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Risk factors for persistence infection of non-typhoidal Salmonella in poultry farms, North-Central Nigeria

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Abstract: Salmonellosis is a bacterial zoonosis with array of health conditions. Non-typhoidal salmonellosis (NTS) have a discrete adaptation to certain animals; in poultry, pullorum and fowl typhoid are its primary disease manifestations. The diseases are prevalent in the Nigerian poultry and have been well studied in Nigeria, but less so in the north central Nigeria (NCN). Using field sampling, laboratory methods and semi structured questionnaire in 1000 poultry farms from NCN, we explore the incidence and risk factors for the persistence of NTS infection in poultry. Approximately 41.6% of the farms have experienced NTS but only 6.3% have current infection with NTS. Farm experience of NTS moderately predicted awareness of salmonellosis. Increasing stock in smallholder farms, self-mixing of concentrate on the farm, usage of stream water, pen odour, non-adherence and partial adherence of farms to recommended poultry vaccination against pullorum and fowl typhoid, and lack of and non-adherence to biosecurity were identified risk factors that increased the odds of NTS infection in poultry. Antibiotic use practice may have reduced the isolation rate of NTS, yet NTS continues to challenge poultry farms in Nigeria. Identified risk practices must be mitigated intentionally and biosecurity and hygiene must improve to reduce the burden of NTS.

Keywords: Non-typhoidal Salmonella; poultry; risk factor; Nigeria; fowl typhoid; pullorum disease.

1. Introduction

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Fowl typhoid and pullorum disease are bacterial infection (salmonellosis) of farmed poultry caused by Salmonella enterica subspecies enterica serovars Gallinarum biovars Gallinarum and Salmonella enterica subspecies enterica serovar Gallinarum biovar Pullorum, respectively, and they are widely distributed globally [1,2]. Recent evidence has also suggested tendency towards increasing antimicrobial resistance in strains of these organisms obtained from poultry [3-5]. Although, its eradication is possible and this have been largely achieved in many commercial poultry in developed countries in Western Europe, the United States of America (USA), Canada, Australia and Japan, its eradication in developing countries, particularly in Africa, Asia and South America remains debatable [6-8].

Salmonellosis is a bacterial zoonoses with considerable public health impacts, and it can be caused by typhoidal and non-typhoidal Salmonella organisms, including those mentioned above [8,9]. According to FoodNet surveillance data, Salmonella causes more disease burden in humans than any other foodborne pathogen, and globally, it causes up to 20 million human cases annually [8-10]. In the USA alone, salmonella-contaminated poultry is responsible for an estimated loss of $2.5 billion annually, or the loss of 15,000 QALYs in annual disease burden [9,10]. This considerable burden of disease is caused by food handling and preparation problems in food service and retail settings, some of which may have been associated with contaminations along the value chain [5,9,10].

The non-typhoidal Salmonella (NTS) refers to the infection produced by all serotypes of Salmonella except for typhoidal and paratyphoidal group. Although, there are at least 2,463 serotypes of Salmonella found to date (over 2,500 by other estimates) [11-14], the laborious traditional phenotypic serotyping method is still popular. It is challenging because it involves more than 150 specific antisera and expert interpreters to analyse the results [12]. In recent times, proposal for genome-based Salmonella serotyping and micro-array methods have been made [12,15]. The symptoms of NTS in humans include diarrhea, vomiting and abdominal cramps which develop 12 to 72 hours after infection. NTS have a discrete adaptation to certain animals such as Salmonella Choleraesuis to pigs, Salmonella Dublin to cattle, Salmonella Abortusovis to sheep, and Salmonella Gallinarum (Salmonella enterica subspecies enterica serovars Gallinarum biovars Gallinarum) and Salmonella Pullorum (Salmonella enterica subspecies enterica serovar Gallinarum biovar Pullorum) to poultry [2,11,16,17].

In Nigeria, the burden of zoonotic salmonellosis is unknown in humans or poultry, however, significant works have been produced on salmonellosis in poultry [3,18-25]. However, these studies have been concentrated in the extreme north and the southern belt of the country. The North Central Nigeria (NCN), which connects the southern belt of the country, where most of the commercial poultry activities occur with the north where most of the indigenous poultry populations predominate, have been less studies to date. This NCN has significant poultry population in excess of 44,789,854, by the year 2020 estimates [26], and account for most of the meat and egg supplies to the Federal Capital Territory and its neighborhood. There is therefore a need to carry out series of empirical study including the risk factor for continuing infections of poultry farms with Salmonella in North Central Nigeria,

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to bridge the existing knowledge gaps that exist in salmonella studies in Nigeria in order to inform policy aimed at reducing the burden of this bacteria zoonosis.

2. Results

This work covered the six states of the North Central zone of Nigeria and the Federal Capital Territory (FCT) (Figure 1). A total of 150 samples were collected from three local government areas (LGAs) in every state surveyed except in Plateau State where 100 samples were collected from two LGAs. In the period under consideration (≤ 15 - 18 months equivalent to the maximum period for current cycle of stocking of poultry chickens), a total of 416 farms (41.6%) have experienced non-typhoidal Salmonella (NTS), as confirmed by validated veterinary laboratory reports or the resident veterinarians, but only 63 farms (6.3%) have current infection with NTS at the time of sample collection based on further laboratory confirmation. All farm-origin samples (n = 1000) were tested first using bacteria cultures, phenotypic and biochemical characterization but only sixty three farms was confirmed positive. Further PCR evaluation of 15 positive samples selected from among the 63 positive isolates revealed three pure culture of Salmonella enterica subsp. enterica serovar Enteritidis strain, with the other samples being mixed infections (Figure S1, Table S1).

