Virulence factors and phylotyping of *Escherichia coli* isolated from non-diarrheic and diarrheic water buffalo calves

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ABSTRACT: *This study aimed to determine the virulence factors, phylogenetic groups, and the relationships between pathovars and phylogenetic groups of E. coli strains isolated from feces of buffalo calves. A total of 217 E. coli strains were obtained from feces after culture and were screened by PCR for detection of virulence factors EAST-1, enterohemolysin, Saa, CNF2, F41, F5, STa, intimin, Stx1 and Stx2. One hundred and thirty-four isolates were positive for one or more virulence factors: eighty-four from diarrheic animals, and fifty from non-diarrheic calves. The pathovars of E. coli identified in diarrheic feces were ETEC (F5+) (2/84), NTEC (16/84), STEC (20/84), EPEC (3/84), EHEC (3/84), and EAEC (EAST-1+) (33/84). Pathovars identified in non-diarrheic animals were NTEC (21/50), STEC (17/50), EHEC (1/50) and EAEC (7/50). E. coli strains positive for EAST-1 (P=0.008) and phylogroup C (P = 0.05) were associated with the presence of diarrhea. Phylogenetic analysis showed that 58.95% of the isolates belonged to phylogroup B1, followed by E (9.70%), B2 (5.90%), C (5.90%), D (5.22%), A (2.24%), and F (1.50%). Phylogroup B1 predominated in pathogenic E. coli isolated from water buffalo, and phylogroup C constituted an enteropathogenic E. coli for water buffalo calves.*
Key words: *buffalo calves, diarrhea, Escherichia coli, pathovars, phylogenetic group.*

Caracterização dos fatores de virulência e filogrupos de *Escherichia coli* isoladas de bezerros bubalinos sem diarreia e diarreicos

RESUMO: *O objetivo foi determinar os fatores de virulência, os grupos filogenéticos e as possíveis relações entre os patovares e os grupos filogenéticos identificados de cepas de Escherichia coli isoladas de fezes de bezerros bubalinos. Um total de 217 amostras de E. coli foram identificadas a partir de cultura das fezes e submetidas a reação em cadeia da polimerase (PCR) para detecção dos fatores de virulência EAST-1, enterohemolisina, Saa, CNF2, F41, F5, STa, intimina, Stx1 e Stx2. Foram identificadas 134 cepas positivas para um ou mais fatores de virulência: 84isoladas de bezerros bubalinos diarreicos e 50 de bezerros bubalinos saudáveis. Os patovares de E. coli obtidos de fezes diarreicas foram ETEC (F5+) (2/84), NTEC (16/84), STEC (20/84), EPEC (3/84), EHEC (3/84), e EAEC (EAST-1+) (33/84). Os patovares isolados de fezes não diarreicas foram NTEC (21/50), STEC (17/50), EHEC (1/50) e EAEC (7/50). Cepas de E. coli positivas para EAST-1 (P = 0,008) e filogrupo C (P = 0,05) foram associadas com a presença de diarreia. A análise de filogrupos revelou que 58,95% dos isolados pertencem ao filogrupo B1, seguido por E (9,70%), B2 (5,90%), C (5,90%), D (5,22%), A (2,24%) e F (1,50%). O filogrupo B1 predomina em cepas de E. coli patogênicas isoladas de bezerros búfalos e o filogrupo C constitui um filogrupo de E. coli patogênica entérica para bezerros.*
Palavras-chave: *bezerros bubalinos; dirreia, Escherichia coli;,grupo filogenético, patovares.*

INTRODUCTION

Diarrhea is an important health concern in young animals, and frequently *E. coli* pathovars such as enterotoxigenic *E. coli* (ETEC), Shiga-toxin producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), and entero-pathogenic *E. coli* (EPEC), are associated with its etiology (CROXEN & FINLAY 2010). Water buffalo calves are susceptible to all *E. coli* pathovars that cause diarrhea in bovine calves, including necrotoxigenic *E. coli* (NTEC) (BORRIELLO et al., 2012).

There are a few studies involving *E. coli* pathovar infections in buffalo calves worldwide (OLIVEIRA et al., 2007; BORRIELLO et al., 2012; MAHANTI et al., 2013; BERALDO et al., 2014). Most previous studies focused on STEC infections, and phylogenetic classification was not performed.

Moreover, a heat stable toxin called EAST-1, primarily detected in strains of entero-aggregative *E. coli* (EAEC) strains, can also occur in other pathovars such as ETEC, EPEC, and EHEC, and has been identified in strains of *E. coli* isolated from cattle and humans with diarrhea (NAGY & FEKETE, 2005; VEILLEUX & DUBREUIL, 2006); although, its ability to cause diarrhea in calves is less known (KOLENDA et al., 2015).

