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Prevalence of *Salmonella* and *Campylobacter* in Chicken among the Different Farming Systems in Papua New Guinea (PNG)

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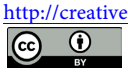

Abstract

This trial was conducted to identify the prevalence of *Salmonella* and *Campylobacter* in different farming practices: commercial, semi-commercial, and subsistence farms in the four regions; Southern, Highland, Momase, and New Guinea Island (NGI) in PNG. The research was conducted at Institute of Medical Research (IMR) at Goroka, Eastern Highland Province. The samples used in the experiment were retrospective samples that were collected in 2011 and 2012 among the four regions and at different farming sites. A total of 333DNA samples were extracted from cloacal swabs. These samples were subjected to molecular assay where DNA extraction was done and analysed using real-time polymerase chain reaction (PCR). From the result, it was evident that in Southern region, significant differences were observed only in commercial and semi-commercial farms (p-value = 0.0030) and semi-commercial and non-commercial farms (p-value = 0.0002). However, in NGI significant differences were not observed in all the farming practices (p-value < 0.05). Significant differences were observed between *Campylobacter* in commercial and non-commercial (p-value = 0.0006) and semi-commercial and non-commercial farm (p-value < 0.0001) in Momase region. The Highlands regions showed significant differences of *Campylobacter* spp. between commercial and semi-commercial (p-value < 0.0001) and commercial and non-commercial (p-value < 0.0001) farming practices.

Subject Areas

Zoology

Keywords

Deoxyribo Nucleic Acid, Polymerase Chain Reaction, *Salmonella*,

Campylobacter, Molecular Assay, Poultry

1. Introduction

Chicken (broiler and layer) farming is one of the agricultural practices that are active throughout the country. In PNG there are basically three types of farming practices occurring and they are commercial farming, semi-commercial, and non-commercial. Commercial farming is owned by big companies, semi-commercial is run by individuals and non-commercial are free range birds that are kept in the village but are reared for eggs and consumption. Chickens are part of the protein constituents in our diets. However, rearing of chicken under three different farming systems has a greater impact on the prevalence of the two bacteria; *Salmonella* and *Campylobacter* in the chicken.

2. Materials and Methods

2.1. Study Design

The study was conducted in Institute of Medical Research (IMR) at Goroka, Eastern Highlands Province. Retrospective poultry samples were used and were originally collected by the National Agriculture Quarantine Inspection Authority (NAQIA) and Department of Agriculture and Livestock (DAL), during their annual surveillance study in poultry survey among the four regions: Southern, Momase, New Guinea Island (NGI), and Highlands, from June 2011 to April 2012. About 385 cloacal swabs were collected and sent to the IMR. The study carried out was designed as a cross-sectional study looking at poultry samples collected over a one year period from different poultry farms in the country. However, no longitudinal analysis was performed due to insufficient collection time points available.

2.2. DNA Extraction

DNA extraction from cloacal swabs were performed using DNeasy Blood and Tissue Kit (QIAGEN Germany), according to the manufacturer's instructions. DNA extraction was done in the biological safety cabinet class II. A total of 200 µl of the sample was transferred into sterile microcentrifuge tube (1.5 ml) (Eppendorf®, Germany). About 20 µl of Proteinase K was added into the reaction tube followed by adding of 200 µl of Buffer AL. The samples were then vortex and incubated in the hot water bath at 56˚C for 10 min. After incubation, 200 µl of ethanol (96% - 100%) was added and the samples were mixed thoroughly by vortexing. After vortexing, all the samples in the reaction tubes were pipette into a new DNeasy mini spin column placed in a 2 ml collection tube. The tubes were then centrifuged at 8000 revolutions per minutes (rpm) (≥6000 g) for 1min. The flow was discarded and the DNeasy spin column tubes were placed into a new 2 ml collection tube. 500 ul of Wash Buffer AW1 was added and the suspension

was centrifuged at 8000 rpm (≥6000 g) for 1min. The flow was discarded and the spin column was placed into a new collection tube. Then 500 µl of Wash Buffer AW2 was added and the samples were centrifuged again for 3 min at 14,000 rpm (20,000 g). The flow was discarded the spin column was placed into a new 1.5 ml labelled microcentrifuge tubes. Finally, 200 µl of elution Buffer AE was directly added into the DNeasy membrane and centrifuged for 1 min at 8000 rpm. The DNeasy spin column was discarded and the DNA extract was ready for real-time PCR.

