

## Prevalence of enterotoxigenic *Escherichia coli* virulence genes from scouring piglets in Zimbabwe

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**Abstract** World-wide, enterotoxigenic *Escherichia coli* (ETEC) and verotoxigenic *E. coli* (VTEC)-induced diarrhea are economically important for porcine producers. Our aim was to investigate the prevalence of toxin and fimbrial genes among *E. coli* isolated from diarrheic piglets from randomly selected piggeries in Zimbabwe. We used multiplex PCR for screening STa, STb, LT, and Stx-2e toxins. Subsequently F4, F5, F6, F18 and F41 fimbriae genes were screened in toxin positive isolates. Toxin positive strains lacking tested fimbriae genes were characterized using transmission electron microscopy, agglutination and agglutination inhibition tests. Approximately 32% of the 1,984 isolates tested positive for STa, STb, LT or Stx-2e genes. Of these, approximately 81% had F4, F5, F6, F18 or F41 fimbriae genes. The remaining toxin

positive strains lacked tested fimbriae genes and appeared to either express F1-like fimbriae, or lacked fimbriae. The data constitute an important framework for implementation of prevention measures, such as using relevant fimbriae-based vaccines against ETEC induced diarrhea or VTEC-induced edema.

**Keywords** Enterotoxigenic *E. coli* · Neonatal scours · Toxin genes · Fimbriae genes

### Introduction

Enterotoxigenic *E. coli* (ETEC)-induced diarrhea poses negative economic implications in the pig industry due to high mortality and reduced growth

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rate (Zhang et al. 2007). Such type of diarrhea is mostly caused by ETEC strains, which account for most gastrointestinal disorders in these animals (Zhang et al. 2007) as a result of heat stable (ST) and heat labile (LT) enterotoxins (Dubreuil 2008; Lothigijs et al. 2008). In addition, ETEC express one or several lectins that can be either fimbrial or non-fimbrial in nature. These adhesins mediate colonization of mucosal epithelium and enable bacteria to proliferate and resist flushing action of gut peristalsis (Dubreuil 2008). The strains of ETEC associated with intestinal colonization in piglets express F4, F5, F6, F17, F18 and F41 fimbriae (Dubreuil 2008). Adherence to mucosal surfaces by adhesins is an initial but essential step in pathogenesis of diarrhea (Li et al. 2007). Thereafter, toxins are responsible for fluid secretion into the gut (Turner et al. 2006). Therefore, prevention of diarrhea is highly possible using fimbrial inhibitors.

In addition to ETEC, there are some strains of verotoxigenic *E. coli* (VTEC) associated with young porcine, which produce enterotoxins and may induce diarrhea and/or edemic disease in pigs (Botteldoorn et al. 2003). Edema disease (ED) frequently causes death in pigs shortly after weaning, due to a plethora of nutritional and environmental factors (Da Silva et al. 2001).

Little is known about fimbriae associated with piglet ETEC-induced scours in Zimbabwe, despite its economic threat to both small scale and commercial pig producers (unpublished information, Central Veterinary Laboratory, Harare, Zimbabwe). Therefore, our study aimed to investigate the prevalence of toxin and fimbrial genes among *E. coli* isolated from piglets with neonatal diarrhea from randomly selected piggeries in Zimbabwe. The information generated is important not only for diagnostic and epidemiological purposes, but for designing effective inhibitors that may prevent neonatal scours among pigs.

## Materials and methods

### Bacterial isolates

A cross-sectional study involving 14 randomly selected pig farms in Mashonaland west and Mashonaland central provinces of Zimbabwe was carried out (Fig. 1). A total of 1,984 *E. coli* strains were isolated from fecal swabs of Large White and Landrace piglets

aged between 4 and 21 days showing clinical signs of scours. The fecal swabs were immediately transported in Amies transport media (Oxoid, Hampshire, England) to Central Veterinary Laboratory, Harare, followed by inoculation on 5% sheep blood agar (Oxoid) plates and incubation at 37°C aerobically overnight. All plates in this study produced almost pure cultures. All lactose-positive colonies were sub-cultured thrice on nutrient agar plates (Oxoid, Hampshire, England) prior to phenotypic characterization. The pure isolates were observed for cultural and biochemical characteristics, including colonial morphology, indole production, citrate utilization, glucose and lactose fermentation, hydrogen sulfate production, Gram stain, oxidase reaction, motility, lysine utilization, methyl-red production, urease production and  $\beta$ -hemolysis (MacFaddin 2000). All *E. coli* isolates were maintained by inoculating in sterile Nunc tubes containing nutrient agar slopes, before storing at 4°C. An inventory with 196 isolates was used in this study based on random selection obtained by choosing isolates from one piglet per sow.

