Risk factors for prevalence of Entero-toxigenic Escherichia coli (ETEC) in diarrhoeic and non-diarrhoeic Neonatal and Weaner Pigs, South Africa

Sub-title: Risk factors for ETEC induced diarrhoea in piglets

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) cause neonatal and post-weaning diarrhoea in pigs. To determine the risk factors, rectal/faecal swabs and visceral organs were analysed microbiologically against risk variables. Seventy-two percent of the young pigs were positive for ETEC toxin genes, and *estB* (38.9%); *estB/STAP* (25%) and *estB/LT* (13.9%) were dominant. Risk factors for ETEC-diarrhoea in pigs include: leaving sick piglet with healthy piglets (OR = 33.52; *P*<0.0001); water spillage in pen (OR = 42.87; *P*<0.0001); hypothermic piglets (OR = 7.29; *P*<0.0001); runt piglets with healthy littermates (OR = 3.65; *P*<0.0001) and prolonged use of antibiotics (OR = 3.05; *P* = 0.05).

Keywords: piglets; weaners; enterotoxigenic Escherichia coli; South Africa; risk factors; environment.

Abbreviations: EAN&PWD; ND; PWD; EAD; G-NW; ETEC, mPCR, MLR

The South African pork industry contributes 1.9% to the agricultural sector and Northern provinces account for approximately 70% of total pork production [1]. Human population rise has caused an increasing demand for pork products hence the increased intensification of pig farming and associated rise in farm management-related pig diseases [2]. Enterotoxigenic Escherichia coli (ETEC)-associated neonatal and post-weaning diarrhoea (EAN&PWD) in pigs are a source of major economic losses with high morbidity, high mortality, retarded growth and tremendous cost of treatment [2]. Neonatal diarrhoea (ND) is usually observed in 1 –4 days old piglets, while post-weaning diarrhoea (PWD) affects piglets in the first 2-3 weeks after weaning with peaks of diarrhoea sometimes occurring 6-8 weeks post-weaning, and even at 12 weeks [3].

Although ETEC associated diarrhoea (EAD) occurs in pigs in South Africa, prevalence reports from South Africa remain scanty. Information on risk factors associated with occurrence of EAD in piglets in South Africa is unknown. Therefore, the objective of this study was to determine the prevalence of ETEC, and identify the risk factors that are associated with ETEC occurrence in piggeries in Gauteng and North West (G-NW) provinces of South Africa. This will contribute to ETEC surveillance and proffer recommendations on the prevention and control of EAN&PWD in South Africa.

This study was conducted in eight piggeries (n=8) of different sizes (16-650 sow units) and productions systems: large scale commercial (> 250 sow unit), medium scale commercial (51 – 250 sow unit) and emerging small scale pig farms (< 50 sow unit) in G-NW, South Africa from August 2015 to June 2016. Twenty-two other samples (visceral organs and faeces of piglets)

which came from pig practitioners across South Africa for molecular characterization were grouped under unidentified pig farms. The total number of pigs tested for ETEC was 250 including 190 neonates and young piglets (≤ 4 weeks) and 60 weaners older than 4 weeks. This study has an ethical approval (Number: V068-15) from the University of Pretoria Ethical Approval Committee. For every farm recruited in this study, rectal swabs were collected from young piglets (1-4 weeks) and weaners (> 4 weeks) using randomized cross-sectional survey approach. Samples were only obtained from farms with EAN&PWD. A few of the sampled piglets also showed signs of edema disease. Management, personnel and animal-associated risk variables for EAN&PWD were collected using a questionnaire/checklist.

A total of two hundred and twenty-eight (228) rectal swabs (plus twenty-two faecal swabs/visceral organs) were obtained. Cotton swabs were used to collect feacal samples from the rectum of diarrhoiec and apparently healthy piglets by gentle massage. Swabs were transported to the laboratory on ice and processed immediately. The rectal swabs were cut with scissors and dropped into 10 ml of buffered peptone water (BPW) enrichment broth and incubated at 37ºC overnight in an orbital shaker. One hundred microliters (100 µl) of enrichment suspension were spread onto Tergitol-7 agar (Oxoid Ltd, England) and incubated overnight at 37ºC for 18-24 hours. Following incubation, genomic DNA was extracted from all Tergitol-7 agar plates showing growth (*E. coli* appear as yellow colonies with yellow halos) and screened for ETEC by mPCR [4]. ETEC was confirmed by culture and multiplex polymerase chain reaction (mPCR).

