Phylogenetic group and virulence gene profile of bovine mastitis *Escherichia coli* isolates from North West Cameroon

Abstract

Escherichia coli, a facultative anaerobic bacterium existing in symbiosis in the gut of warm-blooded animals, has several strains some of which are pathogenic. Its virulence and pathogenicity have been associated with one of several phylogenetic groups. We examined bovine mastitis *E. coli* strains to identify phylogenetic groups and virulence genes in order to understand the public health implications of consuming unpasteurised milk. Thirty-seven *E. coli* isolates previously recovered from mastitis milk and identified by biochemical methods were confirmed by PCR and sequencing. The phylogenetic groups associated with mastitis were determined by the Clermont quadruplex PCR method. We also investigated 23 virulence genes in the isolates. All 37 isolates were confirmed to be *E. coli*. The phylogenetic groups detected were: A (37.8%), B1 (37.8%), F (8.1%), D (2.7%) and E (2.7%). We detected 11 of the 23 virulence genes investigated, and thirty-six (97.3%) isolates harboured at least one virulence gene. The genes detected were *fimH* (91.9%), *traT* (62.2%), *ehlyA* and *stx2* (37.8% each), *eaeA* (35.1%), *stx1* (29.7%), *f17* (16.7%), *kspMII* (13.5%), *iucD* (10.8%), *malX* (8.1%) and *hlyA* (8.1%). Nine virulence gene combinations were identified in phylogroups A and B. The detection of *iucD* and *malX* were significantly associated with *E. coli* that belonged to phylogenetic group F. Most *E. coli* strains in this study are related to intestinal *E. coli* pathotypes, based on the phylogroups and virulence genes suggesting a public health threat. Hence, biosafety measures are recommended in handling raw milk, while consuming unpasteurized milk should be discouraged.

Keywords: *Escherichia coli*, phylogenetic group, virulence genes, bovine mastitis, Cameroon

Introduction

Escherichia coli (*E. coli*) is a facultative anaerobic microorganism that can exist in a symbiotic relationship in the gastrointestinal tract of animals from where it is excreted and could contaminate the soil and water bodies. Exposure to or consumption of contaminated sources can lead to intestinal or extraintestinal diseases in humans and animals, including bovine mastitis. *E. coli* is one of the primary etiologic agents of bovine mastitis, a worldwide production disease that negatively affects the mammary glands of cows. As long as cows produce faeces, it is evident that *E. coli* mastitis will remain [1] Though infection is self-limiting, the time for recovery of the

gland may be long, during which milk composition remains affected, thus extending the economic impact of this pathogen in dairy production [2]. An infection with *E. coli* can lead to severe systemic clinical symptoms like sepsis [3] and a chronic form of mastitis which may be subclinical but elicit recurrent clinical episodes [4, 5]. Apart from the economic consequence of infection, some of its strains, such as enterohaemorrhagic *E. coli* O157:H7, have great zoonotic importance that can be transmitted in mastitic milk [6]. This *E. coli* strain is known to cause haemorrhagic colitis and haemolytic uraemic syndrome in humans [7].

Phylogenetic grouping is widely used for studying *E. coli* (or understanding) *E. coli* population genetics [8, 9]. Based on the genetic substructures, *E. coli* strains can be classified into one of eight phylogenetic groups (phylogroups): A, B1, B2, C, D, E, F [10] and *Escherichia* clade I [11]. Most commensal *E. coli* and intestinal pathogenic *E. coli* (IPEC) strains are more likely to be members of phylogroups A and B1. The majority of *E. coli* strains responsible for extra-intestinal infections belong to phylogroups B2 and, to a lesser extent, D or F [12, 13]. Strains belonging to different phylogroups occupy different ecological niches [14, 15], have different phenotypic and genotypic traits [9] and differ in their ability to cause disease. Thus, phylgrouping helps us understand *E. coli* epidemiology.

Pathogenic *E. coli* strains possess specific virulence factors characteristic of IPEC and extraintestinal pathogenic *E. coli* (ExPEC) [16, 17]. These virulence factors increase the ability of *E. coli* strains to cause distinct diseases and allow their classification into pathotypes [18]. However, mastitis *E. coli* strains are not associated with specific virulence factors [19, 20, 21].

Generally, *E. coli* strains exhibit a combination of virulence factors such as adhesins, toxins, invasins, capsule production, and the ability to resist serum complement and iron uptake systems

(e.g., siderophores). These virulence factors perform different functions to facilitate colonization and host invasion, avoidance or disruption of host defence mechanisms, injury to host tissues, and stimulation of a harmful host inflammatory response [16]. Knowledge of the virulence-associated gene combinations in *E. coli* isolates from mastitis cases may allow an estimation of the risk of disease severity [6, 22] and the most prevalent virulence factors may be targeted for prevention of *E. coli* bovine mastitis [23].