The study of infections with enteric *E. coli* pathovars in buffalo calves is necessary for the development of effective prophylactic and therapeutic protocols for the control of pathogenic *E. coli* infections on buffalo farms (BORRIELLO et al., 2012). In addition, food-producing animals represent an important source of EHEC in the food chain (MARTIN & BEUTIN 2011). EHEC, O157:H7 in particular, causes hemorrhagic colitis and hemolytic uremic syndrome in humans, and contaminated foods of animal origin are considered the main form of EHEC transmission to humans (DOYLE, 1991). Furthermore, contamination with EHEC and STEC in dairy products from buffalo cows represents a potential risk to public health; hence, the frequency of these pathovars in buffalos needs to be determined.

E. coli strains can be classified into one of seven phylogroups: A, B1, B2, C, D, E, and F (CLERMONT et al., 2013). The MLST is the best technique for typing *E. coli*, but the sequence type (ST) provided in such analysis does not directly allow for classification into phylogroups, and it is necessary to determine the correspondence between ST and phylogroups, with the latter performed by means of the "Clermont method". Improved understanding of *E. coli* phylogeny revealed that strains belonging to the different phylogroups are not dispersed randomly, and are associated with the source of isolation. Since its introduction in 2000, phylogenetic typing using polymerase chain reaction (PCR) became widely used due to its simplicity and rapidity. The method was improved in 2013, and, by means of quadruplex PCR, can identify the seven defined phylogroups (CLERMONT et al., 2000; CLERMONT et al., 2013; CLERMONT et al.,2015). Phylogenetic typing using PCR has 80–95% concordance with MLST analysis, showing that such testing can be used to study the genetic diversity of strains of *E. coli* (GORDON et al., 2008; CLERMONT et al., 2013).

Because of the lack of information regarding pathogenic *E. coli* in water buffalos, this study sought to determine the (i) virulence factors involved, (ii) phylogenetic groups involved, and (iii) relationships between pathovars and phylogenetic groups of *E. coli* strains isolated from feces of buffalo calves.

MATERIALS AND METHODS

Animals

Fecal specimens were collected from water buffalo calves on five farms located in Minas Gerais, two from Carmo da Mata, and three from Oliveira counties. All farms bred only Mediterranean and/or Murrah buffalos for milk production. Fecal samples were collected in 2013 from 152 water buffalo calves up to 90 days of age.

Animals were selected randomly, and each buffalo calf was sampled once during a visit to each farm. Feces were classified as diarrheic if they had a watery to paste-like consistency, and as normal if they had a firm consistency, and the perineum and tail of the animals were clean. At the time of sampling, 107 animals were considered diarrheic and 45 non-diarrheic. Samples were collected directly from buffalo calf recta in plastic bags and stored at 4 ºC until bacteriological examination.

Bacteriological examination

Bacterial examination was performed according to COURA et al. (2015a). *E. coli* strains were stored at room temperature (25 ºC) in nutrient agar (NA) for a maximum period of two months before molecular analysis.

DNA extraction and PCR

E. coli isolates in NA were plated onto MacConkey agar and incubated for 18–24 h at 37 ºC. Next, bacterial cells were re-suspended in 100µL of TE (10 mM Tris-HCl; EDTA 1 mM, pH 8.0) in 1.5 mL microtubes, and DNA was extracted according to Pitcher et al.(PITCHER at al., 1989).

The PCR was performed to determine the presence of the following virulence factors: EAST-1 (YAMAMOTO & NAKAZAWA, 1997), EHEC hemolysin (SCHMIDT et al. 1995), Saa(JENKINS et al., 2003), CNF2 (BLANCO et al., 1996), F41, F5, STa, eae, Stx1, and Stx2 (multiplex PCR) (FRANCK et al., 1998). *E. coli* reference strains used as positive controls were: B41 (O101: H−: F41+, F5+, STa+) and EDL 933 (O157: H7; Stx1+, Stx2+, eae+, hlyA$^+$), EAEC O42 (EAST-1+), S5 (CNF2+) and STECLBA05 (Saa+). Ultra-pure water was used for negative controls.

The *E. coli* strains were classified into pathovars according to the virulence factors identified by PCR. *E. coli* strains positive for STa and/or F5

were classified as ETEC; if positive for Stx2 and/or Stx1,they were considered STEC; EHEC strains were identified as positive for Stx and intimin; strains positive for intimin only were classified as EPEC; strains positive for CNF2 were classified as NTEC; Strains positive for EAST-1 only were classified as EAEC pathovars. Atypical combinations of virulence factors of *E. coli* isolates, which did not permit classification as pathovars were classified as "Others".

Phylotyping

Phylotyping of *E. coli* strains was performed as reported previously (COURA et al., 2015b), and according to CLERMONT et al. (2013),and used in the present study for analysis of associations between pathovars, presence of diarrhea, and *E. coli* phylogroups. If a phylogenetic group could not be identified, such *E. coli* isolates were screened for the presence of the *uidA* gene to confirm the detection of *E. coli* (MCDANIELS et al., 1996), as suggested by CLERMONT et al. (2013).