Thirty variables were collected simultaneously during sampling. Data was entered and filtered in Microsoft Excel® and laboratory results were matched with the variables. Filtered data was exported into Stata v9 and analysed using univariable analysis (n = 24) and six were dropped due to collinearity. Variables that were associated with diarrhoea at $P \leq 0.20$ were included in the multivariable logistic regression analysis. All excluded variables were re-tested individually in logistic regression model to determine if they were significant ($p \geq 0.05$). The Hosmer-Lemeshow test was used to determine the goodness-of-fit for the model and outputs were generated as Odd Ratios significant at $p \leq 0.05$. In addition, observational data on season, months, age and clinical predisposition for ETEC prevalence variables were analysed using the two by two tables.

ETEC from cultures and mPCR using different toxin genes confirmed that 72% (180/250) of the piglets and weaners were positive for at least one ETEC-toxin genes and medium scale farms were most affected. The distribution of toxin genes in the ETEC positive samples were as follows: *estB*, 38.9% (70/180); *eltB*, 3.3% (6/180); *estA*, 2.8% (5/180); and *Stx2e,* 5% (9/180).

There was an even distribution (50% apiece) between samples which carried ETEC positive for two or more enterotoxin-encoding genes and those which were positive for only a single toxin gene. Toxin gene combinations includes: *estA/estB,* 25%; *eltB/estB,* 13.9%; *estA/estB/eltB,* 6.1%; *estB/Stx2e,* 2.8% and *estA/estB/Stx2e*2.2% (Figure S2). *Stx2e* was significantly associated with weaners pigs 83.3% (15/18) than pigs aged <1 week up to 4 weeks 16.7% (3/18). Farm prevalence for ETEC varied according to scale of production: 78.8% (26/33) for large-scale commercial; 70% (119/170) for medium-scale commercial and 52% (13/25) for the emerging small-scale pig farms. Significant difference exists between the prevalence of ETEC on large and small-scale farms ($P< 0.05$), but no difference between the large and medium-scale farms ($P = 0.37$) and between the medium and small-scale farms ($P = 0.08$).

The prevalence of ETEC among clinically diarrhoeic pigs was 74.4% and 69.2% in non-diarrhoeic pigs. Piglets aged < 1 up to 4 weeks had 66.3% prevalence while 90.0% of weaners were ETEC positive. There was a significant difference ($P< 0.01$) between the prevalence of ETEC-associated toxin genes between both age categories with pigs **<**1 up to 4 weeks significantly less likely to carry ETEC-associated genes compared with weaner pigs (> 5 weeks) (OR = 4.55, $P<0.0005$, Table 1). ETEC appeared more prevalent in the autumn (prevalence = 78.0%, OR = 2.99, $P = 0.002$) and winter (prevalence = 71.1%, OR = 2.07, $P = 0.04$) than in spring (prevalence = 54.1%, OR = 1.00, P = NA). Similarly, the months of March and June presented with high prevalence and greater odds of ETEC isolation in pig herds (prevalence = 84.2%, OR = 8.46, $P = 0.01$) and (prevalence = 81.8%, OR = 6.88, $P = 0.03$) (Table 1).

