 132 people (13). One hundred fifty modern poultry producers and 3600 beef cattle farms are recorded in this territory (14). However, twenty poultry producers and 320 beef cattle producers are structured in breeding official organizations (Philippe Merot, Personal Communication).

Madagascar is the fifth largest island in the world, with a land mass of 587,000 km2 and 24.24 million inhabitants in 2016 (WorldBank Group, 2015). Its economy is based essentially on agriculture and tourism; producer census was not available at the Direction of Veterinary Services of the Ministry of Livestock Production (Michel Rakotoharinome, Personal Communication).

2.2. Sampling

From February to August 2016, broiler, pigs, and beef cattle farms were sampled in Reunion. Due to a foot-and-mouth outbreak in Mauritius Island, sampling had to be stopped in Reunion for sanitary purposes and reported to August 2017 for beef cattle. In Mayotte, beef cattle and broiler were sampled from September to October 2016, no pig farms were present in this territory due to mostly Muslim community representation; thus, no samples of pigs were collected. In Madagascar, sampling was performed in November 2016. Beef cattle were sampled in Antsirabe, broiler in Mahitsy and pig farms in Imerintsiatosika, known to be key production sites. It is to be noted that broiler and beef cattle farms from Mayotte and Madagascar could also raise few hen and dairy cattle in the exploitation without being the main commercial activity.

In each territory, almost thirty breeding farms of each livestock production sector were targeted for sampling using boot swabs Sterisox®. Number of samples depended on the house's surface area, one Sterisox® being used to cover 100m² of building. If possible all boxes were visited and livestock gathering points (e.g. water pond, watering trough) were also sampled. Number of samples per farm varies between one to five.

All samples were immediately maintained at 4 °C before analyses proceeded within 48 hours after reception (transport within the day for Reunion and within one week for Mayotte and Madagascar).

No ethical approval was needed as noninvasive sampling methods were used to identify farm ESBL sanitary status.

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2.3. Laboratory Investigations

2.3.1. ESBL Phenotype

Sterisox® boot swabs were incubated 20±4h at room temperature with 100 ml of physiological water and 900 µL of Brain-Heart Infusion broth (BioMérieux SA). Ten µL of the enriched suspension was directly streaked onto selective chromogenic agar plates (ChromID-ESBL, Biomérieux, Marcy l'Etoile, France) and incubated overnight at 37°C under aerobic condition. Presumptive ESBL-producers were sub-cultured individually on Drigalski lactose agar, and bacterial species identification performed using MALDI-TOF mass spectrometry (Bruker Daltonics, Breme, Germany).

A positive ESBL phenotype was confirmed by the combination disc test according to the EUCAST guidelines (15). The test was considered positive if the inhibition zone diameter was ≥5 mm larger with clavulanic acid than without.

2.3.2. Characterization of ESBL genes

Ten ESBL-producing isolates were randomly selected by livestock production sector for each territory. Total DNA was extracted using the NucliSens® Easymag® system (Biomérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Extracted eluates were stored at –80°C. Molecular characterization was performed using Check-MDR CT103XL array test (Check-Points Health B.V., Wageningen, Netherlands) for identification of ESBL genes (i.e. BEL, CTX-M1 group, CTX-M2 group, CTX-M9 group, CTX-M8/25 group, GES-ESBL, PER, SHV-ESBL, TEM-ESBL, VEB) and discriminated ESBL and non-ESBL TEM and SHV variants. The assay consisted in a two-step amplification process of the ESBL target sequences, followed by a colorimetric microarray detection of the reaction products. Image analysis and interpretation were provided by Check-Points "5-2-2015" software.

2.4. Questionnaire survey

A semi-directive questionnaire to assess potential risk factors on farms was developed. Data regarding farm building, biosecurity measures, breeding practices including management of knackery, water quality, quarantine, and effluent, vector control, cleaning and disinfection techniques, use of antibiotics, and questions relatives to the breed like housing system and origins of animals were collected (See questionnaire annex). Answers were cross-checked by direct observation and corrected if necessary.

2.5. Risk factors analyzes

A farm was considered positive if almost one boot swab was found positive for ESBL presence in bacteriological analysis. A farm was considered negative if all boot swabs samples were negative for ESBL culture.