The involvement of *E. coli* in bovine mastitis has been reported in several studies from different areas including Cameroon. However, information is scarce on the phylogenetic groups and virulence gene profile of *E. coli* strains associated with bovine mastitis from Cameroon, particularly in the North West region of Cameroon. It has been shown that health status and environmental and geographic conditions could influence the distribution of *E. coli* phylogroups in animals and humans [24, 25]. Bovine mastitis substantially negatively affects dairy production, and *E. coli* is a major bovine mastitis pathogen that can also be transmitted to humans in unpasteurised milk. This study aimed to identify the phylogenetic groups and virulence genes (targeting some virulence genes specific for both IPEC and ExPEC) of bovine mastitis *E. coli* strains. Epidemiological data obtained from this study could be helpful in applying appropriate preventive measures.

2.0 Materials and Methods

2.1 *E. coli* isolates

The *E. coli* isolates for this study were obtained from previous study [26]. The isolates which had been identified only by conventional microbiological and biochemical methods were preserved at -80^{o}C in 50% glycerol broth. The geographical location from which the isolates were recovered, and the period have been described previously [26]. A total of 37 *E. coli* isolates from

thirty-seven quarter milk samples (comprising 7 from clinical mastitis and 30 from subclinical mastitis) were investigated in this study.

2.2 Extraction of *E. coli* genomic DNA

Each *E. coli* isolate was revived by inoculating in 1mL nutrient broth (Liofilchem Diagnostic, Italy) and incubated at 37^{o}C for 24h. DNA was extracted from pelleted cells using QIAamp DNA minikit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The eluted DNA was held at -20^{o}C until used for polymerase chain reaction (PCR) analyses, which included confirmation of *E. coli* identity, phylogrouping and detection of virulence genes.

2.3. Molecular confirmation of *E. coli*

E. coli was confirmed in all putative *E. coli* samples by targeting regions on 23S ribosomal RNA gene specific to *E. coli* using published primers (Eco 223: ATCAACCGAGATTCCCCCAGT and Eco 455: TCACTATCGGTCAGTCAGGAG) and PCR conditions previously described [27]. For all PCR analyses, each reaction mixture was made up of 12.5µL of OneTaq Hot Start 2X Master Mix with Standard Buffer (New England Biolabs, UK), 0.5µL of 20µM of each oligonucleotide primer (synthesized by Inqaba Biotec, South Africa), 5µL DNA template and nuclease-free water (Bioconcept, Switzerland) to top the final reaction volume to 25 µL. DNA amplification was done in a GenAmp PCR system thermal cycler (Applied Biosystems, USA). Amplified PCR products were purified and sequenced by Sanger sequencing at Inqaba Biotec in South Africa to further confirm *E. coli* identification. Bioedit version 7.2.6.1 was used to edit the sequences. Sequence similarity searches were carried out against sequences deposited in the GenBank database using the BLAST search tool (http://www.ncbi.nlm.nih.gov/BLAST).

2.4 Phylogenetic group determination using PCR

DNA of each *E. coli* isolate was subjected to quadruplex PCR targeting four genes: *arpA*, *chuA*, *yjaA* and *tspE4.C2*, using primers and PCR conditions previously described [10] to classify isolates into one of the seven phylogroups A, B1, B2, C, D, E, F or five cryptic clades of *Escherichia*. Isolates that were either phylogroup A/C or D/E were distinguished using C- and E-specific primers, respectively, as described previously [10]. In E- and C-specific PCR reactions, the primers trpBA.f, and trpBA.r were added to provide an internal control [13]. The primer sets used, and the sizes of the expected PCR products are presented in Table 1.

Table 1: Genes and primers used for phylogenetic grouping

Genes	Primer name	Primer sequence (5'-3')	Amplicon (bp)	Reference
arpA	AceK.f	AAC GCT ATT CGC CAG CTT GC	400	[10]
	ArpA1.r	TCT CCC CAT ACC GTA CGC TA		[28]
chuA	chuA.1b	ATG GTA CCG GAC GAA CCA AC	288	[10]
	chuA.2b	TGC CGC CAG TAC CAA AGA CA		[8]
yjaA	yjaA.1b	CAA ACG TGA AGT GTC AGG AG	211	[10]
	yjaA.2b	AAT GCG TTC CTC AAC CTG TG		
tspE4.C2	TspE4.C2.1b	CAC TAT TCG TAA GGT CAT CC	152	[10]
	TspE4.C2.2b	AGT TTA TCG CTG CGG GTC GC		
arpA	ArpAgpE.f	GAT TCC ATC TTG TCA AAA TAT GCC	301	
	ArpAgpE.r	GAA AAG AAA AAG AAT TCC CAA GAG		[29]
trpA	trpAgpC.1	AGT TTT ATG CCC AGT GCG AG	219	
	trpAgpC.2	TCT GCG CCG GTC ACG CCC		
trpA	trpBA.f	CGGCGATAAAGACATCTTCAC	489	[30]
	trpBA.r	GCAACGCGGCCTGGCGGAAG		