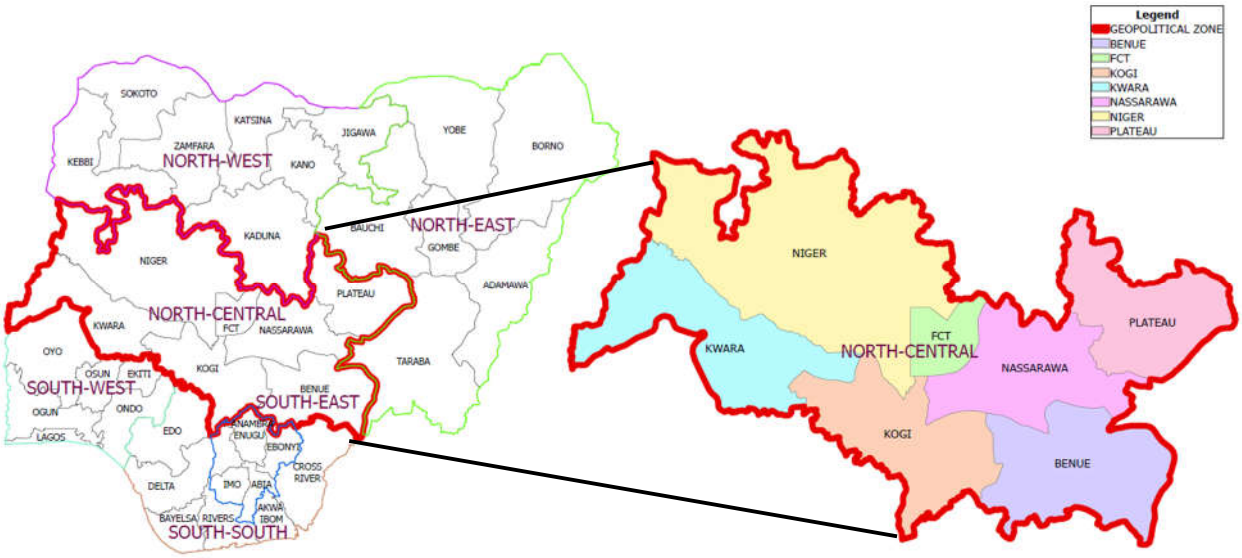

Figure 1. Map of Nigeria with a call-out map of the North-Central Zone.

A total of 569 respondents were male and based on the years of experience in poultry farming, ≤ 2 years, > 2 - ≤ 4 years, > 4 - ≤ 6 years and > 6 years were 22.4%, 31.9%, 23.9% and 21.8% respectively. Majority of the interviewed farmers have tertiary education (50.8%) and only 49.2% having other forms of education up to secondary levels. Among the farms surveyed, 44.4% practiced broiler operations, 22.5% operated layer operations, 29.4 % operated mixed operation (layer and broilers on the farm) (Table 1). Details of other

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descriptive statistics on all farm and field level data were described in Table 1.

Using pairwise correlations, most of the risk and management related variables evaluated against the experience of Salmonella in farms were very weakly or negatively correlated except the awareness of Salmonellosis (NTS) as a potential zoonosis was moderately correlated with the experience of Salmonella in poultry farms (Table 2). The higher the number of poultry chickens in the farm, the higher the odds of NTS in the farms. In particular, having between 500 – 1,000 chickens in the farm has an odds of increasing the risk of infection three folds ($p < 0.001$), and having > 1,000 chickens has an odds of ≈ 4 folds of increasing the risk of persistent infection ($p < 0.001$) (Table 3). Farmers who self-mix concentrate on the farm has a 2-fold odds of persistent NTS infection ($p < 0.001$), and the use of stream water produced the same odds ($p < 0.01$). Chickens in poultry cages have 2-fold odds of persistent NTS infection ($p < 0.001$), and non-adherence of farms to recommended poultry vaccination against pullorum and fowl typhoid increased the odds of NTS infection by > 7 folds ($p < 0.001$), and even partial adherence increased the risk over 4 folds ($p < 0.001$) (Table 3). Farmers who were not implementing and applying the principles of biosecurity strictly have a 2-fold odds of increased NTS infection in their farms (Table 3). The laying stock were approximately two folds as likely to be infected with persistent NTS compared with short-cycled broilers ($p = 0.002$). Finally, farms with no pen odour is 8-folds less likely to experience NTS infection compared with pens with persistent odour ($p < 0.001$) (Table 3).

At the multivariable logistic regression model, the higher the number of poultry chickens in the farm, the higher the odds of NTS in the farms (500 – 1,000 chickens, OR = 2.20, $p < 0.001$; > 1,000 chickens, OR = 2.17, $p = 0.004$). Whereas the dug up well will reduce the odds of infection by half (OR = 0.57, $p = 0.01$), use of stream water as sources of drinking water for poultry birds increased the odds of NTS infection by > 3 folds ($p = 0.005$) (Table 4). Significantly, both the partial and non-adherence of farms to the recommended poultry vaccination against pullorum and fowl typhoid increased the odds of NTS infection in the poultry farms five folds each (Table 4). The Hosmer Lemeshow Goodness of Fit = $\chi 2 = 2.58$; $p = 0.96$; Akaike Information Criterion (AIC) = 945.52; Area under curve (Receiver Operation Characteristics (ROC)) = 0.72 (Figure 2).

Table 1. Descriptive analysis of the respondents' variables on the incidence of non-typhoidal Salmonella in poultry farms, North-Central Nigeria.