Explanatory variables considered for analysis were categorical. If fewer than five observations recorded in a category the variable was excluded. The variable to be explained is ESBL occurrence in the livestock production sector in each territory. Bivariate analyzes were performed using Fisher test ($p<0.05$).

For generalized linear models (GLM), a preliminary step aimed at evaluating association between explicative variables and ESBL farm status with bivariate analyzes in each livestock production sectors (including all three territories). Factors associated with ESBL-positivity with a p-value <0.20 were offered to a full model form multivariate analysis (GLM). The variable territory was not included in models as it was significantly associated with others variables. Interactions between variables were not including in the models. Goodness of fit test were also performed. R software was used to perform statistical analysis (https://www.r-project.org/).

3. Results

3.1. Prevalence Observed, Bacterial Diversity, and Antibiogram Results

In Reunion, higher prevalences were observed in poultry farms (70.0%±16.7%) and pig farms (53.3%±18.2) (Table 1.). Prevalence differed significantly between livestock production type in Reunion (p-value<0.001) with a lower prevalence observed in beef cattle farms (3.7%±5.1%). In Mayotte and Madagascar, no difference in prevalence was observed between exploitation type in each territory (p-value > 0.05).

Comparing prevalence among poultry production in the three territories, no difference was observed (p-value=0.94). In pig production, the prevalence differed significantly between Madagascar and Reunion (p-value<0.005). Finally, in beef cattle the prevalence between the three territories differed significantly (p-value<0.001).

Table 1. Prevalence of ESBLE in livestock production farms of Reunion, Mayotte and Madagascar, 2016-2017.

Territory	N (positive)	EBLSE positive farms	*p*-value
Reunion			<0.001
Poultry	30 (21)	70.0 % [53.3-86.7]	
Pigs	30 (16)	53.3 % [35.1-71.5]	
Beef cattle	54 (2)	03.7% [00.0-08.8]	
Mayotte			0.70
Poultry	23 (17)	73.9 % [55.6-92.2]	
Beef cattle	19 (13)	68.4 % [47.1-89.7]	
Madagascar			0.16
Poultry	30 (21)	70,0% [53.6-86.7]	
Pigs	30 (26)	86,7% [74.3-99.1]	
Beef cattle	30 (20)	66,7% [49.5-83.9]	

In Reunion, four different species were found among Enterobacteriacae isolates with two species in poultry farms, three species in pig and two beef cattle farms (Table 2.).

In Mayotte, Enterobacteriacae diversity was reduced to Escherichia coli and Enterobacter cloacae complex in both poultry and beef cattle production.

In Madagascar, an important diversity of species was found among Enterobacteriacae isolates with about six identified in all type of production. Species diversity varied according to the production type with five species identified in pig production, three in beef cattle and poultry production.

The main represented species in all territories and all type of production was Escherichia coli with 88.0% (n=307) of all Enterobacteriaceae isolates (N=349), 94.8% (n=291) out of them being ESBL producers (Table 2).

163 **Table 2.** Diversity of the ESBL bacterial species isolated in samples from livestock production farms of Reunion, Mayotte and Madagascar, 2016-2017.

		Reunion						Mayotte				Madagascar					
		Poultry		Pig		Cattle		Poultry		Cattle		Poultry		Pig		Cattle	
	N (% ESBL)	**n**	**ESBL (%)**	**n**	**ESBL (%)**	**n**	**ESBL (%)**	**n**	**ESBL (%)**	**n**	**ESBL (%)**	**n**	**ESBL (%)**	**n**	**ESBL (%)**	**n**	**ESBL**
Citrobacter freundii	6 (100.0%)			2	2 (100.0%)									4	4 (100.0%)		
Escherichia coli	307 (94.8%)	145	136 (93.8%) (93,8%)	45	40 (88.9%)	2	2 (100.0)	19	19 (100.0%)	17	17 (100.0%)	28	28 (100.0%) (100,0%)	29	28 (96.6%)	22	22 (100
Escherichia hermannii	2 (100.0%)															2	2 (100.
Enterobacter cloacae complex	17 (82.4%)	1	1 (100.0%)			1	0 (00.0%)	1	1 (100.0%)	1	1 (100.0%)	1	1 (100.0%)	6	6 (100.0%)	2	2 (100.
Klebsiella pneumoniae	11 (100.0%)			2	2 (100.0%)							2	2 (100.0%)	7	7 (100.0%)		
Morganella morganii	6 (100.0%)													6	6 (100.0%)		