Twenty risk factors significant at $P\leq0.20$ in the univariable analysis were included in the final multivariable logistic regression (MLR) model (Table 2). Only seven (7) variables were retained as significant ($P\leq0.05$) in the final MLR model. Five (5) increased the odds of ETEC diarrhoea in pig farms including: leaving a sick piglet in the pen or not separating sick piglets from apparently healthy ones (OR = 33.52; $CI_{95\%}$ = 6.41, 175.33; $P<0.001$); water spillage on the floor of the pen (OR = 42.87; $CI_{95\%}$ = 7.00, 262.44; $P<0.001$); the continuous use of antibiotics (OR = 3.05; $CI_{95\%}$ = 0.99, 9.42; $P = 0.05$); the lack of heated areas for the piglets (OR = 7.29; $CI_{95\%}$ = 2.39, 22.27; $P<0.001$) and leaving a runt piglet in the pen (OR = 3.65; $CI_{95\%}$ = 1.77, 7.51; $P<0.001$). Two factors including dirty piglets (OR = 0.15; $CI_{95\%}$ = 0.047, 0.50; $P=0.002$) and a pen with a dirty floor (OR = 0.03; $CI_{95\%}$ = 0.01, 0.22; $P<0.001$) marginally reduced (protective) the odds of ETEC diarrhoea (Table 3).

Table 1. Seasonal, monthly, age and clinical predisposition for ETEC prevalence

	Total Samples	Total Positives per farm	Percentage positive per category	RR	OR	95% CI	*P*-value
Diarrhoea/ no diarrhoea							
Diarrhoeic	133	99	74.4	1.08	1.29	0.74, 2.26	0.37
Non-Diarrhoeic	117	81	69.2	1.00	1.00	-	NA
Total (n)	250	180	72.0				
Age							
1day – 4 weeks	190	126	66.3	1.00	1.00	-	NA
Above 4 weeks	60	54	90.0	1.36	4.55	1.94, 12.22	<0.0005
Total	250	180	72.0				
Sampling Season							
Winter	76	54	71.1	1.31	2.07	1.02, 4.25	0.04
Spring	61	33	54.1	1.00	1.00	-	NA
Autumn	91	71	78.0	1.44	2.99	1.48, 6.15	0.002
Total	228	158	69.3				
Sampling Months							
August	54	36	66.7	1.78	3.26	0.68, 18.24	0.14
September	8	3	37.5	1.00	1.00	-	NA
October	53	30	56.6	1.51	2.15	0.45, 11.91	0.35
March	57	48	84.2	2.25	8.46	1.67, 49.93	0.01
April	34	23	67.7	1.80	3.37	0.66, 19.96	0.15
June	22	18	81.8	2.18	6.88	1.14, 49.44	0.03
Total	228	158	69.3				

RR-Relative risk; OR-Odds ratio; 95% CI-95% confidence interval

Table 2. Quantitative variables tested for association with ETEC-related diarrhoea in pig farms using univariable analysis

Tested Variables for risk of ETEC in pigs	Odds ratio	95% Confidence interval	*P*-value
Animal factors (n = 228)			
Antibiotic used in farms routinely	2.20	1.12, 4.30	0.02
Dirty piglets observed in the pen	2.01	1.13,3.57	0.02
Dirty sow observed in the pen	1.27	0.71,2.29	0.42
Dirty sow nipples	0.96	0.56,1.67	0.90
Sick piglet in pen	7.79	3.55,17.10	0.00
Cold piglet	2.92	1.67,5.08	0.00
Runt or no runt in pen	3.58	2.02,6.37	0.00
Management/attendant factors (n = 228)			
Attendant stay in one house	1.83	0.97,3.46	0.06
Shoe/boot dirty	3.41	1.92,6.08	0.00
Clothes/overall dirty	8.14	4.03,16.43	0.00
Attendant have boot	0.41	0.19,0.85	0.02
Attendant have overall	0.42	0.24,0.75	0.00
Adequate creep area	0.73	0.28,1.89	0.52
Dirty feed trough	7.03	3.65,13.55	0.00
Availability of creep feed	0.24	0.12,0.49	0.00
Floor feeding	8.14	4.03,16.43	0.00
A feed as only feed supply in pen	2.68	1.21,5.94	0.02
Environmental factors (n = 228)			
Perforated flooring	0.38	0.22,0.64	0.00
Dirty pen wall	3.28	1.87,5.76	0.00
Dirty pen floor	1.95	1.07,3.56	0.03
Water spill in pen	5.47	2.70,11.10	0.00
Temperature control	0.39	0.23,0.67	0.00
Heated laying place for piglets	1.13	0.46,2.78	0.79
Wet cleaning of pen	3.46	1.79,6.68	0.00

Six variables were dropped due to collinearity leaving a total of 24 variables for analysis.