Variable* (n)	Categories	Proportion (%)	95% Confidence Interval
States (1000)	Kwara	15.00	12.78 – 17.22
	Nasarawa	15.00	12.78 – 17.22
	Kogi	15.00	12.78 – 17.22
	Niger	15.00	12.78 – 17.22
	Plateau	10.00	8.14 – 11.86
	Benue	15.00	12.78 – 17.22
	FCT	15.00	12.78 – 17.22
Experience confirmed cases of salmonellosis in the last 1-2 years (1000)	No	58.40	55.27 – 61.48
	Yes	41.60	38.54 – 44.66
Current infection with Salmonella (1000)	Negative	93.70	92.19 – 95.21

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	Positive	6.30	4.79 – 7.81
Gender (1000)	Male	56.90	53.83 – 59.97
	Female	43.10	40.02 – 46.17
Length of years in the poultry farm (1000)	≤ 2years	22.40	19.81 – 24.99
	> 2 - ≤ 4years	31.90	29.01 – 34.79
	> 4 - ≤ 6years	23.90	21.25 – 26.55
	>6years	21.80	19.23 – 24.36
Educational level of the poultry farmer (1000)	Primary	8.80	7.04 – 10.56
	Secondary	38.10	35.08 – 41.12
	Tertiary	50.80	47.70 – 53.90
	Others	2.30	1.37 – 3.23
Type of poultry (1000)	Broilers	44.40	41.31 – 47.48
	Layers	22.50	19.91 – 25.09
	Others	3.70	25.28 – 4.87
	Mixed	29.40	26.57 – 32.23
Number of chickens (1000)	≤200	34.90	31.94 – 37.86
	201 – 500	27.50	24.73 – 30.27
	501 - 1000	25.90	23.18 – 28.62
	≥1000	11.70	9.70 – 13.70
Source/type of feed (999)	Concentrate	59.46	56.41 – 62.51
	Mix	23.72	21.08 – 26.37
	Self-compounded	16.82	14.49 – 19.14
Source of water for chickens (999)	Borehole	46.05	42.95 – 49.14
	Tap borne (municipal)	20.22	17.73 – 22.72
	Well	29.53	26.70 – 32.36
	Stream	4.00	2.79 – 5.22
	Other	0.20	0.07 – 0.48
Pen type (998)	Standard block	30.06	27.21 – 32.91
	Dwarf block	41.98	38.92 – 45.05
	Zinc type	24.64	21.97 – 27.33
	Others	3.31	2.20 – 4.42
System of management (1000)	Deep litter	64.20	61.22 – 67.18
	Battery Cage	31.80	28.91 – 34.69
	Others	4.00	2.78 – 5.22
Type of litter material used (1000)	Sawdust	42.90	38.83 – 45.97
	Wood shavings	30.20	27.35 – 35.05
	Sand	11.70	9.70 – 13.70
	Cement floor	14.00	11.85 – 16.15
	Others	1.20	0.52 – 1.88
Litter management (1000)	Poor	65.20	62.24 – 68.16
	Fair	9.50	7.68 – 11.32
	Good	25.30	22.60 – 28.00
Pen odor (1000)	No	41.60	38.54 – 44.66
	Yes	58.40	55.34 – 61.46
Stocking density (chickens per square meter of available floor space) (998)	12-14	17.43	15.08 – 19.79
	14-16	18.24	15.84 – 20.64
	16-18	22.04	19.47 – 24.62
	18-20	11.52	9.54 – 13.51
	20 and above	6.71	5.16 – 8.27

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	Unknown	24.05	21.39 – 26.70
Adherence to vaccination (1000)	No	8.10	6.41 – 9.79
	Yes	64.40	61.43 – 67.37
	Partial	27.50	24.73 – 30.27
Practiced biosecurity (1000)	No	11.40	9.43 – 13.37
	Yes	55.50	52.41 – 58.59
	Partial	33.10	30.18 – 36.02
Ever heard of salmonellosis (1000)	No	34.90	31.94 – 37.86
	Yes	64.90	61.94 – 67.86
	Don't know	0.20	0.08 – 0.48
Experience confirmed cases of salmonellosis in the last 1-2 years (1000)	No	30.90	28.03 – 33.77
	Yes	41.60	38.54 – 44.66
	Don't know	27.50	24.73 – 30.27
When salmonellosis or mixed infection was experienced in the farm, how was it handled? Or what protocol was used? (1000)	Antibiotics	0.70	0.18 – 1.21
	Vaccination	36.90	33.90 – 39.90
	Antibiotics combined with vaccination	11.50	9.52 – 13.48
	Culling	27.00	24.24 – 29.76
	Sales	13.20	11.10 – 15.30
	Others	10.60	8.69 – 12.51
	No response	0.10	0.09 – 0.30
Have the knowledge (awareness) of salmonellosis as a zoonotic disease (1000)	No	38.00	34.99 – 41.01
	Yes	60.80	57.77 – 63.83
	No response	1.20	0.66 – 2.11
Source of Knowledge (1000)	Electronic media	11.00	0.45 – 1.75
	Print media	35.40	32.43 – 38.37
	Extension agent	86.00	6.86 – 10.34
	Vet/AHO	9.40	7.59 – 11.21
	Other farmers	26.10	23.37 – 28.83
	Hospital	15.80	13.54 – 18.07
	Other sources	3.60	2.44 – 4.76
Ever taken samples to veterinary service (1000)	No	36.00	33.02 – 38.98
	Yes	62.10	59.09 – 65.11
	No response	1.90	1.20 – 2.97
Access to professional support (1000)	No	26.70	23.95 – 29.44
	Yes	33.90	30.96 – 36.84
	Not always	37.40	34.40 – 40.40
	Others	2.00	1.13 – 2.87

All analysis was conducted using the method of Agresti and Coull [27] and reported using the Binomial Wald method. *Categorization of Variables based on selected industry standards and peer reviewed literature (Table S2).