2.6 Detection of genes associated with virulence by PCR

All *E. coli* isolates were screened for virulence genetic markers using conditions described previously for targeting *papEF*, *sfa*/*focDE*, *afa*, and *hlyA* (multiplex PCR) [31], *cnf* and *iucD* (duplex PCR) [31], *bfpA* [32], *f*17 [33] and *clpG* [34].

The detection of *traT* (singleplex), *kpsMII*/*papC* (duplex PCR), and *fimH*/*malX* (duplex PCR) was done using PCR conditions previously described by Johnson and Stell [35] with slight modifications. For all reactions, preheating of the mixture was done for 5min at 95^{o}C, and

extension was done for 30s (for *traT)*, 30s (for *kpsMII*/*papC*), and 1min (for *fimH*/*malX*) at 68^{o}C. Previously described conditions were used to investigate the presence of *aggR* [36], *lt*/*st* (duplex PCR) [37] and *f5*/*f41* (duplex PCR) [38]. Multiplex PCR was performed to detect *stx1*, *stx2*, and *ehlyA* genes under the following optimized conditions: 95^{o}C for 5min; 35 cycles of 94^{o} C for 1min, 52^{o}C for 1min, and 68^{o}C for 1min; and a final extension of 68^{o}C for 5min. A singleplex PCR was performed for *eaeA* gene using these optimized conditions: 95^{o}C for 5min; 35 cycles of 94^{o}C for 1min, 58^{o}C for 1min, and 72^{o}C for 30s; and a final extension of 72^{o}C for 5min. The primer sets used and the expected PCR product sizes are presented in Table 2.

PCR products mixed with DNA loading dye (New England Biolabs, UK) in the ratio 5:1 were electrophoresed in 1.5% agarose gels, then stained with ethidium bromide (Sigma-Aldrich, E-8751, Germany), destained with distilled water, and photographed by use of an ultraviolet transilluminator and digital capture system (Gel DOC^{XR} Imaging System, Bio-Rad, USA). The sizes of the amplicons were determined by comparing them with a 100-bp DNA ladder (New England Biolabs, UK).

Table 2: Genes associated with virulence and primers used for PCR amplification

	Target protein	Gene	Primer name	Primer sequence (5'-3')	Amplicon (bp)	Reference
Adhesins/ colonization factors	Type I fimbriae	*fimH*	FimH f FimH r	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508	[35]
	F5(K99) fimbriae	*f5*	F5 f F5 r	TGGGACTACCAATGCTTCTG TATCCACCATTAGACGGAGC	450	[39]
	F17 fimbriae	*f17*	F17 f F17 r	GCAGAAAATTCAATTTATCCTTGG CTGATAAGCGATGGTGTAATTAAC	537	[33]