### Genotyping of *E. coli* isolates

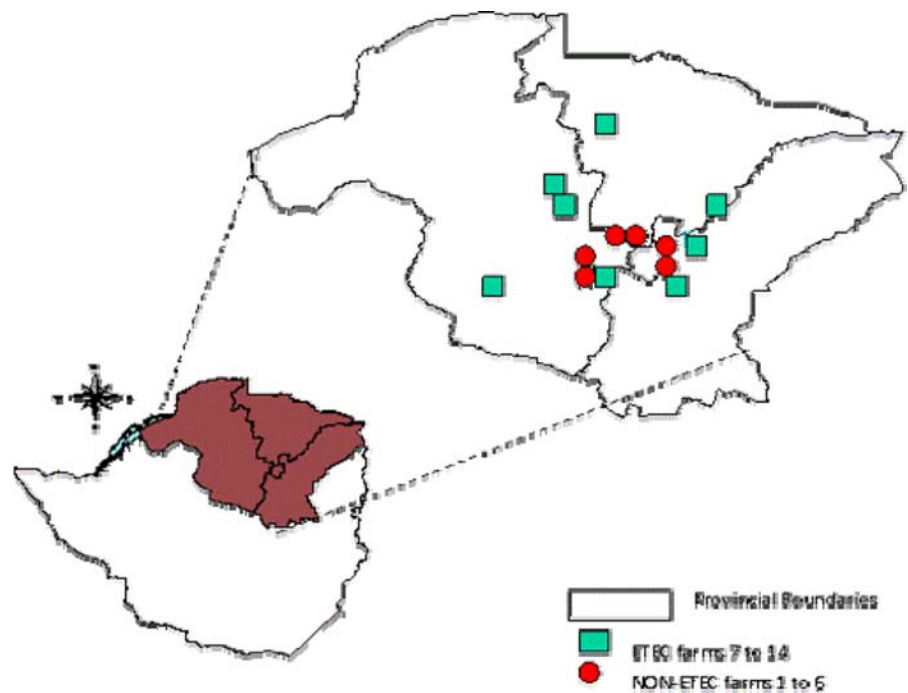
#### DNA extraction

*E. coli* preserved on nutrient agar slopes were sub-cultured on nutrient agar plates and incubated aerobically at 37°C for 24 h. A colony from nutrient agar was inoculated into 1 ml LB (Luria-Bertani, i.e. 1% w/v bactotryptone, 0.5% w/v yeast extract and 1% w/v NaCl, pH 7.3) medium. The medium was incubated overnight aerobically at 37°C while shaking in a Gyrotory<sup>R</sup> water bath shaker (Model G76, New Brunswick Scientific Co., Inc. Edison, N.J. U.S.A) at speed level 4. Total genomic DNA was extracted from overnight cultures using Wizard<sup>R</sup> Genomic DNA extraction kit according to the manufacturer's instructions (Promega, Madison, USA).

#### PCR amplification of toxin and fimbriae genes

Multiplex PCR reactions for toxin genes (STb, STa, Stx-2e, LT) were carried out using *Ex Taq TaKaRa* DNA polymerase (Takara Shuzo, Japan) and primers shown in Table 1. The 50  $\mu$ l reaction mixtures contained the following: 1x PCR buffer (10 mM Tris

**Fig. 1** Map showing randomly selected Zimbabwean farms used in this study and distribution of ETEC among neonatal piglets with scours (1 cm represents 154.91 km). The detached part highlights ETEC and non-EETEC farms and is not drawn to scale



pH 8.3, 50 mM KCl), 0.25 mM of each dNTP, 0.4  $\mu$ M of each primer, 2.5 U *Ex Taq TaKaRa* DNA polymerase, 10  $\mu$ l template DNA (diluted 1:10 v/v) and 28.8  $\mu$ l of Milli-Q water. The PCR mixtures were subjected to the following programme on a thermocycler (Perkin Elmer, Gene Amp PCR System 9600):

pre-denaturation for 3 min at 94°C; 30 cycles of amplification using the following conditions: denaturation for 30 s at 90°C, annealing for 30 s at 55°C, primer extension for 1 min at 72°C; and final extension for 10 min at 72°C. The following *E. coli* strains were used as references for detecting STb,