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Table 2. Pairwise correlation of selected variables for incidence of Non-Typhoidal Salmonella in poultry farms, North-Central Nigeria.

	Experienced Salmonella	Gender	Length of farming	Education level	Type of farms	No of chickens	Feed source	Water source	Management system	Litter management	Pen odor	Stocking density	Adherence to vaccination	Practice biosecurity	Heard of Salmonella	Knowledge of Salmonella
Experienced Salmonella	1.000															
Gender	-0.003	1.000														
Length of farming	0.041	0.083*	1.000													
Education level	0.017	0.032	0.234*	1.000												
Type of farms	0.097*	0.084*	0.189*	0.120*	1.000											
No of chickens	0.233*	0.084*	0.145*	0.080*	0.149*	1.000										
Feed source	-0.156*	-0.004	0.099	0.004	0.095*	-0.079*	1.000									
Water source	-0.172*	0.009	0.090*	-0.068*	0.025	-0.157*	0.257*	1.000								
Management system	-0.125*	-0.022	-0.014	0.008	-0.096	-0.237	0.100	0.136*	1.000							
Litter management	-0.071*	-0.051	-0.116*	-0.151*	-0.049	-0.108*	0.177*	0.136*	0.044	1.000						
Pen odor	0.029	-0.005	0.003	-0.021	-0.007	0.014	0.075*	0.232*	0.086*	0.152*	1.000					
Stocking density	-0.110*	0.011	0.063*	-0.022	-0.063*	-0.009	0.053	0.021	0.056	0.093*	-0.006	1.000				
Adherence to vaccination	0.178*	0.116*	0.074*	0.109*	0.071*	0.219*	-0.237	-0.165*	-0.059*	-0.224*	-0.017	-0.127*	1.000			
Practice biosecurity	0.143*	0.046	0.141*	0.110*	0.050	0.084*	-0.051	-0.180*	0.037	-0.267*	-0.143*	-0.065*	0.322*	1.000		
Heard of Salmonella	0.478*	0.011	0.026	0.081	0.123*	0.196*	-0.198*	-0.174*	-0.054	-0.126*	0.038	-0.046	-0.227*	0.172*	1.000	
Knowledge of Salmonella	0.343*	-0.003	-0.066*	-0.084*	0.101*	0.221*	-0.122*	-0.209*	-0.057	-0.042	-0.017	-0.053	0.119*	0.170*	0.456*	1.000

* Significant at $p = 0.05$. Only 'Heard of Salmonella' variable was moderately correlated with 'Experienced Salmonella', while the 'Knowledge of Salmonella' was weakly predicted by the 'Experienced Salmonella'. All other variables were poorly or negatively correlated with the experience of Salmonella.

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Table 3. Univariable analysis for contamination of poultry farms with Non-Typhoidal Salmonella (NTS) in North-Central Nigeria

Variable	Category	OR (95% CI)	Chi Square value	*p*-value*
Length of poultry farming by the farmer	< 2 years	1.00	2.54	Ref
	2 – 4 years	0.87 (0.61; 1.23)		0.43
	> 4 – 6 years	0.99 (0.69; 1.44)		0.98
	> 6 years	1.15 (0.79; 1.68)		0.47
Level of education of the poultry farmer	Primary	1.00	3.90	Ref
	Secondary	0.79 (0.49; 1.26)		0.32
	Tertiary	0.91 (0.58; 1.43)		0.68
	Other forms (skill learning, etc.)	0.42 (0.15; 1.18)		0.10
Number of chickens in the farm	< 200	1.00	60.09	Ref
	201 – 500	1.47 (1.05; 2.06)		0.03
	501 – 1000	2.93 (2.10; 4.11)		< 0.001
	> 1000	3.79 (2.45; 5.87)		< 0.001
Source of feed	Multi-sourced commercial	1.00	41.28	Ref
	Bought-in concentrate and mix	1.87 (1.38; 2.54)		< 0.001
	Self-compounded	0.47 (0.32; 0.70)		< 0.001
Source of water	Borehole	1.00	59.83	Ref
	Pipe-borne municipal water	1.53 (1.10; 2.13)		0.01
	Dug-up well	0.42 (0.30; 0.58)		< 0.001
	Stream	2.33 (1.19; 4.58)		0.01
Pen type	Standard type house (fully built)	1.00	8.81	Ref
	Dwarf block with side nets	0.90 (0.67; 1.22)		0.51
	Zinc-sided (roofing sheet) house	0.61 (0.43; 0.86)		0.005
	Other forms of buildings	0.77 (0.37; 1.61)		0.49
Management system	Deep litter	1.00	16.10	Ref
	Battery cage	1.74 (1.33; 2.28		< 0.001
	Others (semi-intensive etc.)	1.25 (0.66; 2.40)		0.49
Litter management	Good	1.00	11.13	Ref
	Poor	1.14 (0.74; 1.75)		0.59
	Fair	0.62 (0.46; 0.84)		0.002
Litter materials used	Saw dust	1.00	4.62	Ref
	Wood shavings	1.00 (0.74; 1.35)		0.99
	Sand (non-cemented floor)	0.87 (0.57; 1.33)		0.53
	Cemented floor	1.33 (0.91; 1.95)		0.14
	Other types (straw, etc.)	2.03 (0.63; 6.51)		0.23
Pen odour	Yes	1.00	0.72	Ref
	No	0.13 (0.87; 1.46)		0.36
Stocking density (chickens per square	12 – 14	1.00	3.59	Ref
	15 – 16	0.84 (0.55; 1.27)		0.40

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meter of available floor space)	17 – 18	0.83 (0.55; 1.23)		0.35
	19 – 20	0.68 (0.43; 1.10)		0.12
	> 20	0.64 (0.36; 1.14)		0.13
Adherence to vaccination	Yes	1.00	46.85	Ref
	No	7.43 (3.65; 15.10)		< 0.001
	Partial	4.36 (2.09; 9.10)		< 0.001
Implementation and adherence to biosecurity	Yes	1.00	20.84	Ref
	No	1.99 (1.30; 3.06)		0.002
	Partial	1.14 (0.72; 1.79)		0.58
Types of chickens in the poultry farm	Broiler	1.00	14.71	Ref
	Laying stock	1.87 (1.35; 2.59)		< 0.001
	Other species/stock	1.07 (0.54; 2.14)		0.85
	Mixed	1.30 (0.96; 1.76)		0.09

*p-values were obtained through Wald test.