Table 3: Multivariate analysis of risk factors associated with 5diarrhoea in piglets and weaners

Tested Variables for risk of ETEC in pigs	Odds ratio	95% Confidence interval	*P*-value
Antibiotics used	3.05	0.99,9.42	0.05
Dirty piglets	0.15	0.05,0.50	0.002
Sick piglet in pen	33.52	6.41,175.33	<0.001
Cold piglet	7.29	2.39,22.27	<0.001
Runt of no runt in pen	3.65	1.77,7.51	<0.001
Dirty pen floor	0.31	0.01,0.22	<0.001
Water spill in pen	42.87	7.00,262.44	<0.001

Although, few studies have reported on the occurrence of ETEC in piglets in South Africa [5], data on risk factors associated with EAN&PWD in South African pig farms remains scanty. The findings of this study are consistent with previous report which showed that ETEC occurs in diarrhoeic and apparently healthy pigs in South Africa [5]. The overall prevalence of 72% was higher than previously reported values (40.5 – 67 %) [5]. This high ETEC prevalence may be ascribed to targeted sampling used in this work.

Younger piglets (< 4 weeks) are significantly less likely to have ETEC compared with older weaners (> 4 weeks) perhaps due to stress associated with weaning-relocation, mixing with other piglets from other sows and diet change. While the work did not specifically target seasons, prevalence pattern was evidently aligned with the seasons covered (Table 1). Other reports which have confirmed that EAN&PWD outbreaks peaked in cold months including autumn and winter [6]. The colder months expose the piglets and newly weaned pigs to immense stress and challenge their immune system through impairment of colostral immunoglobulins acquisition [6]. A retrospective study in Canada on pig diarrhoea reported a higher probability of diagnosing ETEC and other diarrhoeagenic pathogens in winter [6]. Furthermore, seasonal and diurnal effects and temperature variations may have effect on EAN&PWD.

Hypothermic piglets were more predisposed to risk of EAN&PWD (OR = 7.29; P<0.0001, Table 3). Cold, particularly when associated with extreme weather condition and wet floor significantly lead to aggravated stress in young pigs and have increasing risk of high susceptibility to diarrhoeagenic pathogens such as ETEC, *C. perfringens* type A, rotavirus, and *Cystoisospora suis* (formerly *Isospora suis*) [6]. Because piglets huddle more closely in order to warm up and generate body heat, ETEC pathogens are transmitted among litter mates and spread easily especially between sick and healthy piglets. Although the mean ambient temperature was not measured, a recent study had shown that 92% of small-scale and medium-scale farmers in South Africa do not provide extra heat source for their piglets during the colder months [1] whereas piglets need an ambient temperature of approximately 32°C which should be reduced step-wisely until it reaches 27°C at approximately 4-5 weeks.

In this study, *estB* toxin gene was the most prevalent gene in EAN&PWD. Other workers have confirmed the predominance of *estB* virulence gene in pig ETEC and suggested its implication in the pathogenesis of the ETEC diarrhoea [7]. STb is known to cause more severe diarrhoea in older pigs and weaners, we detected a higher incidence in piglets younger than 4 weeks in our study. While pathogenic potential of E. coli can be inferred based on virulence genes [8], whether combinations of virulence genes aggravate or reduce the pathogenic potentials of the isolates from this study was not investigated (Table S1). However, these genes were present in both the

diarrhoeic and non-diarrheic piglets and weaners in various proportions (Table S1). Although previous studies have associated the Stx2e toxin primarily with oedema disease in pigs, it was found either as a single variant or in combination with STb and STb/STa pathotypes in piglets and weaners (Figure S2). Other workers have indicated that some ETEC strains may carry the Stx2e variants responsible for shiga-toxin in addition to diarrhoeagenic associated toxin genes [9] with piglets developing both oedema disease and diarrhoea.