164 No phenotypic resistance to ertapenem (ETP) was identified in ESBL isolates (Table 3.). Resistance to nalidixic acid (NA) was elevated in ESBL producing *E. coli* in beef cattle from Reunion (50.0%) and in Madagascar both in poultry (28.6%) and pig (25.0%) farms. Resistance to ofloxacin (OFX) was the most elevated in ESBL producing *E. coli* in pig production both in Madagascar (21.4%) and Reunion (25.0%). Resistance to gentamicin (GEN) was elevated in ESBL producing K. pneumoniae in Madagascar. No resistant profile to amikacin (AKN) was identified in all territories. In ESBL producing *E. coli* triméthoprime/sulfaméthoxazole (SXT) resistance was higher in Reunion both in poultry and pig production (61.2% and 87.5% respectively). ESBL producing *E. coli* most resistant profiles to tetracycline (TE) were observed in Madagascar (i.e. 92.9% in broiler, 75.0% in pigs and 50.0% in beef cattle).

Table 3. Antibiogram results of ESBL Enterobacteriaceae in samples from livestock production farms of Reunion, Mayotte, and Madagascar, 2016-2017.

	ETP			**NA**			**OFX**			**GEN**			**AKN**			**SXT**			**TE**			
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	ND
Reunion Broiler																						
E. coli (N=134)	134 (100.0%)	0 (00.0%)	0 (00.0%)	106 (79.1%)	5 (03.7%)	23 (17.2%)	130 (97.0%)	2 (01.5%)	2 (01.5%)	128 (95.5%)	0 (00.0%)	6 (04.5%)	134 (100.0%)	0 (00.0%)	0 (00.0%)	52 (38.8%)	0 (00.0%)	82 (61.2%)	22 (16.4%)	0 (00.0%)	49 (36.6%)	63 (47.0%)
E. cloacae (N=1)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	
Pig																						
E. coli (N=40)	39 (97.5%)	1 (02.5%)	0 (00.0%)	29 (72.5%)	1 (02.5%)	10 (25.0%)	30 (75.0%)	0 (00.0%)	10 (25.0%)	35 (87.5%)	0 (00.0%)	5 (12.5%)	40 (100.0%)	0 (00.0%)	0 (00.0%)	5 (12.5%)	0 (00.0%)	35 (87.5%)	5 (12.5%)	1 (02.5%)	23 (57.5%)	11 (27.5%)
C. freundii (N=2)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	
K. pneumoniae (N=2)	1 (50.0%)	1 (50.0%)	0 (00.0%)	1 (50.0%)	0 (00.0%)	1 (50.0%)	1 (50.0%)	0 (00.0%)	1 (50.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	1 (50.0%)	2 (100.0%)	1 (50.0%)	1 (50.0%)	0 (00.0%)	1 (50.0%)	
Beef cattle																						
E. coli (N=2)	2 (100.0%)	0 (00.0%)	0 (00.0%)	1 (50.0%)	0 (00.0%)	1 (50.0%)	1 (50.0%)	1 (50.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	
Mayotte Broiler																						
E. coli (N=19)	19 (100.0%)	0 (00.0%)	0 (00.0%)	14 (73.7%)	4 (21.1%)	1 (05.3%)	19 (100.0%)	0 (00.0%)	0 (00.0%)	18 (94.7%)	0 (00.0%)	1 (05.3%)	19 (100.0%)	0 (00.0%)	0 (00.0%)	14 (73.7%)	0 (00.0%)	5 (26.3%)	3 (15.8%)	0 (00.0%)	16 (84.2%)	
E. cloacae (N=1)	1 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	
Beef cattle																						
E. coli (N=16)	16 (100.0%)	0 (00.0%)	0 (00.0%)	7 (43.8%)	5 (31.3%)	4 (25.0%)	14 (87.5%)	2 (12.5%)	0 (00.0%)	12 (75.0%)	0 (00.0%)	4 (25.0%)	16 (100.0%)	0 (00.0%)	0 (00.0%)	15 (93.8%)	0 (00.0%)	1 (06.3%)	12 (75.0%)	0 (00.0%)	4 (25.0%)	