Table 4. Multivariable analysis for contamination of poultry farms with non-typhoidal Salmonella (NTS) in North-Central Nigeria

Variable	**Category**	**Crude OR (95% CI)**	**Adjusted OR (95% CI)**	***p*-value***
Number of chickens in the farm	< 200	1.00	1.00	Ref
	201 - 500	1.41 (0.95; 2.10)	1.42 (0.92; 2.20)	0.11
	501 – 1000	2.82 (1.92; 4.15)	2.20 (1.44; 3.37)	**< 0.001**
	> 1000	3.32 (2.03; 5.44)	2.17 (1.28; 3.71)	**0.004**
Source of feed	Multi-sourced commercial	1.00	1.00	Ref
	Bought-in concentrate and mix	1.55 (0.92; 1.92)	1.49 (0.99; 2.25)	0.07
	Self-compounded	0.54 (0.35; 0.84)	0.70 (0.42; 1.18)	0.18
Source of water	Borehole	1.00	1.00	Ref
	Pipe-borne municipal water	1.33 (0.92; 1.92)	1.49 (0.99; 2.25)	0.06
	Dug-up well	0.43 (0.29; 0.62)	0.57 (0.37; 0. 87)	**0.01**
	Stream	2.18 (1.03; 4.60)	3.31 (1.45; 7.58)	**0.005**
Litter management	Good	1.00	1.00	Ref
	Poor	1.03 (0.65; 1.64)	1.16 (0.67; 2.01)	0.59
	Fair	0.55 (0.38; 0.80)	0.67 (0.44; 1.02)	0.06
Pen odour	No	1.00	1.00	Ref
	Yes	1.26 (0.94; 1.69)	1.56 (1.12; 2.18)	**< 0.01**
Adherence to vaccination (*Fowl typhoid and fowl cholera (pullorum)*)	Yes	1.00	1.00	Ref
	No	8.33 (3.49; 19.84)	5.18 (1.96; 13.66)	**< 0.001**
	Partial	5.09 (2.07; 12.51)	5.10 (1.85; 14.04)	**0.002**
	Yes	1.00	1.00	Ref
	No	2.08 (1.26; 3.41)	1.54 (0.87; 2.72)	0.14

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Implementation and adherence to biosecurity	Partial	1.14 (0.67; 1.94)	0.73 (0.40; 1.33)	0.31

*p-values were obtained through Wald test. Akaike Information Criterion (AIC) = 945.52; Hosmer Lemeshow Goodness of Fit = X^2 = 2.58; p-value = 0.96; Area under curve (Receiver Operation Characteristics (ROC)) = 0.72.

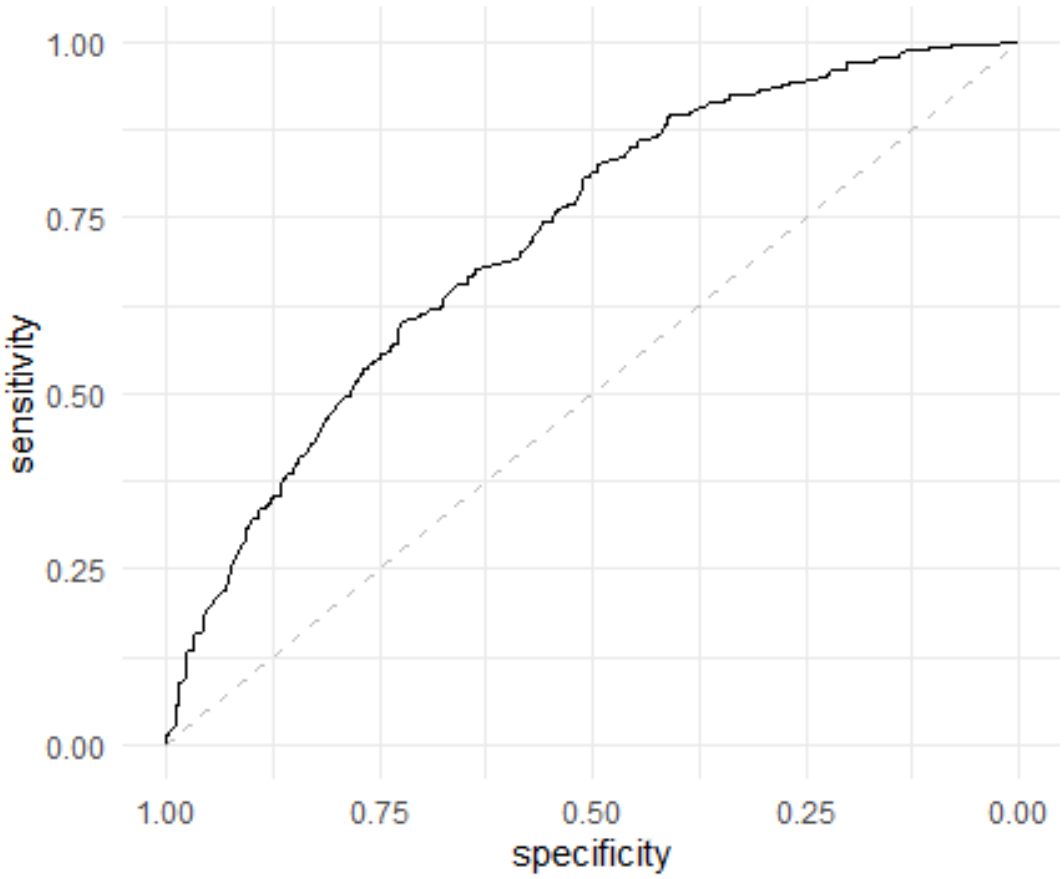

Figure 2. Receiver Operation Characteristics of risk factor model for persistence infection of non-typhoidal Salmonella in poultry farms, North-Central Nigeria