2.3. Primer and Probes

Salmonella and *Campylobacter* primers used in real-time PCR are listed below (Table 1). Primers for *Salmonella* are specific for outer membrane protein "ompF" gene and primers for *Campylobacter* are mainly for 16S rRNA gene.

2.4. Real-Time PCR

Nucleic acid extracted from cloacal swabs was tested for *Salmonella* and *Campylobacter* spp. using real-time PCR analysis. PCR preparation was done by creating a plate map followed with master-mix reaction preparation (Table 2).

All the reagents were thawed down in the bio-safety hood cabinet and pipette into the reaction Eppendorf tube (1.5 ml) (Interpath Australia). The reaction tube was then centrifuge using the micro-centrifuge. 18 µl of the reaction solution was aliquot into each Hard-Shell® PCR Plates 96-well white (Bio-Rad Australia)

Table 1. Probes and primers nucleotide sequencing.

Organism	Oligonucleotide	Nucleotide Sequence	Reference
Salmonella	F primers	5'CCTGGCAGCGGTGATCC'3	(Tatavarthy and Cannons, 2010) [1]
	R primers	5'AAATTTCTGCTGCGTTTGCG'3	(Tatavarthy and Cannons, 2010) [1]
	Probe	5'TGCCCTGCTGGCTGCTGCA'3	(Tatavarthy and Cannons, 2010) [1]
Campylobacter	F primers	5'CTGCTTAACACAAGTTGAGTA GG'3	(Josefsen *et al.*, 2004) [2]
	R primers	5'TTCCTTAGGTACCGTCAGAA'3	(Josefsen *et al.*, 2004) [2]
	Probes	5'CCTCCACGCGGCGTTGCTGC'3	(Josefsen *et al.*, 2004) [2]

Table 2. Master-mix preparation.

No: of samples: 12				
Reagents	Volume for 1 rxn (µl)	Vol. for master + 10% (µl)	Vol. for master + 1 rxn (µl)	Vol. for master + 2 rxn (µl)
2 × Quantitect master-mix	10	121	120	130
Forward primer (20 µl)	0.2	2.42	2.4	2.6
Reverse primer (20 µl)	7.4	89.54	88.8	96.2
Total	17.8	215.38	213.6	231.4
Template DNA (per rxn): 2 µl				

on the green bio-freezer rack and capped with Optical Flat 8-Cap Strips (Bio-Rad Australia). The PCR plates were then covered with aluminium foil and transferred to Dangerous Pathogen Laboratory (DPL) for addition of templates and the controls. Extracted DNA (templates), positive control, and non-template control were added to the PCR well strips respectively at a volume of 2 µl each. The samples were then transferred to the wet lab for molecular analysis using real-time PCR.

The PCR strips were placed in the max-centrifuge (Thermo IEC, Centra Gp8R). The rpm was set to 520 for 1 min. After centrifuging the strips were wiped with tissue and placed in the CFX 96 Real-time PCR system C1000 Thermol Cycler. *Salmonella* and *Campylobacter* have the same PCR cycling parameters: Initial heating occurred at 50˚C for 2 min followed by denaturation at 95˚C at 15 sec and 40 cycles of annealing temperature at 94˚C for 1 min and extension temperature at 60˚C for 1 min (Table 3). The amplification read at (FAM) Fluorophores and the final results amplified after 2 hrs 6 min.

2.5. Statistical Analysis

All statistical analysis was performed using Real-Stats in Microsoft Excel. Comparison of *Salmonella* and *Campylobacter* spp. in different farming methods and in different regions was subjected to chi-square test. Further analysis was done using Fisher's Exact Test to determine the significance difference of *Salmonella* and *Campylobacter* among each of the farming practices, and regions. A p-value of 0.05 or less was considered significant.

3. Results

3.1. Southern Region

Prevalence of *Salmonella* and *Campylobacter* spp. were compared among different poultry farming methods: commercial, semi-commercial and non-commercial farming methods. A total of 8 cloacal swabs were collected from commercial farms, 6 from semi-commercial and 33 from non-commercial farms. *Salmonella* was not observed among the farming methods. However, *Campylobacter* was observed at 83.3% (5/6) in semi-commercial farms and 6.1% (2/33) in non-commercial farms but absent in commercial farms (Table 4).