E. cloacae (N=1)	1 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)
Madagascar Broiler																					
E. coli (N=28)	28 (100.0%)	0 (00.0%)	0 (00.0%)	13 (46.4%)	7 (25.0%)	8 (28.6%)	22 (78.6%)	3 (10.7%)	3 (10.7%)	27 (96.4%)	0 (00.0%)	1 (03.6%)	28 (100.0%)	0 (00.0%)	0 (00.0%)	27 (96.4%)	0 (00.0%)	1 (03.6%)	1 (03.6%)	1 (03.6%)	26 (92.9%)
E. cloacae (N=1)	1 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)
K. pneumoniae (N=2)	2 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)	1 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	1 (100.0%)
Pig																					
E. coli (N=28)	28 (100.0%)	0 (00.0%)	0 (00.0%)	13 (46.4%)	8 (28.6%)	7 (25.0%)	20 (71.4%)	2 (07.1%)	6 (21.4%)	28 (100.0%)	0 (00.0%)	0 (00.0%)	28 (100.0%)	0 (00.0%)	0 (00.0%)	28 (100.0%)	0 (00.0%)	0 (00.0%)	7 (25.0%)	0 (00.0%)	21 (75.0%)
E. cloacae (N=6)	6 (100.0%)	0 (00.0%)	0 (00.0%)	2 (33.3%)	2 (33.3%)	2 (33.3%)	6 (100.0%)	0 (00.0%)	0 (00.0%)	4 (66.7%)	0 (00.0%)	2 (33.3%)	6 (100.0%)	0 (00.0%)	0 (00.0%)	4 (66.7%)	0 (00.0%)	2 (33.3%)	0 (00.0%)	0 (00.0%)	6 (100.0%)
C. freundii (N=4)	4 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	4 (100.0%)	0 (00.0%)	0 (00.0%)	4 (100.0%)	1 (25.0%)	0 (00.0%)	3 (75.0%)	4 (100.0%)	0 (00.0%)	0 (00.0%)	1 (25.0%)	0 (00.0%)	3 (75.0%)	0 (00.0%)	0 (00.0%)	4 (100.0%)
M. morganii (N=6)	6 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	6 (100.0%)	0 (00.0%)	0 (00.0%)	6 (100.0%)	0 (00.0%)	0 (00.0%)	6 (100.0%)	6 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	6 (100.0%)	0 (00.0%)	0 (00.0%)	6 (100.0%)
K. pneumoniae (N=7)	7 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	3 (42.9%)	4 (57.1%)	5 (71.4%)	0 (00.0%)	2 (28.6%)	0 (00.0%)	0 (00.0%)	7 (100.0%)	7 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	7 (100.0%)	0 (00.0%)	0 (00.0%)	7 (100.0%)
Beef cattle																					
E. coli (N=22)	22 (100.0%)	0 (00.0%)	0 (00.0%)	15 (68.2%)	3 (13.6%)	4 (18.2%)	18 (81.8%)	3 (13.6%)	1 (04.5%)	21 (95.5%)	0 (00.0%)	1 (04.5%)	22 (100.0%)	0 (00.0%)	0 (00.0%)	21 (95.5%)	0 (00.0%)	1 (04.5%)	11 (50.0%)	0 (00.0%)	11 (50.0%)
E. cloacae (N=2)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)
E. hermannii (N=2)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	0 (00.0%)	2 (100.0%)

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3.2. ESBL Enzymes Identification

The most common ESBL gene identified in all territories and production type was the CTX-M-1 subtype which accounted for 54.4% (n=49) of all *E. coli* isolates tested (N=90), followed by CTX-M- 15 (31.1%, n=28) (Table 4.). The higher diversity in ESBL gene was found in poultry production from all IOC territories.

Table 4. ESBL genes identified in ten *E. coli* isolated from poultry, pig and beef cattle production farms in Reunion, Mayotte and Madagascar, 2016-2017.