	F41 fimbriae	*f41*	F41f F41r	GAGGGACTTTCATCTTTTAG AGTCCATTCCATTTAATGGC	431	[40]
	P fimbriae	*papEF*	PapEF f PapEF r	GCAACAGCAACGCTGGTTGCATCAT AGAGAGAGCCACTCTTATACGGACA	336	[31]
		papC	PapC f PapC r	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328	[41]
	S and FIC fimbriae Sfa/FocDE[h] region	*Sfa/focDE*	sfa f sfa r	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410	[42]
	Bundle –forming pili	*bfpA*	EP1 EP2	AAT GGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	326	[43]
	Coli-surface associated (CS31A)	*clp*G	clpG1 clpG2	GGGCGCTCTCTCCTTCAAC CGCCCTAATTGCTGGCGAC	402	[34]
	Afimbrial adhesin	*afa*	Afa f Afa r	GCTGGGCAGCAAACTATAACTCTC CATCAAGCTGTTTGTTCGTCGCCG	750	[42]
	Intimin	*eaeA*	EaeA f EaeA r	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	384	
Toxins	Shiga toxin 1	*stx1*	Stx1 f Stx1 r	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	180	[44]
	Shiga toxin 2	*stx2*	Stx2 f Stx2 r	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255	
	Cytotoxic necrotic factor 1 & 2	*cnf*	Cnf f Cnf r	CTGGACTCGAGGTGGTGG CTCCTGTCAACCACAGCC	533	[45]
	Enterohaemolysin	*ehlyA*	EhlyA f EhlyA r	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534	[44]
	α- Haemolysin	*hlyA*	HlyA f HlyA r	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177	[31]
	Haet stable toxin	*st*	St f St r	TCTGTATTGTCTTTTTCACCTTTC TTAATAGCACCCGGTACAAGC	165	
	Heat labile toxin	*lt*	Lb f Lb r	ATGAGTACTTCGATAGAGG ATGGTATTCCACCTAACGC	279	[37]
Iron uptake systems	Aerobactin	*iucD*	iucD f iucD r	TACCGGATTGTCATATGCAGACCGT AATATCTTCCTCCAGTCCGGAGAAG	602	[31]
Factors conferring serum resistance	TraT lipoprotein	*traT*	TratT f TraT r	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	290	[35]
	Group II capsule	*kpsMII*	kpsII f kpsII r	GCGCATTTGCTGATACTGTTG CATCCAGACGATAAGCATGAGCA	272	
Others	Aggregative Regulon (AggR) transcription regulator protein	*aggR*	aggR.F aggR.R	GCAATCAGATTAARCAGCGATACA CATTCTTGATTGCATAAGGATCTGG	426	[36]
	Maltose regulatory	*malX*	RPAi f RPAi r	GGACATCCTGTTACAGCGCGCA TCGCCACCAATCACAGCCGAAC	930	[35]

3.0 Results and discussion

3.1 Results

3.1.1 Molecular confirmation of *E. coli* isolates

E. coli-specific target on 23S ribosomal RNA gene was detected in all 37 isolates (including seven from clinical and 30 from subclinical mastitis) (Figure 1) to confirm *E. coli*. Thirty-one selected PCR amplified products sequenced, further confirmed the identification of *E. coli.* The sequences had homology ranging from 98.71 to 100% with Genbank sequences. All 31 sequences were deposited in the Genbank under accession numbers OR654111 to OR654141.

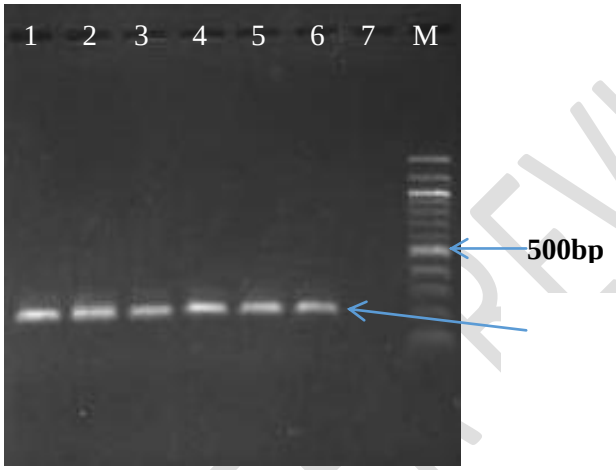

Figure 1: Electrophoretic separation of amplified PCR products of singleplex 23S ribosomal RNA PCR. 100bp DNA Ladder (lane M), positive samples (lanes 1-6), negative control (lane 7)

3.1.2 *E. coli* phylogenetic groups

The Clermont PCR method performed for the determination of *E. coli* phylogroups showed that phylogroups A (37.8%; 14/37) and B1 (37.8%; 14/37) had a high proportion, followed by group F (8.1%, 3/37). The groups with the most diminutive proportions were D (2.7%, 1/37), E (2.7%, 1/37) and *Escherichia* clade I or II (2.7%, 1/37). Some isolates (8.1%, 3/37) could not be classified under any phylogroup.

Table 3 depicts that, among the clinical isolates, the majority (71.4%) belonged to phylogroup A and the majority (46.7%) of the subclinical isolates belonged to phylogroup B1 and the differences were slightly significant (p = 0.046).

Table 3: Phylogenetic group distribution of isolates by mastitis type

Mastitis type	Number examined	Number (%) of isolates in phylogenetic group							*P*-value
		A	B1	D	E	F	Clade I or II	Unknown	
Clinical	7	5 (71.4)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	**0.046***
Subclinical	30	9 (30.0)	14 (46.7)	0 (0.0)	1 (3.3)	3 (10.0)	1 (3.3)	2 (6.7)	**0.046***

*Statistically significant variables ($P < 0.05$)

3.1.3 Prevalence and distribution of virulence genes

Analysis by PCR of the 37 *E. coli* isolates showed that 11 genes were detected out of the 23 virulence factor genes investigated. The most prevalent gene was the *fimH* gene (91.9%), followed by the *traT* gene (62.2%) and *ehlyA* and *stx2* genes (37.8% each) (Table 4).