**Table 1** Primers used in multiplex PCR reactions for toxin and fimbrial genes of *E. coli* isolates in this study

Primer target gene	Forward and reverse primer codes	Nucleotide sequence (5' to 3')	Amplicon size
STb	STb-1	TGCCTATGCATCTACACAAT	113
	STb-2	CTCCAGCAGTACCATCTCTA	
StaP	STa-1	CAACTGAATCACTTGACTCTT	158
	STa-2	TTAATAACATCCAGCACAGG	
F5	K99-1	AATACTTGTTCCAGGGAGAAA	230
	K99-2	AACTTTGTGGTTAACTTCCT	
LT	LT-1	GGCGTTACTATCCTCTCTAT	272
	LT-2	TGGTCTCGGTCAGATATGT	
F18	F18-1	TGGTAACGTATCAGCAACTA	313
	F18-2	ACTTACAGTGCTATTCGACG	
F6	987P-1	GTAACCCACCGTTTGTATC	409
	987P-2	AAGTTACTGCCAGTCTATGC	
F4	K88-1	GAATCTGTCCGAGAATATCA	499
	K88-2	GTTGGTACAGGTCTTAATGG	
F41	F41-1	AGTATCTGGTTCAGTGATGG	612
	F41-2	CCACTATAAGAGGTTGAAGC	
Stx-2e	STx2e-1	AATAGTATACGGACAGCGAT	733
	STx2e-2	TCTGACATTCTGGTTGACTC	

STa, LT and Stx-2e toxin genes: *E. coli* 107/86 (Stx-2e), *E. coli* 91KP01 (LT, STb, STa), *E. coli* B41 (STa) and *E. coli* E68 (LT, STb). The multiplex PCR amplicons were separated in 1.5% agarose gels (San Vertech, Nusieve 3.1 agarose). Lambda ( $\lambda$ ) DNA digested with PstI was used as molecular weight marker.

Strains that showed expected PCR products for tested toxin genes were considered to be ETEC or VTEC, hence they were subjected to multiplex PCR for fimbriae genes (F4, F5, F6, F18, F41). The reaction conditions were similar to multiplex PCR for toxin genes, with the exception of primers (Table 1) and volume of Milli-Q water. The following reference strains were included in every PCR run: *E. coli* B41 (F5, F41), *E. coli* 107/86 (F18), *E. coli* 91KP01 (F4) and *E. coli* 987P (F6). *E. coli* HB101 was used as negative control.

### Serotyping of fimbrial antigens

PCR amplicons that were positive for tested fimbriae were subjected to serotyping to determine gene expression. For this purpose, anti-F4, anti-F5, anti-F6, anti-F18 and anti-F41 antisera from VETWEY FIMBEX kits (Central Veterinary Laboratory, Weybridge, UK) were used. The *E. coli* cells were grown in LB and Minca (0.45% w/v  $\text{KH}_2\text{PO}_4$ , 0.57% w/v  $\text{K}_2\text{HPO}_4$ , 0.2% w/v  $\text{NH}_4\text{Cl}$ , 0.02% w/v  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 0.0005% w/v  $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ , 0.0005% w/v citric acid, 0.0011% w/v  $\text{CaCl}_2$  and 2.5 ml/liter 1.0 M glucose, pH 7.3) broths, incubated overnight at 37°C, washed 3 times in phosphate buffered saline (10 mM potassium phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4), followed

by centrifugation at 1,700 g in a refrigerated centrifuge. The optical density of ETEC cells was measured in a spectrophotometer (Uvikon 933, Bausch and Lomb, double beam UV/Vis) and cell density was adjusted to  $1 \times 10^8$  cfu/ml. Equal volumes of bacterial cell suspension and undiluted specific antisera were mixed on clean glass slides, followed by rocking for 3 min. Bacterial cells were also treated without antisera in order to rule out autoagglutination. Bacterial aggregation indicated a positive reaction.