This work has confirmed that five (5) and two (2) factors served as risk or mitigating/protective factors respectively (Table 3). Water spillage in farrowing pens was the most significant factor associated with increased risk of EAN&PWD (OR = 42.87; $P < 0.0001$). Because heat is generated in the creep areas for regular provision of warmth (27 – 32°C), spillage of water/wet floors provides an opportunity for high humidity and favourable environment suitable for ETEC multiplication. The primary role of cleaning is to reduce the microbial load and pathogen transmission in the pen. However constant wet cleaning and water spillage is counter-productive in the farrowing unit. Furthermore, the presence of a sick (diarrhoeic) piglet in a farrowing pen with other litter mates significantly increased the plausibility of recording cases of EAN&PWD by up to 34 times ($P < 0.0001$).

Pens with hypothermic (cold) piglets are at higher risk of more cases of EAN&PWD (OR = 7.29; $P < 0.0001$). A previous study showed that 92% of farmers in South Africa do not provide adequate heat source for their piglets particularly during the cold winter months which result in increased mortalities [1]. This observation might be linked with high prevalence of ETEC diarrhoea in piglets during the colder months. Similarly, farrowing pens with runts have association with ETEC diarrhoea (OR ≈ 3.7; $P<0.001$). Runts are weaker piglets among litter mates which are constantly bullied when sucking and may sometimes be deprived of colostrum at birth with consequent poorer immunity against diseases and infections. Runts may sometimes die from diarrhoea and starvation, and may be a source of infection for healthier piglets. As such, presence of runts may be an indication of ETEC in the pen as well as a predisposition for more cases of ETEC diarrhoea in piglets. Finally, this work has revealed that an indiscriminate use of antibiotics (misuse, abuse and overuse, oftentimes as prophylaxis) was associated with risk of ETEC diarrhoea in piglets (OR ≈ 3.1; $P =0.05$). This observation has been reported as a major reason for occurrence of antimicrobial resistance on pig farms [7]. Whereas these antimicrobials may have been effective against the *E. coli* strains, previous overuse and abuse may lead to resistance with implications for prolonged cases of non-resolving diarrhoea and other infections [3,7].

Two factors, dirty piglets (OR = 0.2, *P* =0.002) and dirty pen floors (OR = 0.3, *P*<0.001) served as protective factor against ETEC-associated diarrhoea. Oftentimes, dry cleaning of farrowing pens results in dry to dusty environment where bacterial growth are not encouraged but with predisposition to helminth infections and mange mite infestations [10]. In addition, within the smallholder farming system, poorly designed pen floors are uneasy to clean and piglets often appear dustier. It's advisable to pay attention to floor design while mitigating against enteric pathogens of piglets and weaners.

Certain conclusions can be drawn from this work: (a) STb alone and in combination with other toxins has been found in high frequency in South African piglets and weaners and future E. coli vaccine production and management procedure must take this into consideration; (b) Pig farmers in South Africa, and especially where the study was conducted should adopt good farm practice that eliminate the promote the earlier mentioned risk factors and may utilize probiotics. Finally, piglets are sensitive to cold-stress and so environmental temperature should be clement enough to prevent hypothermia and suitable enough to enable all physiological processes in the piglets.

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Conflicts of interest

None declared

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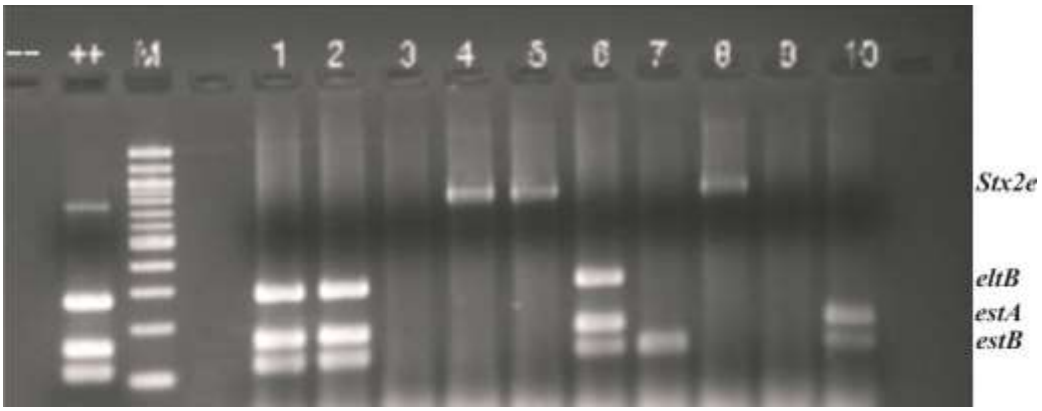
Supplementary materials