Significant differences was observed only in commercial and semi-commercial farms (p-value = 0.0030) and semi-commercial and non-commercial farms (p-value = 0.0002).

3.2. New Guinea Island (NGI)

A total of 57 cloacal swabs were collected from different poultry farming methods in the NGI region. *Salmonella* was positive at 5.9% (1/17) in semi-commercial and 2.6% (1/38) in non-commercial farms. *Campylobacter* was observed at 50.0% (1/2), 29.4% (5/17) and 21.1% (8/38) in commercial, semi-commercial and non-commercial farms respectively (Table 5).

Table 3. Real-time PCR cycling parameters.

Stage	Temperature	Time
Initial heat activation	50˚C	2 min
Denaturation	95˚C	15 sec
40 cycle of:		
Annealing	94˚C	60 sec
Extension	60˚C	60 sec

Table 4. Prevalence of *Salmonella* and *Campylobacter* spp. by different farming methods in the Southern region.

Farming methods	Total samples tested	*Salmonella*		*Campylobacter*	
		Positive	%	Positive	%
Commercial	8	0	0.0	0	0.0
Semi-commercial	6	0	0.0	5	83.3
Non-commercial	33	0	0.0	2	6.1
Total	47	0	0.0	7	14.9

Table 5. Prevalence of *Salmonella* and *Campylobacter* spp. among farming methods in NGI region.

Farming methods	Total samples tested	*Salmonella*		*Campylobacter*	
		Positive	%	Positive	%
Commercial	2	0	0.0	1	50.0
Semi-commercial	17	1	5.9	5	29.4
Non-commercial	38	1	2.6	8	21.1
Total	57	2	3.5	14	24.6

3.3. Momase

A total of 150 cloacal swabs samples were collected and tested among different poultry farming methods in the Momase region. *Salmonella* spp. was not observed in the different farming methods. Semi-commercial farms had the highest prevalence of *Campylobacter* spp. at 57.7% (45/78), with commercial farms at 52.0% (13/25) and non-commercial farms at 12.7% (6/47) (**Table 6**).

Significant differences were observed between *Campylobacter* in commercial and non-commercial (p-value = 0.0006) and semi-commercial and non-commercial farm (p-value < 0.0001).

3.4. Highlands

A total of 131 cloacal swabs were collected from poultry farms in the Highlands region. Twenty four swabs were collected from commercial farms, 62 from semi-commercial and 45 from non-commercial farms. *Salmonella* spp. was observed in both semi-commercial and non-commercial farms at 1.6% (1/62) and

Table 6. Prevalence of *Salmonella* and *Campylobacter* spp. among poultry farming methods in Momase region.

		Salmonella		*Campylobacter*	
		Positive		Positive	
Farming methods	Total samples tested	n	%	n	%
Commercial	25	0	0.0	13	52.0
Semi-commercial	78	0	0.0	45	57.7
Non-commercial	47	0	0.0	6	12.7
Total	150	0	0.0	64	42.7

2.2% (1/45) respectively. *Campylobacter* spp. was found to have a higher prevalence rate in semi-commercial farms at 56.5% (35/62) compared to commercial farms at 8.3% (2/24) and least prevalent in non-commercial farms at 17.8% (8/45) (Table 7).

Significant differences were observed for *Campylobacter* spp. between commercial and semi-commercial (p-value < 0.0001) and commercial and non-commercial (p-value < 0.0001) farming practices in Highlands region.

3.5. *Salmonella* and *Campylobacter* spp. among Commercial, Semi-Commercial, and Non-Commercial Farms in the Country

All data from different farming methods and data from all the regions were pooled, and subjected to chi-square test. Semi-commercial farms were observed to have higher carriage rates of *Campylobacter* spp. at 52.1% (85/163) compared to commercial farms at 27.1% (16/59) and non-commercial farms at 9.8% (16/163). *Salmonella* spp. was present only in semi-commercial farm and non-commercial farms at 1.2% (2/163) and 1.2% (2/163) respectively, but absent in commercial farms (Table 8).

Significant differences were observed for *Campylobacter* spp. between commercial and semi-commercial farms (p-value = 0.0012) and semi-commercial and non-commercial farm (p-value < 0.0001).