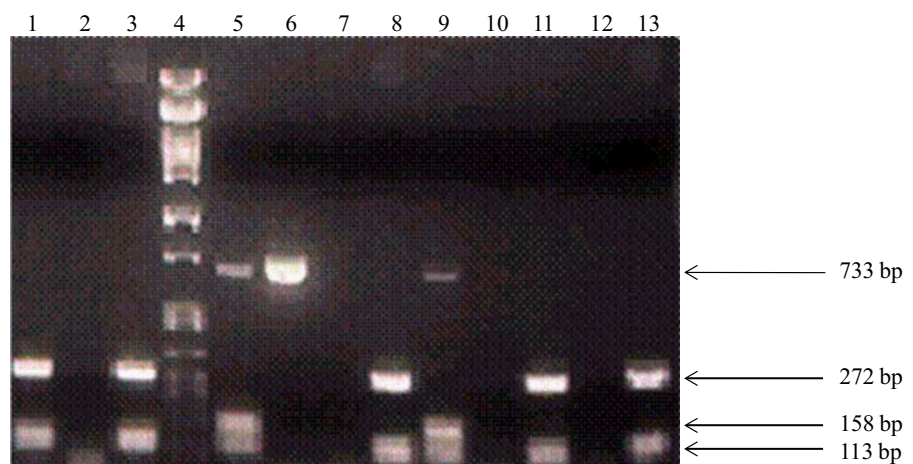
### Agglutination tests

ETEC or VTEC strains that gave negative results for fimbriae genes were considered to have 'unknown' fimbriae, hence they were characterized using rabbit, human (A, B, AB and O), goose and guinea pig erythrocytes. Briefly, whole blood from various animal species was centrifuged at 4°C for 10 min at 400 g. The erythrocytes were washed three times with phosphate buffered saline and adjusted to 4% (v/v). The bacterial cells were prepared as described above. Equal volumes of erythrocytes and bacterial cells were mixed and rocked on glass slides for 3 min, followed by observation for agglutination. Bacterial cell suspensions were similarly tested for agglutination using *Saccharomyces cerevisiae* (1% in PBS).

### Negative staining for transmission electron microscopy

*E. coli* strains that were negative for tested fimbriae genes were characterized by negative staining and

**Fig. 2** Typical result from multiplex PCR for toxin genes. Lanes: 1, 3, 8, 11, 13: field isolates positive for LT and STb genes; 4:  $\lambda$  PSTI molecular weight marker; 5, 9: field strains positive for STa, STb and Stx-2e genes; 6: reference strain 107/86 with Stx-2e gene; 2, 7, 10, 12: field strains that were negative for LT, Stx-2e, STa and STb genes



**Table 2** Prevalence of individual toxin genes from *E. coli* strains isolated from neonatal piglets with scours

Farm identity <sup>1</sup>	STa	STb	LT	Stx-2e
7	3	8	5	4
8	13	0	0	0
9	2	0	0	1
10	0	7	8	0
11	0	0	1	1
12	0	0	0	9
13	0	13	13	0
14	0	3	5	2
Total	18 (18.4%)	31 (31.6%)	32 (32.7%)	17 (17.3%)

<sup>1</sup>: The farm identity is arbitrary for confidential purposes. See Fig. 1 for the locations of different farms. Percentages for individual toxin genes are based on n=98, whereby n represents the total number of toxin genes (17+32+31+18).

transmission electron microscopy (TEM) as described by Houf et al. (2005). Briefly, *E. coli* cell suspensions (50 µl) were placed on pieces of Parafilm. Pioloform- and carbon-coated copper grids (400 mesh) were floated on the droplets for 10 min with coated surfaces facing down, followed by rinsing of grids with physiological saline for 10 sec and staining with 2% uranyl acetate. The stained grids were observed using TEM (Phillips EM208S).

## Results

### Initial characterization of isolates

First, visual characterization for cultural and biochemical properties of purified isolates was performed. β-Hemolysis positive isolates showed a zone of complete erythrocyte lysis around bacterial colonies after overnight incubation at 37°C on 5% sheep blood agar plates. *E. coli* isolates were positive

for indole, Methyl-red, lysine utilization and motility tests, whilst citrate utilization, urease and hydrogen sulphide production tests were negative.