Territory / Production type	***E. coli* tested**	**ESBL genes identified (%)**						
		CTX-M-1 group				**CTX-M-9 group**	**SHV**	**TEM**
Subtype		**CTX-M-1**	**CTX-M-3**	**CTX-M-15**	**CTX-M-32**			
Reunion								
Poultry	32	29 (90.6%)					1 (3.1%)	2 (6.3%)
Pigs	10	7 (70.0%)	1 (10.0%)	2 (20.0%)				
Beef cattle	2	-	-	-	-	-	-	-
Mayotte								
Poultry	9	7 (77.8%)	1 (11.1%)	1 (11.1%)				
Beef cattle	7	1 (14.3%)		5 (71.4%)	1 (14.3%)			
Madagascar								
Poultry	10	5 (50.0%)		2 (20.0%)		3 (30.0%)		
Pigs	9			9 (100.0%)				
Beef cattle	9			9 (100.0%)				

3.3. Explanatory Factors of ESBL Occurrence in Livestock Sectors Production in Reunion, Madagascar and Mayotte, 2016-2017

Univariate ORs for the occurrence of ESBL in each livestock production sectors and territory was presented in (Table 5.). Recent building age was associated with an increased risk of ESBL occurrence in broiler production in Reunion. In pig production, changing shoes/boots before entering the building was observed as a risk factor of ESBL occurrence whereas rodent control by an external society and two disinfections between next batch were associated with a decreased probability of ESBL occurrence.

In Madagascar, absence of chick introduction in the farm (self-production) in broiler farms was a protective factor of ESBL occurrence. Clearing around the farm was associated with a decreased risk of ESBL occurrence in beef cattle production.

Table 5. Bivariate explanatory factors of ESBL occurrence in livestock from Reunion, Mayotte and Madagascar, 2016-2017.

Country	**Livestock**	**Variable**	**OR, IC95%**	***p*-value**
Reunion	Broiler	Recent building age (<1999)	12.72 [1.25-671.77]	0.01
	Pigs	Change clothes before entering house/pen	6.52 [0.92-80.50]	0.05
		Change shoes/boots before entering house/pen	13.62 [1.35-716.37]	0.01
		Rodent control by a society	0.11 [0.01-0.75]	0.01
		Lightning in the building	0.18 [0.01-2.13]	0.04
		Two desinfections before next batch	0 [0-0.92]	0.04
	Beef cattle cows	-	-	-
Madagascar	Broiler	Chicks produced in the farm	0 [0.00-0.91]	0.02
	Pigs	Use of antibiotic for prophylaxy	0.09 [0.00-1.36]	0.05
	Beef cattle	Clearing around the farm	0 [0.00-0.94]	0.03
		Clean condition around the farm	0 [0-1.94]	0.003
Mayotte	Broiler	Distance from another poultry farm (>500 m)	13.39 [0.79-883.37]	0.04
	Beef cattle	-	-	-

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Generalized linear models explaining ESBL occurrence (all territories included) varied between livestock production sectors (Table 6). In broiler, "water quality control" was identified as a protective factor (OR: 0.12); the best model selected the variables "distance to another farm", "foot bath at entrance", "water quality" and "water storage tank" (AIC: 93.98).

In pig production, "other farmers visiting the farm", "soaking the surface", "detergent use for cleaning" and "antibiotic use recently" were identified in the best model (AIC: 65.09).

For beef cattle, the best model kept "livestock size", "antibiotic use", "disinfestation", "clearing around the farm", "pet presence" and "water storage tank" (AIC: 83.53).

Table 6. Best model explaining ESBL occurrence in poultry, pig and cow production (including all territories), 2016-2017.

Dependent variables	Independent variables	Adj. OR (CI95%)	*p*-value	AIC
Broiler occurence (**a**)	Distance elev oth species >500m	3.18 (0.65-15.56)	0.15	93.68
	Distance elev oth species <500m	0.99 (0.26-4.39)	0.99	
	Foot bath at room entrance	5.89 (0.61-57.17)	0.13	
	Water quality control	0.12 (0.02-0.82)	0.03	
	Water storage tank presence	2.58 (0.85-7.79)	0.09	
Pig occurrence* (**b**)	Other farmers visiting	14.15 (1.17-171.35)	0.04	65.09
	Soaking surface	22.34 (1.51-330.98)	0.02	
	Detergent use for cleaning	0.12 (0.02-0.75)	0.02	
	Antibiotic use recently (< 1 year)	8.82 (1.09-71.4)	0.04	
Beef cattle occurrence (**c**)	Livestock size > 25	0.07 (0.02-0.28)	<0.001	83.53
	Antibiotic use recently (< 1 year)	3.94 (1.04-14.98)	0.04	
	Disinfestation	0.19 (0.04-0.91)	0.04	
	Clearing around the farm	0.19 (0.04,0.91)	0.09	
	Water storage tank presence	0.38 (0.11-1.35)	0.14	
	Pet presence	6.87 (1.13-41.67)	0.04	

***Reunion and Madagascar only,** (**a**) Intercept = 0.01376, null deviance : 99.832, model d.f.= 4; (**b**) Intercept = -2.7919, null deviance : 73.304, model d.f.= 3; (**c**) Intercept = 0.9959, null deviance : 132.027, model d.f.= 5.