3. Discussion

The total burden of zoonotic salmonellosis in humans or poultry in Nigeria is unknown, however, as highlighted earlier, significant research efforts have been made on salmonellosis in poultry in Nigeria [3,18-25]. Such studies have been skewed and concentrated in the extreme north and the southern belt of the country. Whether these observation is related to the dispersal of Veterinary schools in Nigeria is not evaluated in this study. However, the North Central Nigeria (NCN), connects the southern belt of the country, where most of the commercial poultry activities occur with the north where most of the indigenous poultry populations predominate; and is a significant zone in the Nigerian

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poultry value chain (a hub for production, processing and marketing of poultry resources among others). The NCN serves the Federal Capital Territory and burgeoning neighborhood. In this regard, this work is timely and meet the need to prevent food-borne zoonoses and related infections in the North-Central belt of Nigeria (Figure 1; [28]).

In this study, bacteria culture and phenotypic and biochemical characterization was used as the basis for identification and confirmation, with additional test using the polymerase chain reaction (PCR) were used to for identification; it has been confirmed that these methods (culture and phenotypic and biochemical characterization) were very sensitive and specific for the identification of NTS and they compare favorably with PCR and ELISA [2,29,30]. We obtained samples from broiler, layers and mixed farms but did not consider the hatcheries and parent/grandparent farms because these latter farms need special permission to access and may have to be considered separately. In Trinidad and Tobago, 23 different Salmonellae have been found in broiler production with prevalence of between 8.9 – 20.5% [5]. Similarly, in a recent survey in Great Britain involving 23 commercial broiler hatcheries, a prevalence of between 0 – 35% were obtained with the chick handling areas, hatcher areas, macerator area, tray wash/storage areas, external areas and other waste handling areas being more contaminated in the hatchery operations [31].

In our study, 56.9% of the farmers were male, a confirmation of male-domination of the smallholder poultry practices [32,33]. The years of experience appear evenly distributed though the majority clustered into less than 6 years of practice. It may appear that the dwindling economy and prevailing influence of unemployment may have forced many individuals into poultry husbandry and this may need independent evaluation. Only 50.8% of the participants have tertiary education (Table 1). The point prevalence of NTS in the surveyed smallholder poultry farms was 6.3%. However, based on laboratory and clinic-pathological records of farms, we confirmed that 41.6% of the farms have been infected with NTS in the last 15 – 18 months. The obvious disparity may not be unconnected with the non-discriminatory use of antimicrobials in poultry farms. Previous work have confirmed that the uncontrolled implementation of antibiotic treatments of flocks may reduce the likelihood of cultivating Salmonella from seropositive birds and farms [34]. We recommend that a specialized study should be carried out for the hatcheries, parent and grandparent farms in Nigeria, to ascertain whether there are some links in the transmission and dispersal of NTS in the poultry value chain. This may assist in classifying the hatcheries based on their sanitary compliance in reducing the burden of NTS and provide guidance in intensifying tailor-made biosecurity protocols and disinfection procedures in farms. Similarly, the suppression of diagnosis in poultry farms may not be unconnected to the antimicrobial use practices [34-36]. While it has been established that vaccines for Salmonella are not capable of eradicating infection from flocks but can increase the threshold for infection, reduce the level of shedding of the organism and reduce vertical transmission in poultry that results in contamination of hatching or table eggs [2], only 27% of the farmers adhered to the protocol of culling of infected flocks. Others continue to practice non-recommended practices against NTS eradication including the administration of antibiotics (0.7%), vaccination (36.9%), combination of antibiotics and vaccination (11.5%), and sale of infected poultry to the consumers (13.2%). These practices have the likelihood of further horizontal transmission of NTS to other farms and potential for zoonosis. To control NTS in non-infected farms, vaccination protocol is an aid to other eradication and control measures such as culling, all in-all out production, biosecurity and farm hygiene. In the surveyed farms in this study, only 64.4% adhered to vaccination protocol and only 55.5% of the farmers implement and adhered to biosecurity practices (Table 1).

In our observation, the source of water and litter materials vary from farm to farm and there was wide disparity in adherence to sanitary practices (Table 1). Because extension agents play significant roles as the source of knowledge to the farmers (86%), and access to veterinary professionals and paraprofessionals not always guaranteed (33.9%), the extension agents can be used as agents of change in risk communication and community engagement in spreading awareness and targeted messaging to farmers on the risk

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of salmonellosis. For effectiveness and efficiency, the extension agents will need to be trained in relevant animal health matters, as anecdotal evidence revealed that most of the extension agents were skewed towards plant production. In addition, the awareness of Salmonella was moderately correlated with the experienced of Salmonella in the farm (Table 2), an indication that the previous or current experience of NTS in the farm is a positive predictor for awareness of Salmonella infection.