Tables S1. Distribution of virulence genes in sample variables used for statistical analysis.

Age group	Total Samples	Total Positives	**STB**	**STAP**	**LT**	**STx2E**	**STB/STAP**	**STB/LT**	**STB/STX2E**	**STB/STAP/LT**	**STB/STAP/STX2E**	**% Postive**
1-4 Weeks	190	126	**63**	**0**	**5**	**0**	**31**	**22**	**3**	**2**	**0**	**66.32**
Above 4 Weeks	60	54	**7**	**5**	**1**	**9**	**14**	**3**	**2**	**9**	**4**	**90.0**
Total	**250**	**180**	**70**	**5**	**6**	**9**	**45**	**25**	**5**	**11**	**4**	
Diarrhoiec and Non-Diarrhoiec	Total Piglets	Total Positive	**STB**	**STAP**	**LT**	**STx2E**	**STB/STAP**	**STB/LT**	**STB/STX2E**	**STB/STAP/LT**	**STB/STAP/STX2E**	**% Postive**
Diarrhoeic	133	99	**44**	**2**	**4**	**5**	**23**	**15**	**2**	**3**	**1**	**70.43**
Non-Diarrhoiec	117	81	**26**	**3**	**2**	**4**	**22**	**10**	**3**	**8**	**3**	**68.14**
Total	**250**	**180**	**70**	**5**	**6**	**9**	**45**	**25**	**5**	**11**	**4**	
Sampling Months	Total Piglets	Total Positive	**STB**	**STAP**	**LT**	**STx2E**	**STB/STAP**	**STB/LT**	**STB/STX2E**	**STB/STAP/LT**	**STB/STAP/STX2E**	**% Postive**
August	54	36	**19**	**0**	**5**	**0**	**0**	**11**	**0**	**1**	**0**	**66.67**
September	8	3	**1**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**37.5**
October	53	30	**10**	**0**	**0**	**0**	**12**	**7**	**1**	**0**	**0**	**56.6**
March	57	48	**20**	**0**	**0**	**0**	**14**	**5**	**3**	**6**	**0**	**84.21**
April	34	23	**9**	**2**	**0**	**0**	**9**	**0**	**1**	**0**	**2**	**67.65**
June	22	18	**8**	**2**	**0**	**0**	**6**	**0**	**0**	**1**	**1**	**81.82**
Total	**228**	**158**	**67**	**4**	**6**	**0**	**41**	**24**	**5**	**8**	**3**	
Sampling Season	Total Piglets	Total Positive	**STB**	**STAP**	**LT**	**STx2E**	**STB/STAP**	**STB/LT**	**STB/STX2E**	**STB/STAP/LT**	**STB/STAP/STX2E**	**% Postive**
Winter	76	54	27	2	5	0	6	11	0	2	1	70
Spring	61	33	11	0	1	0	12	8	1	0	0	54
Autumn	91	71	29	2	0	0	23	5	4	6	2	78
Total	**228**	**158**	**67**	**4**	**6**	**0**	**41**	**24**	**5**	**8**	**3**	

Figure S2. Results showing mPCR amplicons of different band sizes on agarose gel electrophoresis



- - : negative control; ++: positive control; M: 100bp DNA ladder, Lane 1-10 represent the isolates tested using multiplex PCR: *Stx2e* (bp 733); *eltb* (bp 272); *estA* (bp 158) and *estB* (bp 113) are revealed as bounds along the gel.