## Genotyping

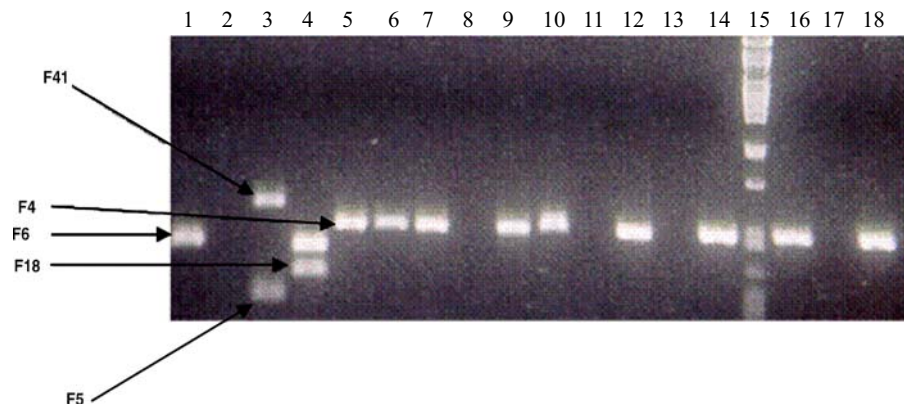
### Toxin genes

An example of results from multiplex PCR for toxin genes is shown in Fig. 2. Of the 196 *E. coli* isolates examined by multiplex PCR, 63 (32%) tested positive for at least one of the tested toxin genes, hence they were classified as ETEC or VTEC. STb and LT genes constituted the highest number of toxin genes (32% and 33% respectively; Table 2).

Only 8 of the 14 farms possessed ETEC toxin genes (Fig. 1). These farms were relatively further away from Harare. Farm 13 had the highest number of toxin genes. Both ETEC (STa, STb, LT) and VTEC (Stx-2e) isolates were recovered from farm 7 (Table 2).

**Fig. 3** Typical multiplex PCR for fimbriae genes.

Lanes 1: F6 positive reference strain; 2: negative control isolate; 3: F5 and F41 positive reference strain; 4: F6 and F18 positive field isolate; lanes 5–7, 9–10, 12, 14, 16, 18: F4 positive field isolates; lanes 8, 11, 13, 17: fimbriae negative field isolate; lane 15: λ PstI molecular weight marker



**Table 3** Prevalence of individual fimbriae genes in *E. coli* strains isolated from neonatal piglets with scours

Farm identity <sup>1</sup>	F5	F4	F41	F18	F6
7	13	0	13	0	0
9	2	0	2	0	0
10	0	0	0	0	0
11	0	0	0	0	0
12	0	0	0	11	0
13	0	12	0	0	0
14	0	4	0	2	0
Total	15 (22.3%)	19 (28.4%)	15 (22.3%)	17 (25.4%)	1 (1.5%)

<sup>1</sup>: cfr Table 2.

### Fimbriae genes and combination with toxin genes

An example of results from multiplex PCR for fimbriae genes is shown in Fig. 3. The prevalence of fimbriae genes for toxin positive isolates was as follows: F4 were the most important fimbriae, followed by F18, F5 and F41, and F6 (Table 3). F5 and F41 fimbriae genes were always found together (Table 3) and in conjunction with STa gene (Table 4). Likewise, F4 fimbriae genes were mostly associated with LT and STb genes (Table 4). Of the toxin positive isolates, 19% (12 out of 63) were negative for tested fimbriae genes (Table 4).

The Stx-2e toxin genes were always found in F18-positive strains (Table 4). Most ETEC had more than one type of enterotoxin genes. All ETEC isolates that carried STa gene also carried both genes for F5 and F41 fimbriae (Table 4).

Some farms had restricted fimbriae genes; for instance farms 13 (F4 genes), 12 (F18 fimbriae genes), and 8 and 9 (F5 and F41 genes) (Table 3). Most F18 genes (11 out of 17) were found in farm 12, followed

by farm 7 (4 out of 17) and farm 14 (2 out of 17). Most F5 and F41 genes (13 out of 15) were found in farm 8 whilst only 2 out of 15 were found in farm 9.

### Serotyping of fimbriae

The results of serotyping were similar to those of multiplex PCR for fimbriae genes, except for F18 that showed negative results with anti-F18 antisera. All 15 *E. coli* isolates that were positive for F5 and F41 genes agglutinated with anti-F5 and F41 antisera respectively. Similarly, 19 *E. coli* isolates that were positive for F4 genes agglutinated anti-F4 antisera.

### Hemolytic activity

Of the F18-positive strains, 15 produced beta-hemolysin on 5% sheep blood agar, whilst two strains were negative for beta-hemolysin (Table 5). All nineteen F4-positive strains produced  $\beta$ -hemolysin. Contrary to these results, all F5- and F41-positive strains were negative for  $\beta$ -hemolysin (Table 5).