Based on the distribution of virulence genes among the different phylogenetic groups, the prevalence of *iucD* ($p = 0.001$) and *malX* ($p = 0.000$) in phylogroup F was statistically significant (Table 4). In addition, the prevalence of *kspMII* ($p = 0.002$) was statistically significant among clinical mastitis isolates (Table 4).

Table 4: Distribution of virulence genes detected in mastitis *Escherichia coli* among the different phylogroups and type of mastitis isolate

Virulence gene	Occurrence of virulence factor genes by phylogroups (%)							Total (%) (n=37)	*P value*[1]	Type of mastitis isolate (%)		*P value*[2]
	A (n=14)	B1 (n =14)	D (n=1)	E (n=1)	F (n= 3)	*Escherichia* clade I or II (n= 1)	Unclassified isolates (n=3)			Clinical (n=7)	Subclinical (n=30)	
eaeA	7 (50.0)	3 (21.4)	1 (100.0)	-	-	1 (100.0)	1 (33.3)	13 (35.1)	0.217	4 (57.1)	9 (30.0)	0.213
ehlyA	7 (50.0)	3 (21.4)	-	1 (100.0)	1 (33.3)	-	2 (66.7)	14 (37.8)	0.398	2 (28.6)	12 (40.0)	0.687
f17	2 (14.3)	4 (28.6)	-	-	-	-	-	6 (16.2)	0.772	1 (14.3)	5 (20.0)	1.000
fimH	13 (92.9)	13 (92.9)	1 (100.0)	1 (100.0)	3 (100.0)	1 (100.0)	2 (66.7)	34 (91.9)	0.672	7 (100.0)	27 (90.0)	1.000
iucD	-	-	-	-	3 (100.0)	-	1 (33.3)	4 (10.8)	**0.001***	0 (0.0)	4 (13.3)	0.570
kspMII	3 (21.4)	1 (7.1)	1 (100.0)	-	-	-	-	5 (13.5)	0.420	4 (57.1)	1 (3.3)	**0.002***
malX	-	-	-	-	3 (100.0)	-	-	3 (8.1)	**0.000***	0 (0.0)	3 (10.0)	1.000
stx1	6 (42.9)	2 (14.3)	-	1 (100.0)	1 (33.3)	-	1 (33.3)	11 (29.7)	0.361	1 (14.3)	10 (33.3)	0.649
stx2	7 (50.0)	3 (21.4)	1 (100.0)	1 (100.0)	1 (33.3)	-	1 (33.3)	14 (37.8)	0.398	2 (28.6)	12 (40.0)	0.687
traT	8 (57.1)	9 (64.3)	1 (100.0)	1 (100.0)	3 (100.0)	-	1 (33.3)	23 (62.2)	0.561	3 (42.9)	20 (66.7)	0.390
hlyA	-	3(21.4)	-	-	-	-	-	3 (8.1)	0.521	0 (0.0)	3 (10.0)	1.000

-, not detected; [1], *p-value* of the association of each virulence gene among the different phylogenetic groups; [2], *p-value* of the association of each virulence gene with mastitis type; *, Significant variables ($P < 0.05$)

Among the 37 *E. coli* isolates, one isolate had none of the genes investigated, 36 (97.3%) harboured at least one gene, while one isolate of the 36 isolates harboured 7 of the genes detected (Figure 2). A wide virulence gene profile was determined among the *E. coli* isolates, with the most frequent gene combinations being: *fimH-hlyA-traT, ehlyA-fimH-stx1-stx2-traT* and *eaeA-ehly-fimH- stx1-stx2-traT* that were detected in three (8.1%; 3/37) isolates each (Table 5).