4. Discussion

ESBL prevalences estimated in IO farms in our study tend to be high. In broiler production prevalence ranged from 70% (Madagascar, Reunion) to 79.9% (Mayotte) which is higher than 50.0% reported in 2012 in Germany (16), but similar to 70.0% reported in Japan in 2007 (16). In India, in 2014, among 87.0% of ESBL were detected in broiler farms and 42.0% in layers farms (17). High prevalence was also observed in Dutch boiler farms (100%, n=50) in 2010-2011 (18) with on average 32 days of antibiotic administration in broiler farms in Netherland (19). The elevated prevalence observed in IO could be due to a large use of antibiotic drugs but currently, in 2018, no data regarding antibiotic consumption in IOC livestock is available.

In pig farms production, the prevalence differed significantly between territories from 53.3% in Reunion to 86.7% in Madagascar both higher than the 8.3% reported in pigs in Japan in 2007 (20) but similar to 88.2% of ESBL positive farms observed in 2012 in Germany (16). The elevated prevalence observed in Madagascar tends indicating important antibiotic use and/or misuse of antibiotics (including over the counter). For instance, in Danish pig production farms in 2010-2011, 20% of prevalence was observed if no cephalosporin was used (C3G and C4G) within the preceding year and 79% if those antibiotics were used (11) highlighting that a reduced use of antibiotic could quickly be favorable.

In Reunion, the prevalence of ESBL in beef cattle farms tends to be lower than data reported in comparable designed study in Germany (Bavaria) from 2011 to 2012, with 73.3% of beef cattle farms tested positive (21) and 54.4% in 2012 in Mecklenburg-Vorpommern (16). In Japan prevalence of 40.0% in beef cattle farms was observed in 2007 (20). These data are comparable to prevalence

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observed in our study in Mayotte and Madagascar. Beef cattle prevalence in Reunion was low but could be higher in dairy cattle as reported in Switzerland (OR: 5.95).

The main represented bacterial species among ESBL isolates was *E. coli* as observed in other studies (e.g. (18, 22, 23)). This species normally accounts for up to 1% of the colonic bacteria in cattle (24). This species can serve as a reservoir of antibiotic resistance genes within the digestive tract (25). Thus, *E. coli* was finally identified as logical indicator of the extent of antibiotic resistance within microbial populations digestive tract (26) and could be also used as an indicator of ESBL trends in epidemiological surveillance in Indian Ocean and worldwide.

No phenotypic resistance to ertapenem in ESBL isolates was identified which is in accordance with the absence of detection of carbapenemase producing Enterobacteriacae (CPE) in livestock in Indian Ocean in 2018 (1). However, use of CPE selective media would be more suitable for CPE detection in livestock. Resistance to nalidixic acid was elevated in ESBL producing *E. coli* in beef cattle from Reunion (50.0%) and in Madagascar both in poultry (28.6%) and pig (25.0%) productions. However, isolates resistant to nalidixic acid were not resistant to ofloxacin as observed in majority of cases (27). Finally, fluoroquinolone resistance was elevated in ESBL producing *E. coli* in pig production both in Madagascar (21.4%) and Reunion (25.0%) which could indicate a specific use of this antibiotic class use in both territories. Resistance to fluoroquinolone could be lower in Mayotte as no resistance to ofloxacin was observed but it should be confirmed as sample was reduced. Finally, resistance to fluoroquinolone observed in pig production could indicate past or present use of this critically important antimicrobial drug. Pig production was identified as the most important antibiotic consumer worldwide (28) but consumption estimates of each antibiotic classes in Indian Ocean territories is not available. French national data indicated that fluoroquinolones use in cattle production was the most important before pigs and poultry production but trends could differ in French oversea territories (29). Resistance to gentamicin was elevated in ESBL producing K. pneumoniae in Madagascar as already observed in humans in 2008 (30). Resistance to tetracycline was also high in this territory which could point out a drug overuse, particularly for widely available oral agents (1).