It should be noted that pathogen population increases with farm intensification and crowding of poultry per unit space [37], thus the results were not surprising that having 500 - 1000 chickens in the farm, (Adjusted Odds Ratio (AOR = 2.2, $p = 0.004$) and having more than 1000 chickens (AOR = 2.2, $p < 0.001$) were associated with higher risks of infection with NTS in poultry. Similarly the use of dug up well (AOR = 0.57, $p = 0.01$) and the use of stream water to serve the chickens (AOR = 3.3, $p = 0.05$) predicted risks of infection with NTS infection. While it is expected that ground water should increase the risk [38], the well water decreased the risk by half. We are aware that most dug-up well are regularly treated with chlorine, and this may have positively influence the reduction in the burden of risk observed in this case. Pen odour (AOR = 1.56, $p < 0.01$) increased the risk by almost two fold, and it will rather be an indication of the poor hygiene practices and poor litter management in the farm rather than itself being a risk factor. Finally, the non-adherence to pullorum and fowl typhoid vaccination (AOR = 5.2, $p < 0.001$), and partial adherence to vaccination (AOR = 5.1, $p = 0.002$) both significantly increased the risk of infection with NTS infection in poultry. As outlined above, vaccination against Salmonella infection in poultry is not capable of eradicating infection from flocks but only offer an extra layer of protection, we advocate the compliance to vaccines against pullorum and fowl typhoid in poultry in Nigeria [2]. This should be supported with other practices as emphasized in the standard protocol for control and eradication of NTS in poultry [2].

4. Materials and Methods

4.1 Selection of states and sampling sites

The North-Central Nigeria (NCN) comprises of 6 states and the Federal Capital Territory (FCT) with a total poultry population of 44,789,854 including the indigenous chickens (n = 13,556,352) and the improved exotic breeds (n = 31,233,502) [26]. The states in this geopolitical zone include: Kogi, Niger, Nasarawa, Kwara, Benue, Plateau and the FCT (Figure 1). The selection of this study site was informed by the lack of empirical data sources on non-typhoidal Salmonella (NTS) from NCN, and the need to aggregate the risk factors for persistence of non-typhoidal Salmonella in poultry farms, North-Central Nigeria.

4.2 Development of questionnaire and training of data collectors

Through literature review and probing questions to veterinarians and animal health assistants, a list of previously identified risk factors for Salmonella in poultry in Nigeria was developed ([39,40]; Table S3). A semi-structured questionnaire was prepared based on this list of identified risk factors and drivers of NTS infection in farms. Although the question was prepared in English, and approximately 90% of all respondents have at least secondary level of education, respondents were allowed to choose a convenient language of communication during the interview. All communication was in English language and local dialects where the respondents needed to effectively communicate or provide further inputs. The questionnaire targeted data on location, demographic, years of experience, type of management and chickens kept, housing and farm environment details, awareness of Salmonella, case and mortality patterns and some economic variables, as well as access to professional support.

Hired research assistants (HRA/data collectors) (n = 21) were recruited from the localities of the sampling sites in each of the states. The lead researcher (AOS) organized a training for the HRA on the objectives of the study, how to avoid bias during the field data collection and how to include internal quality control to enhance data validity. Five

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of the trained HRA/data collector conducted the role play and served as respondents. Feedbacks from the role play was used to improve the questionnaire. All questions were checked for consistencies, avoidance of ambiguity and misinterpretation. The pre-tested questions were printed in hard copies for the use of data collectors in the field.

4.3 Field sampling and laboratory analysis

A maximum number of poultry farms were targeted for sampling per each state (n = 150, except for Plateau State where 100 farms were visited). At the same period when samples are collected in sterile sample container per farm, a lead person (typically, the farm manager, farm owner or his/her designate) was interviewed using the pretested questionnaire. The farms were randomly selected and recruited once it qualifies for the definition of a poultry farm, without bias to the bird types available in the farm or the farm size. All samples were transported on ice to the laboratory, and a total of 1000 samples and 1000 questionnaire were collected. These samples included: cloacal swabs and swabs of organs and tissues [2], and were identified using the bacterial culture methods described below at the STEP-B laboratory of the Federal University of Technology Minna, Niger State and Central Research and Diagnostic Laboratory, Ilorin.

4.4 Bacteriological culture, phenotypic and biochemical characterization

Collected and transported fecal swabs were cultured for identification. About 25g of each sample was weighed (Metler Electronic scale) and added to 225 mL 0.1% peptone water, and was incubated overnight at 37°C (Gallenkemp(R)). After incubation, 0.1 mL of each sample was transferred to 10 mL Rappaport-Vassiliadis Soy Peptone (RVS) Broth (Merck, Germany) and incubated overnight at 41.5°C. Following the incubation, samples were cultured on Xylose Lysine Desoxycholate (XLD) agar (Merck, Germany) and incubated overnight at 37°C. Red colonies with a black center were subcultured in nutrient agar (NA) (Merck, Germany) to perform Gram staining and biochemical tests (Sobur et al., 2019). Gram staining identied morphological characteristics and biochemical identication included sugar fermentation, Voges Proskauer (VP) test, indole, and methyl red (MR) test [2,41].

4.5 Molecular analysis

Following bacteriological culture, selected bacterial culture positive isolates were subjected to further molecular characterization as described below.

4.5.1 DNA extraction

DNA was extracted using the protocol stated by Zhang et al. [42]. Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 °C. The grown cultures were centrifuged at 4600g for 5 min. The resulting pellets were re-suspended in 520 µl of TE buffer (10 mM Tris HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and vortexed. The suspension was incubated for 10 min at 65 °C and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer.