**Table 4** Various combinations of toxin genes elaborated by ETEC isolates and their associated fimbriae genes

Toxin gene combinations	Associated fimbriae	
	Number of isolates	% of isolates
STa, STb, Stx-2e	3	5
Stx-2e	13	21
STa	15	24
LT, STb	21	33
STa, STb, LT	7	11
LT	3	5
LT, Stx-2e	1	1
Total	63	100

Nil\*: Negative for F4, F5, F6, F18 and F41

**Table 5** Association of beta-hemolysin and fimbriae genes

Fimbriae type	Isolates with fimbriae genes	$\beta$ -hemolytic isolates
F5	15	0
F4	19	19
F41	15	0
F18	17	15
F6	1	1

#### Negative staining for transmission electron microscopy

Toxin-positive *E. coli* that were negative for F4, F5, F6, F18 and F41 were characterized using TEM. Eight out of twelve (67%) of the characterized *E. coli* had rigid fimbriae of approximately 7 nm in diameter and up to 2  $\mu$ m long, whilst 4 out of 12 (33%) did not have any fimbriae (Fig. 4).

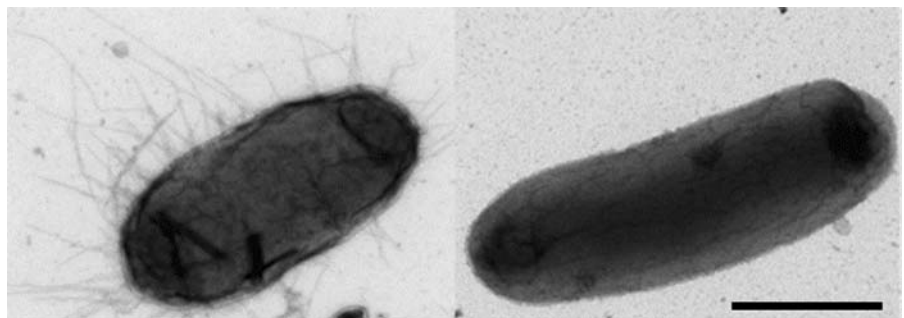
#### Agglutination tests with erythrocytes and yeast cells

Most fimbriated ETEC isolates that lacked genes for tested fimbriae, hemagglutinated rabbit, human (A, B, AB and O), goose and guinea pig erythrocytes (8 out of 12) at 4°C and 25°C. The above hemagglutination was inhibited by 0.1 M mannose and not by 0.1 M N-acetylglucosamine. The same isolates agglutinated *S. cerevisiae* and the agglutination was inhibited by 0.1 M mannose and not by 0.1 M N-acetylglucosamine. All non-fimbriated *E. coli* isolates characterized did not agglutinate yeast and tested erythrocytes.

## Discussion

The multiplex PCR used in this study for detection of fimbriae and toxin genes was valuable and sensitive

**Fig. 4** Representative electron micrographs of negatively stained *E. coli* strains expressing (left) or not expressing (right) type 1 fimbriae. Bar: 1  $\mu$ m



for identification of virulence markers of *E. coli* strains isolated from scouring piglets. This shows preliminary epidemiological information about the economic importance of *E. coli* as the main causative agent of scours among piglets in Zimbabwe. The majority of ETEC strains that cause diarrhea in piglets in Zimbabwe present similar fimbriae as those in other geographical zones (Taillon et al. 2008). This is relevant for designing vaccines against pig enteric infections for application in Zimbabwe. In general, an excellent correlation existed between results obtained by multiplex PCR and serotyping with the exception of F18 genes, which were not expressed, probably because special culture conditions were required (Wittig et al. 1994).

The *E. coli* isolates with enterotoxin genes were more prevalent compared to shiga toxin gene. This finding is consistent with the notion that ETEC are the most common *E. coli* pathotype causing neonatal diarrhea worldwide (Zhang et al. 2007). The most significant contributors to piglet ETEC diarrhea were LT and STb. The STb toxin gene has been primarily found in association with ETEC strains from scouring piglets (Erume et al. 2008) and genes for LT and STb are usually found on the same plasmid (Wasteson and Olsvik 1991). Interestingly, a few Stx-2e toxin genes were present among *E. coli* isolates from diarrheic piglets without any clinical signs of edema disease. However, since Stx-2e was associated with F18 fimbriae genes, these are variants of F18ac, which are more typical of ETEC responsible for diarrhea in piglets (Fekete et al. 2002). In addition, occurrence of Stx-2e toxin genes in conjunction with F18 fimbriae genes indicates that these pathogens persist in porcine population in Zimbabwe despite comparatively low incidence of edema disease in the country (M. Mavengwa, University of Zimbabwe, personal communication).