The most common ESBL gene identified in *E. coli* isolates tested was the CTX-M-1 subtype (54.4%) as observed in food-producing animals in European countries (31). It is now well established that CTX-M beta-lactamase is largely situated on plasmids, which allows the horizontal transfer between enterobacteriaceae (32) and explains the actual epidemic spread of this enzyme all over the world.

Overall ESBL gene diversity observed was reduced in our study, it could be due to a small sample of *E. coli* tested (N=10). The more diverse ESBL genes identified was in poultry production with almost three different genes detected in each territory. Most of ESBL genes detected in poultry were of the CTX-M1 group but SHV-ESBL and TEM-ESBL genes were also identified as in Dutch broilers (18).

In pig production, ESBL gene diversity was higher in Reunion with CTX-M-1, 3 and 32; the situation differed from Switzerland and Danish pig production where only CTX-M1 subtype was mostly represented (11, 23). In Madagascar, all *E. coli* tested in pig and beef cattle belonged to the CTX-M-15 subtype which is the main subtype observed in humans (33, 34) and could point circulation of ESBL between reservoirs. In beef cattle from Mayotte, ESBL *E. coli* belonged to the CTX-M-1 subtype whereas more diverse genes, including SHV, were detected in cattle from Switzerland (23).

Finally, specific ESBL gene observed in each production type from each territory (e.g. CTX-M-1 in pigs from Reunion and CTX-M-15 in Madagascar) could point a common past source of contamination by exploitation type with diffusion probably due to contact and introduction of ESBL carriers in the farm, as reported with CTX-M-14 in cattle from the United Kingdom (10). Thus, low ESBL gene diversity in each production type and territory seems pointing low introduction/exchanges of ESBL from other reservoirs and environment as stated by Dorado-Garcia (35). However, the identification of CTX-M-15 in pig, beef cattle and humans in Madagascar could point a common past ESBL source with transmission and diffusion between reservoirs. Thus, further

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investigations, including complete genome sequencing, is needed to evaluate the hypothesis of ESBL transmission and diffusion between reservoirs in this territory.

4.1. Risk factors of ESBL occurrence in IOC

4.1.1. Univariate

In Reunion, "recent age of the building" for broiler production was significantly explaining ESBL occurrence in univariate analysis. This factor is difficult to explain as biosecurity measure should be improved in recent farms, however, it could point an increasing antibiotic drug use in modern exploitations. No study has been performed regarding antibiotic consumption and use in IOC farms. In Reunion in pig exploitations, "change of shoes/boots before entering the building" was significantly explaining ESBL occurrence as observed in poultry production in Vietnam (12). This result could be related to a confusing explanatory factor not identified in both studies. Both "rodent control" and "two disinfections before next batch" factors were significantly reducing ESBL occurrence in pig production in Reunion related to biosecurity and hygiene measures known to help controlling disease and antibiotic resistance spread (36). In this way, ESBL introduction into farms by rodent should be investigated.

In Madagascar, in broiler production, chick production in the farm for raising chicken was identified as significantly reducing occurrence of ESBL. This is in accordance with data from the Netherlands indicating that ESBL-producing *E. coli* were introduced in the Dutch poultry production chain through imported day-old grandparent chickens indicating a vertical ESBL transmission (37). In beef cattle production in Madagascar, "clearing around the farm" reduced significantly ESBL occurrence in exploitations, effect of this measure was difficult to explain and could be related to a confusion factor not identified here.

4.2.2. Generalized linear models

In poultry, water quality control was a protective factor of ESBL occurrence. This observation is in accordance with studies on Campylobacter spp. which showed that electrolyzed water or chlorinated-water allowed reducing bacterial presence during poultry farm washing (38, 39). Resistant genes (i.e. vanA) were identified in drinking water biofilms (40) and rural surface water may become a large pool of antibiotic residues and resistant bacteria (41). As highlighted by Cox and Pavic (2009), in order to minimize transmission of enteropathogens, drinking water should be of potable quality and water should be treated with chemicals, or by filtration or reverse osmosis, to ensure freedom from enteric pathogens (42). Public water supply was identified as a protective factor of ESBL *E. coli* in pigs farms (compared to private sources) (43); this highlights the importance of controlling water quality on the farm. However, the role of water quality in the occurrence and maintenance of antibiotic resistance (both genes and resistant bacteria) in livestock production is still under investigated (most study focusing antibiotic spread thanks to wastewater) and further studies are needed.