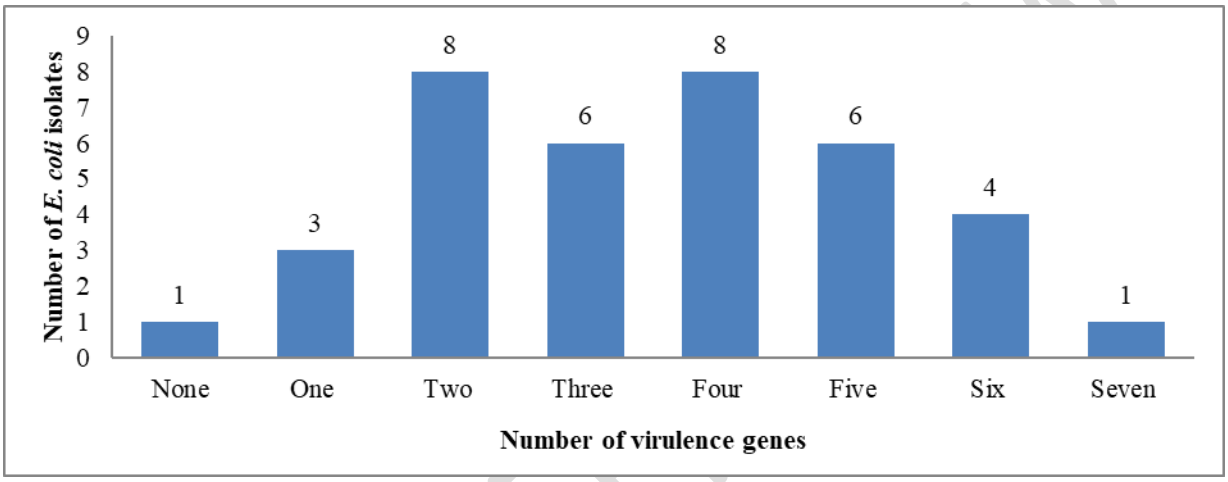

Figure 2: Number of virulence genes detected in *E. coli* strains

Table 5: Combinations of virulence genes detected in the mastitis *E. coli* isolates

Virulence profile	Gene combination	Number of isolates in phylogroup							Total number (%) of isolates	Number of isolates in the different mastitis types	
		A	B1	D	E	F	Clade I/II	Unknown		Clinical	Subclinical
I	*ehlyA*	0	0	0	0	0	0	1	1 (2.8)	0	1
II	*fimH*	1	0	0	0	0	0	0	1 (2.8)	0	1
III	*traT*	0	1	0	0	0	0	0	1 (2.8)	0	1
IV	*eaeA + fimH*	0	0	0	0	0	1	1	2 (5.4)	1	0
V	*fimH + kspMII*	2	0	0	0	0	0	0	2 (5.6)	2	0
VI	*fimH + f17*	0	2	0	0	0	0	0	2 (5.6)	0	2
VII	*fimH + traT*	0	2	0	0	0	0	0	2 (5.6)	0	2
VIII	*eaeA + f17 + fimH*	0	1	0	0	0	0	0	1 (2.8)	0	1
IX	*f17 + fimH + traT*	1	0	0	0	0	0	0	1 (2.8)	0	1
X	***fimH + -hlyA + traT***	0	3	0	0	0	0	0	**3 (8.3)**	0	3
XI	*fimH + kspMII + traT*	0	1	0	0	0	0	0	1 (2.8)	0	1
XII	*eaeA + ehly + f17 + fimH*	1	0	0	0	0	0	0	1 (2.8)	1	0
XIII	*eaeA + f17 + fimH + stx1*	0	1	0	0	0	0	0	1 (2.8)	0	1
XIV	*eaeA + fimH + kspMII + traT*	1	0	0	0	0	0	0	1 (2.8)	1	0
XV	*eaeA + fimH + stx2 + traT*	1	0	0	0	0	0	0	1 (2.8)	0	1
XVI	*ehlyA + fimH + stx2 + traT*	0	2	0	0	0	0	0	2 (5.6)	0	2
XVII	*fimH + iucD + malX + traT*	0	0	0	0	2	0	0	2 (5.6)	0	2
XVIII	*eaeA + ehly + fimH + stx1 + stx2*	1	1	0	0	0	0	0	2 (5.6)	0	2
XIX	*eaeA + fimH + kspMII + stx2 + traT*	0	0	1	0	0	0	0	1 (2.8)	1	0
XX	***ehlyA + fimH + stx1 + stx2 + traT***	2	0	0	1	0	0	0	**3 (8.3)**	1	2
XXI	***eaeA + ehly + fimH + stx1 + stx2 + traT***	3	0	0	0	0	0	0	**3 (8.3)**	0	3
XXII	*ehlyA + fimH + iucD + stx1 + stx2+ traT*	0	0	0	0	0	0	1	1 (2.8)	0	1
XXIII	*ehlyA + fimH + iucD + malX + stx1 + stx2 + traT*	0	0	0	0	1	0	0	1 (2.8)	0	1
	Total	**13**	**14**	**1**	**1**	**3**	**1**	**3**	**36 (100)**	**7**	**29**

3.2 Discussion

To our knowledge, this is the first study in Cameroon, particularly the North West region, which determines the frequency of phylogenetic groups and virulence factor genes of mastitis *E. coli*. Phylogenetic grouping is essential for epidemiologic studies. Determination of phylogroups of *E. coli* using a simple extended quadruplex PCR phylogroup assignment method proposed by Clermont *et al.* [10] has gained popularity.