Interestingly, most ETEC isolates with LT genes were also positive for STb gene. This notion was

expressed by other researchers who indicated that LT and STb are expressed in severe ETEC diarrhea (Erume et al. 2008).

The outcome of multiplex PCR for fimbriae genes provides evidence for the occurrence of F4, F5, F6, F18 and F41 fimbriae genes in piggeries in Zimbabwe. These fimbriae are highly associated with ETEC diarrhea in piglets in experimental animals and in the field (Vu Khac et al. 2006). F4 genes were most prevalent, as demonstrated previously in *E. coli* diarrhea (Zhang et al. 2007). F41 was always found in association with F5 because these genes are co-expressed (Martin et al. 2002). Although genes encoding F6 expression and toxin STa are localised on a plasmid of 35–40 mega Da (Schifferli et al. 1991), in this study F6 was found in association with LT gene. Despite the presence of two isolates with F6 genes from farm 14, fimbriae-based vaccines for use in Zimbabwean piggeries should also incorporate F6, as these fimbriae may become important in future.

ETEC-induced neonatal piglet diarrhea was observed in piggeries 7 to 14, which are further away from Harare. The organization of these piggeries showed less hygienic accommodation, coupled with continuous furrowing, inadequate cleaning and poor environmental temperature control. In addition, piglets from these farms were purchased from various sources whose hygienic status was poor and diet was undefined.

Most ETEC strains with F18 genes produced beta-hemolysin on 5% sheep blood agar because adhesion and hemolysin genes are linked to the same large (98MDA) self-transmissible plasmid (Fekete et al. 2002). However, a few strains with F18 genes were negative for beta-hemolysin, probably because there was loss of hemolytic properties under *in vitro* conditions due to co-deletion of the linkage gene (Wittig et al. 1994). All F4 positive *E. coli* produced beta-hemolysin, indicating a strong correlation between F4 fimbriae and hemolysin production. On the contrary, all F41 and F5 fimbriae positive *E. coli* were negative for beta-hemolysin.

Most fimbriated ETEC isolates that tested negative for fimbriae genes hemagglutinated rabbit, human (A, B, AB and O), goose and guinea pig erythrocytes (8 out of 12) at both 4°C and 25°C, with the strongest hemagglutination at 4°C as previously observed (Van Driessche et al. 2000). Hemagglutination indicates the presence of specific receptors on erythrocytes for fimbrial lectins expressed on *E. coli* isolates. Howev-

er, all agglutination was inhibited by 0.1 M mannose and not by 0.1 M N-acetylglucosamine, suggesting the presence of F1 (or 'common') fimbriae (Van Driessche et al. 2000). This was in agreement with TEM results that showed rigid fimbriae of approximately 7 nm diameter, implying F1 fimbriae. F1 fimbriae are often present on both commensal and enterotoxigenic strains, hence their role in pathogenesis of diarrhea is still controversial. Some ETEC that were negative for fimbriae genes did not show fimbriae under the electron microscope, probably due to unsuitable culture conditions for expression or the presence of unidentified membrane-bound adhesins.

This study was performed to determine the prevalence of virulence genes associated with neonatal scours among piglets in Zimbabwe in order: to understand the epidemiology of porcine *E. coli* strains that provoke scours, to use relevant vaccines against scours due to ETEC and to include new fimbriae in existing vaccines so as to improve their protective effect. For this purpose, multiplex PCR used in this study for detection of fimbriae and toxin genes was fast and sensitive for identification of virulent markers of *E. coli* strains from pigs. The results show preliminary epidemiological information about the presence of known virulent factors in Zimbabwean piggeries. Therefore, vaccination or vaccine development based on F4, F5, F6, F18 and F41 antigens continues to be appropriate for controlling ETEC infections. This is the first extensive study regarding porcine enterotoxigenic *E. coli* in Zimbabwe. It constitutes an important database for implementation of prevention measures. Further analysis of a larger number of strains might be important in future.

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