3.6. *Salmonella* and *Campylobacter* spp. among the Four Regions: Southern, NGI, Momase, and Highlands

Data from the three different farming practices was pooled, and the presence of *Salmonella* and *Campylobacter* spp. was analysed between the different regions: Southern, Momase, NGI, and Highlands by chi-square analysis followed by Fisher's exact test. *Campylobacter* was observed in all four regions while *Salmonella* was largely absent. The Momase and Highlands regions had the higher prevalence rate of *Campylobacter* at 42.7% (64/150) and 34.4% (45/131) respectively compared to the NGI and Southern regions at 24.6% (14/57) and 14.9% (7/47) respectively (Table 9). *Salmonella* was observed but at a very low prevalence rate only in NGI region and Highlands region at 3.5% (2/57) and 1.5% (2/131) respectively. Significant differences in *Salmonella* between regions were not observed.

Table 7. Prevalence of *Salmonella* and *Campylobacter* spp. among poultry farming methods in Highlands region.

		Salmonella		*Campylobacter*	
		Positive		Positive	
Farming methods	Total samples tested	n	%	n	%
Commercial	24	0	0.0	2	8.3
Semi-commercial	62	1	1.6	35	56.5
Non-commercial	45	1	2.2	8	17.8
Total	131	2	1.5	45	34.4

Table 8. Prevalence of *Salmonella* and *Campylobacter* spp. among farming methods in the country.

		Salmonella		*Campylobacter*	
		Positive		Positive	
Farming methods	Total samples tested	n	%	n	%
Commercial	59	0	0.0	16	27.1
Semi-commercial	163	2	1.2	85	52.1
Non-commercial	163	2	1.2	16	9.8
Total	385	5	1.3	117	30.4

Table 9. Prevalence of *Salmonella* and *Campylobacter* spp. among the four regions in the country.

		Salmonella		*Campylobacter*	
Regions	Total samples tested	Positive	%	Positive	%
Southern	47	0	0	7	14.9
NGI	57	2	3.5	14	24.6
Momase	150	0	0	64	42.7
Highlands	131	2	1.5	45	34.4
Total	385	4	1.0	130	33.8

Significant differences were observed for *Campylobacter* between the Southern and Momase regions (p-value = 0.0008), Southern and Highlands regions (p-value = 0.0132), and Momase and NGI regions (p-value = 0.0313). Significant differences in *Salmonella* between regions were not detected.

4. Discussion

4.1. Prevalence of *Salmonella* and *Campylobacter* among Different Farming Methods

Campylobacter and *Salmonella* were both detected from cloacal samples collected among the three different poultry farming methods in the country. There

is a high prevalence of *Campylobacter* in semi-commercial (52.5%) and commercial (27.1%) farms and least in non-commercial or free range system (9.8%) in the present study. The differences in the prevalence of *Campylobacter* among the farming methods could be as a result of different agricultural activities such as farming methods, vaccines, deep litter management and biosecurity practices in the farm.

Vaccination of chickens is regarded as an additional measure to increase resistance of birds against *Salmonella* infection and decrease shedding of viable organisms (Dewaele *et al.*, 2012 [3]; Hassan and Curtiss, 1996 [4]). Studies have proven that vaccination works by reducing the prevalence of *Salmonella* in breeder hens and the progeny or by increasing the passive immunity of the birds (broiler and layer) and blocking the horizontal transmission of *Salmonella* to the flocks (Dórea *et al.*, 2010) [5]. However, no suitable vaccine is readily available for Campylobacter infection (Ahmed *et al.*, 2013 [6]; Schroeder *et al.*, 2014 [7]). Hence, chicken are highly susceptible to *Campylobacter* transmission and colonization than *Salmonella.*

Under normal poultry farming condition, chicks usually become infected with *Campylobacter* at about 2 - 3 weeks of age (Hodgins *et al.*, 2015 [8]; Lin, 2009 [9]). Maternal antibodies providing passive immunity are transferred to the avian embryos from serum to egg yolks (Hamal *et al.*, 2006) [10]. The level of antibodies is very high at 3 to 4 days after hatching and thereafter gradually decreases to undetectable levels at 2 to 3 week (Sahin *et al.*, 2001) [11]. Once the chicken is infected with *Campylobacter*, the organisms usually spread so rapidly that close to 100% birds are reported to be colonized for a shorter period of time (Hamal *et al.*, 2006) [10]. The pathogen survives in the chicken gut throughout the grow-out period (Hodgins *et al.*, 2015) [8].