In pig production, recent antibiotic use, soaking surface and farm visited by others farmers were identified as risk factors and detergent use for cleaning was a protective factor of ESBL occurrence in our model.

In Dutch pigs farms, the presence of ESBL *E. coli* carrying pigs was not related to total antibiotic use but strongly determined by the presence or absence of cephalosporin use at the farm (43). However, in our study, no information regarding the antibiotic type used was available but further investigation should be needed as well.

We identified use of detergent for cleaning as a protective factor of ESBL occurrence. On dairy farms, risk factors associated with hygiene paucity were identified (e.g. storage of slurry in a pit, infrequent cleaning of feeding equipment) (10). Cleaning and disinfection processes could be a milestone in ESBL occurrence with ESBL eradication obtained in pig farms under specific disinfection procedures (36). Soaking surface practice in pig farm production could be associated with wrong

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biosecurity practices; for instance, let water for a too short period could not allow complete cleaning. Thus, publications regarding biosecurity and hygiene measures reducing ESBL occurrence are scarce and further investigations should also be necessary.

To our knowledge, visiting farms by others farmers has never been identified as a risk factor of ESBL introduction. In Adler et al. (2015) frequent visits of the veterinarian increased the prevalence ratio in cattle farms in Israel (44). Visitors could contribute to ESBL introduction and could carry/share material that favor transmission pathways.

In beef cattle farms, recent antibiotic use, pet presence in the farm were risk factors whereas livestock size, disinfection and brushing around the farm were protective factors of ESBL occurrence in our model.

Antibiotic use is well identified to exert selection pressure on Enterobacteriaceae. In United Kingdom, 3rd or 4th generation cephalosporin use in dairy farms in the last 12 months were nearly 4 times more likely to have ESBL *E. coli* (10) and prophylaxis purposes increased ESBL carriage in Israeli cattle (44). In our study, recent antibiotic use was identified as a risk factor of ESBL occurrence but other factors such as frequency of use, type of antibiotic used and reasons for treatment in Madagascar, Reunion and Mayotte should be investigated.

Pet presence in the farm was a risk factor of ESBL. This funding was in accordance with Santman-Berends et al., 2017 (45) which found cat presence as an explanatory factor of ESBL occurrence in organic herds. This observation could be due to the fact that pets could be both given antibiotics by owners or play a role of reservoir/vector of ESBL from the close environment.

Paradoxically, herd size was identified as a protective factor in our study. Adler et al. (2017) also reported a decreased crowdedness associated with ESBL carriage reduction in Israeli cattle farms (44). The cattle herd size was also identified as a risk factor (46) and probably be due to higher animal traffic with more cattle in the biggest farm with risk of EBLSE carrier introduction. Thus, big farms could apply stricter biosecurity and cleaning practices in our study.

As discussed before, disinfection seems to be a milestone in ESBL management. However, publications regarding biosecurity and hygiene measures reducing ESBL occurrence are scarce particularly in cattle production. Furthermore, only beef cattle production was investigated in the three territories but ESBL occurrence could be higher in dairy cattle as in Switzerland (46).

5. Conclusions

Finally, this study in IOC commercial farms pointed elevated ESBL prevalence in all production type, except beef cattle in Reunion. It highlighted probable antibiotic overuse/misuse in exploitation contributing in ESBL selection and needs to evaluate consumption and use of antibiotic drugs in IOC territories. Concrete protective and risk factors of ESBL occurrence (e.g. pet presence, detergent use for cleaning) were identified, even if further investigations are needed to reinforce these results. This study is the first to explore tools for management of antibiotic resistance in IO farms and pointed needs in biosecurity and hygiene measures identification contributing to antibiotic resistance reduction in livestock. Last but not least, it provides interesting hypothesis to explore about ESBL transmission between food-producing animals and humans in Madagascar.6. Patents

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Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used E.C. and O.B. conceived and designed the experiments; M.L., M.J. collected samples in the field; A.L., G.M., A.E. S.R performed the laboratory experiments; N.G. analyzed the data and provided analysis tools; N.G. wrote the paper." Authorship must be limited to those who have contributed substantially to the work reported.

Conflicts of Interest: "The authors declare no conflict of interest. "

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