According to our results, among the 37 *E. coli* isolates (comprising 7 from clinical mastitis and 30 from subclinical mastitis) majority (75.6%, 28/37) belonged to phylogroup A (37.8%, 14/37) and B1 (37.8%, 14/37). Similar findings have been reported in Iran [46] and China [47]. Of the seven clinical mastitis isolates, majority (5/7, 71.4%) belonged to phylogroup A. This corroborates reports from Finland [20] China [48], Brazil [49] and Bangladesh [50] that reported *E. coli* phylogroup A to be most prevalent among clinical mastitis. However, other studies in China [51], Switzerland [52] and Brazil [53] have reported a majority of *E. coli* isolates in clinical mastitis to belong to phylogroup B1. In Brazil, a study on clinical isolates even reported the same proportion of phylogroup A and B1 [54] and were the most common phylogroups. The difference in the proportions could be associated with differences in geographical locations. Several reports have indicated that *E. coli* of bovine origin were assigned mainly to phylogroups A and B1 [55, 56, 57], which may explain why the majority of *E. coli* that infect the bovine mammary gland belonged to phylogroups A and B1 since *E. coli* is an opportunistic environmental mastitis pathogen.

Together with phylogroups A and B1, which were the majority, we identified other phylogroups such as F (8.1% 3/37), D (2.7%, 1/37) and E (2.7%, 1/37), which also caused bovine mastitis. These phylogroups were also reported in other studies that evaluated *E. coli* isolated from bovine mastitis. Ghanbarpour and Oswald [46] reported 16.5% (n=127) of both clinical and subclinical isolates belonging to phylogroup D. Among clinical mastitis *E. coli*

isolates, Guerra *et al.* [54] reported phylogroups D (4.0%; 2/50), F (8.0%; 4/50) and clade I or II (2.0%; 1/50), Nuesch-Inderbinen *et al.* [52] reported phylogroups D (9.8%; 8/82), E (2.4%; 2/82) and F (2.4%; 2/82), and Guerra *et al.* [51] reported group E (6.1%; 7/82).

This study found that 8.1% (3/37) of *E. coli* isolates from bovine mastitis were not assigned to any phylogroup using the Clermont quadruplex PCR method. Guerra *et al*. [53] in Brazil and Lan *et al.* [51] in China also reported 0.9% (1/114) and 5.4% (5/92) respectively of mastitis *E. coli* isolates that were not assigned to any phylogroup using this method. This could be because the strains are sporadic or the strains result from recombination events where the donor and recipient originated from two different phylogroups [10]. Another reason may be due to the frequent gain and loss of genes in the highly variable gene content *E. coli* [15].

The establishment of specific diseases by *E. coli* in humans and animals is characterized by mechanisms, represented mainly by adherence of the pathogen to host cells, toxin production, iron uptake, and the ability to resist the serum immune response [16] which are controlled by specific virulence genes. There is no specific profile of virulence genes in *E. coli* related to bovine mastitis [20, 58]. This study determined a diverse virulence gene combination among *E. coli* mastitis isolates (Table 4). The most common adhesins found in all *E. coli* isolates are type 1 fimbriae (*fimH* gene) [59]. The *fimH* gene (91.9%; 34/37) was the most common virulence gene detected among the isolates in this study. This finding is in agreement with studies that reported *fimH* detection of 100% (27/27) in Finland [20], 89.9% (71/79) in China [48] and 100% (114/114) in Brazil [53] among mastitis *E. coli* isolates as the most commonly detected virulence gene. Other studies have reported the detection of 76.7% in China [60] and 15% in Jordan [61] of the *fimH* gene but it was not the most frequently detected gene.

The second most frequently detected gene was *traT* (62.2%). To resist the host´s complement system during infection, bacteria either utilize polysaccharides (e.g. capsules) or outer membrane proteins (e.g. TraT) [62]. The *traT* gene of *E. coli* encodes the outer lipoprotein of the membrane that interacts with the complement system, which is considered a serum factor of resistance [35]. The gene has been associated with serum resistance in humans and avian isolates [63]. Other studies have detected the *traT* gene as the second most prevalent gene [53, 61]. The *traT* gene has been detected as the most frequent virulence gene among *E. coli* isolates from mastitis dairy cows having prevalence between 36.8% [22] and 95.1% [60], although the importance of this gene, as a primary virulence mechanism of *E. coli* in mammary infections is not clear [64, 65]. However, the presence of the *tra*T gene was correlated to *E. coli* causing clinical mastitis, compared to isolates from faeces, suggesting a role in mastitis pathogenesis [66].