Poultry litter provides an ideal environment for *Campylobacter* growth however other researchers have observed lower prevalence of *Salmonella* in litter. Environmental factors in the poultry house play an important role on the growth of *Campylobacter* in the litter. Since *Campylobacter* is sensitive to drying, increased temperature, lower ventilation rate and high humidity with an increasing pH in the litter encourages higher growth of *Campylobacter* (Chinivasagam *et al.*, 2009) [12]. This could be the reason for the non-detection of *Campylobacter* in non-commercial poultry farms. Litter management in the farm is a very important agricultural practice to reduce prevalence of *Campylobacter* (Line and Bailey, 2006) [13].

Urea and uric acids make up 70% of the nitrogen content of poultry litter and are readily degraded to ammonia (Newell *et al.*, 2011 [14]; Rothrock *et al.*, 2008 [15]). Ammonia emissions beyond the threshold level of 25 ppm in the poultry house causes complication in chicken's health. Birds starting to develop stressful conditions, infection occurs in respiratory system and eventually decreases immunity. Thus leaving the birds susceptible to bacterial colonization and infection (Line and Bailey, 2006) [13].

Prevalence of *Salmonella* and C*ampylobacter* in poultry is determined by the on-farm biosecurity practices (Colles *et al.*, 2015 [16]; Hald *et al.*, 2000 [17]). Biosecurity is a set of preventative measures to reduce the risk of transmission in poultry farms in three main areas: isolation, mobility, and cleanliness and hygiene. Effective biosecurity measures to control *Salmonella* and *Campylobacter* in poultry farms include all-in-all-out system followed by disinfection of sheds, restricted movement of birds, workers and equipment, lack of contact with migratory birds, use of feed pellets, chlorination of drinking water, proper litter management, washing and sanitizing of hatching eggs, and purification of air in hatching cabinets (Colles *et al.*, 2015 [16]; Conan *et al.*, 2012 [18]). The prevalence of *Salmonella* and *Campylobacter* in the farm is not guaranteed to be eliminated completely, but lowering the prevalence is an important strategy for reducing contamination of birds entering the processing plant and minimising risk of contaminated meat and egg products entering the food chain (McCrea *et al.*, 2006 [19]; Trampel *et al.*, 2014 [20]).

Poultry housing system in PNG provides free access for movement of insects such as Synanthropic flies, in particular the house fly Musca domestica and flies belonging to Diptera: Brachycera spp. which have been associated with animal and human activities (Hald *et al.*, 2008 [17]; Trampel etal., 2014 [20]). Darkling beetles (Hazeleger *et al.*, 2008) [21] and rodents are ubiquitous and thrives wherever food stocks and habitats are plentiful and can live in close proximity to humans and livestock (Meerburg *et al.*, 2006) [22].

4.2. Prevalence of *Salmonella* and *Campylobacter* spp. in Different Regions

Salmonella and *Campylobacter* positivity were identified among the four regions in the country, Southern, Momase, NGI and the Highlands regions. *Campylobacter* was found to be more prevalent than *Salmonella*. It was observed that *Campylobacter* had a higher prevalence in the Momase and Highland regions at 42.7% and 34.4% respectively compared to the NGI (24.6%) and Southern (14.9%) regions. *Salmonella* was observed only in NGI and Highlands regions at (3.5%) and (1.5%). Currently there is no information available about the poultry farming practices in each of the regions. However, it is assumed that higher prevalence of *Campylobacter* in Momase and Highlands regions is believed to be associated with poultry farms having poor biosecurity practices.

5. Conclusion

In conclusion, *Salmonella* and *Campylobacter* was observed in poultry farms in PNG, however at low prevalence as compared to other developed countries. *Campylobacter* is more prevalent than *Salmonella*. Semi-commercial and commercial farms were found to have more carrierssss of *Campylobacter* than in non-commercial or free-range farm. A higher prevalence of *Campylobacter* was also observed in Momase and Highlands region.

Conflicts of Interest

The authors declare no conflicts of interest.

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