4.5.2 Polymerase chain reaction

PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each of 16S rRNA gene PCR forward and reverse primers: (27F, 5′-AGAGTTTGATCMTGGCTCAG-3′ and

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1525R, 5′-AAGGAGGTGATCCAGCC-3′.) and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds; and a final termination at 72°C for 10 mins. And chill at 4°C.GEL (Ahmed et al., 2009; Biase et al., 2002).

The integrity of the amplified gene fragment was checked on a 1.5% Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet transillumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

4.6 Definition of case and control farms

For the purpose of risk factor evaluation, a case farm was defined as poultry farm from which a biological sample collected from a suspect/no suspect clinical case, tested in the laboratory according to the protocol mentioned above, and was consistently positive with the test methods (culture and biochemical confirmation) in accordance with the international regulations for confirmed positive case of poultry salmonellosis (fowl typhoid and pullorum diseases) [2]. In the alternative, poultry farms, which has also experienced salmonellosis non-typhoidal Salmonella (NTS), within the period under consideration (≤ 15 - 18 months equivalent to the maximum period for current cycle of stocking of poultry chickens), and has been confirmed both clinico-pathologically and through laboratory confirmation were included as case farms. For this work, a total of 416 case farms have experienced NTS and tested for poultry salmonellosis in the last ≤ 15 months. A control farm is described as a farm where sample was collected and tested as described for the case farm above but was negative by all test protocols. Such farms must be negative through clinico-pathological as well as laboratory diagnostic tests. A total of 584 farms have not experience poultry salmonellosis in the last batch of chickens present in their farms (≤ 15 months).

4.7 Statistical analysis

Data were cleaned in Microsoft Excel 2018 and imported to Stata v 15 (Stata Corporation, College Station, 4905 Lakeway Dr., Texas, US) for analysis. Initially, we conducted descriptive statistics on all farm and field level data to determine their proportions, standard errors (SE) and 95% confidence intervals (CI95%) for each variable, using the method of Agresti and Coull, [27]. Categorical variables were also summarised as proportions. The disease prevalence was computed as the number of farms reporting to have reported or had NTS divided by total number of study farms as a percentage. We aggregate selected risk-related variables and run comparisons using pairwise correlation to determine whether there were significant correlations among the variables. Since the observations were not independent, a logistic regression model was used to investigate the association between the various potential risk factors and the outcome variable (defined as a farm having experienced NTS or not, and confirmed through clinical and laboratory diagnosis). The predictor variables used in the analysis were listed in Tables 1 - 3. The effect of each

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independent variable was first run in the univariable logistic regression model. Variables associated with the outcome (non-typhoidal salmonella (NTS) infection) at $P \leq 0.2$ were considered for inclusion in the multivariable logistic regression model. Independent variables were tested for pairwise associations, using a two-tailed chi-square test. The model was progressively simplified using the backward stepwise elimination method. Backward stepwise regression is a stepwise regression approach that begins with a full (saturated) model and at each step gradually eliminates variables from the regression model to find a reduced model that best explains the data. The stepwise approach is useful because it reduces the number of predictors, reducing the multicollinearity problem and it is one of the ways to resolve the overfitting. Variables that were found not to have strong evidence of an association, Wald test P-value (>0.05), with the outcome were excluded one at a time with the least statistically significant excluded at each step. To check that variables removed did not have a huge effect on the model, the log likelihood ratio test was calculated each time.

The Hosmer and Lemeshow test goodness of fit test was used to show well the data fits the model. Model discrimination was assessed by using area under the receiver operating characteristic curve (AUROC). AUROC is used to compare the goodness of fit of logistic regression models, where values for the measure range from 0.5 to 1.0. A value of 0.5 indicates that the model is no better than chance at making a prediction of membership in a group and a value of 1.0 indicates that the model perfectly identifies those within a group and those not. At each stage of backward stepwise elimination, the models' discrimination and overall fit was assessed. All Analyses were carried out in Stata v 15 (Stata Corporation, College Station, Texas, US). A statistical significant level was set at $P<0.05$.

5. Conclusions

Antibiotic use practice may have reduce isolation rate of NTS as observed in this study, yet NTS continues to challenge poultry farms in Nigeria. Identified risk factors and farm practices must be mitigated intentionally and biosecurity and hygiene must improve in order to reduce the burden of NTS. Finally, full compliance with vaccination protocols against pullorum and fowl typhoid in poultry combined with other control measures will assist in eradicating infection with NTS from poultry flocks in Nigeria.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1,
Figure S1. PCR Gel picture of Salmonella enterica isolated from North-Central Nigeria;
Table S1. Molecular identification of NTS salmonella spp;
Table S2: Categorization of Variables based on selected industry standards and peer reviewed literature;
Table S3: Sample Questionnaire for risk factor data collection in the field

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used Conceptualization, A.O.S., A.J. and F.O.F.; methodology, A.O.S., J.O., A.U., L.O.A. and F.O.F.; software, J.O., A.U., L.O.A. and F.O.F.; validation, J.O., A.U. and F.O.F.; formal analysis, A.O.S., J.O., A.U., L.O.A. and F.O.F.; investigation, A.O.S., J.O., A.U. and F.O.F.; resources, A.O.S. and F.O.F.; data curation, A.O.S., A.U., L.O.A. and F.O.F.; writing—original draft preparation, A.O.S., J.O. and F.O.F.; writing—review and editing, all authors; visualization, J.O., A.U. and F.O.F.; supervision, A.J. and F.O.F.; project administration, F.O.F.; funding acquisition, A.O.S. and F.O.F. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study subject to the explanation of the detailed study and receipt of formal or written consent to participate.

Data Availability Statement: This work is part of the PhD study of A.O.S. All data associated with this work and other component of the PhD study will be permanently archived with the Department of Veterinary Tropical Diseases, University of Pretoria, South Africa, and will be made available as open access, including the final thesis.

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