Among the isolates, *ehlyA* and *stx2* (37.8%, 14/37 each), *eaeA* (35.1%, 13/37) and *stx1* (29.7%, 11/37) were also frequently detected. The *ehlyA* gene codes the toxin enterohaemolytic *E. coli* (EHEC)-haemolysin (similar in functions as α-haemolysin produced by ExPEC), a cytolytic protein toxin that lyses the erythrocytes of mammals and even fish [67]. Other studies have recorded *ehlyA* detection between 0% [20, 47] and 19.1% [68] among mastitis *E. coli* isolates. The *stx* genes code shiga toxin (stx1 and stx2); an exotoxin produced by shiga-toxigenic *E. coli* (STEC) strains and this toxin is the main virulence factor of these strains which acts by inhibiting protein synthesis in endothelial and other cells [69, 70]. Shiga toxins (stx1 and stx2) exhibit their effect in human beings and not cattle [71]. In this study, 15 (40.5%) strains harboured *stx* genes (Table 4). The gene *eaeA* codes for intimin, an attaching and effacing protein which is necessary for the characteristic intimate attachment of uropathogenic *E. coli* (UPEC), enteropathogenic *E. coli* (EPEC) and Shiga-toxigenic *E. coli* (STEC) strains to epithelial cells [16, 72]. In line with the detection of these

genes in our study, other studies have reported the detection of *stx2* between 0% [48, 52] and 55.3% [68], *eaeA* between 0% [52, 60] and 89.3% [68], and *stx1* between 0% [48, 60] and 93% [61].

The presence of virulence genes associated with IPEC is undoubtedly true because cattle are the primary reservoir of IPEC strains, such as STEC [52]. The presence of these virulence genes among *E. coli* in milk (particularly in subclinical mastitis) increases the risk of severe infections in humans, highlighting the danger of consuming raw milk.

Other genes detected in our study included *f17* (16.7%), *kspMII* (13.5%), *iucD* (10.8%), *malX* (8.1%) and *hlyA* (8.1%). According to our result, the gene that codes for capsule synthesis (*kspMII*) was detected more frequently among *E. coli* that caused clinical mastitis (57.1%) compared to subclinical mastitis (3.3%), and the difference was statistically significant (p = 0.002). Previous studies have only reported investigation of *kspMII* among clinical mastitis *E. coli* isolates, and our result was higher than previous results (range: 2.4 – 12.0%) [52, 53]. This may mean that *kspMII* is an important virulence factor in *E. coli* pathogenicity of clinical mastitis. On another light, the gene that codes for aerobactin (siderophore) biosynthesis (*iucD* gene) and phosphotransferase enzyme II (*malX* gene) were detected more frequently among mastitis *E. coli* isolates that belonged to phylogroup F compared to the other phylogroups. The differences were statistically significant (*iucD*: p = 0.001 and *malX*: p = 0.000). *MalX* is part of a pathogenicity island associated with extraintestinal infections, and the enzyme it encodes utilizes mainly glucose and maltose [73]. *IucD* is also known to be associated with extraintestinal pathogenic *E. coli* (ExPEC) infections [74-77]. Phylogroup F is among the phylogroups mostly associated with ExPEC infections [12, 13]. Thus, this may explain why these genes are significantly associated with phylogroup F. To the best of our knowledge, the investigation and detection of *malX* among mastitis *E. coli* isolates are reported for the first time in Cameroon. The differences in the presence of virulence genes in

mastitis *E. coli* isolates could be due to differences in sample type, geographical area and number of isolates studied.

4.0 Conclusion

We found that *E. coli* phylogroups A (37.8%; 14/37) and B1 (37.8%; 14/37) were the most frequent cause of mastitis. Thirty six (97.3%) isolates haboured at least one of the virulence genes investigated. Twenty-three virulence gene profiles were determined and the most frequently detected genes were *fimH* (91.9%), followed by *traT* (62.2%), *ehlyA* and *stx2* (37.8% each), *eaeA* (35.1%, 13/37) and *stx1* (29.7%, 11/37). A diverse virulence gene combination (09) was identified in phylogroups A and B. The detection of *iucD* and *malX* was significantly associated ($P < 0.05$) with *E. coli* that belonged to phylogroup F.

The detection of virulence genes, as investigated in this study, does not say whether these genes are actually expressed. Hence, a study designed in this area to investigate the expression of these genes among mastitis *E. coli* isolates will throw more light on their involvement in the pathogenesis of *E. coli* mastitis. However, the presence of these genes among mastitis *E. coli* isolates indicates their potential to cause infections, especially in humans, as genes associated with IPEC strains were detected. Thus, it is important to apply biosafety measures when handling milk and prohibit raw milk consumption.

Data availability

All data used to support the findings of this study are included in the article.

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