Statistical analysis

All data analyses were carried out using Stata Statistical Software (STATACORP, 2011). Associations involving fecal consistency, pathovars, and phylogroups, were studied using the Chi-square test or Fisher's exact test. Results were expressed as P values. Results were considered statistically significant at P≤0.05. Correspondence analysis (CA) (GREENACRE & BLASIUS,2006) was used to study pathovar categories and phylogroups. In CA, relationships between categories were represented in two-dimensional graphs, with the value of the third dimension shown in parenthesis. The relatedness between pathovars and phylogroups was demonstrated by evaluating which variables plotted closely together (HAIR,2009).

RESULTS

In total, 217 *E. coli* strains were analyzed by PCR screening for virulence factors. One hundred and thirty-four isolates (61.75%) were positive for one or more virulence factors: 84 (62.68 %) from diarrheic feces, and 50 (37.32%) from normal feces. Figure 1 illustrates the virulence factors and pathovars of *E. coli* strains identified in diarrheic and fecal samples. The identification of *E. coli* strains positive for EAST-1 (EAEC) was associated with the presence of diarrhea (P=0.008).

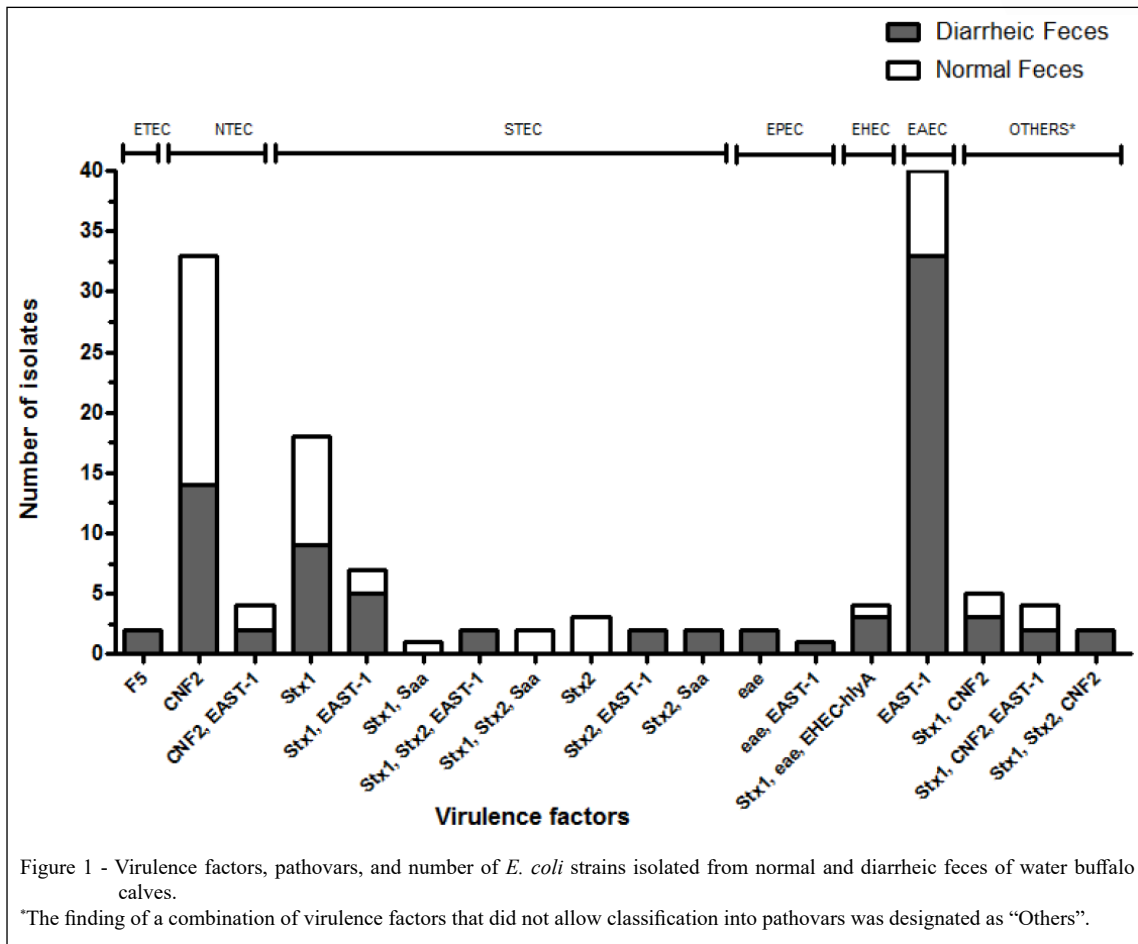Phylogenetic analysis of the 134 *E. coli* strains isolated from buffalo calves showed that 58.95% of the isolates belonged to phylogroup B1, followed by E (9.70%), B2 (5.90%), C (5.90%), D (5.22%), A (2.24%), and F (1.50%). Fourteen strains were assigned as "unknown" because they were positive for the four genes arpA/chuA/yjaA/TspE4.C2, and were screened for the *uidA* gene, confirming the detection of *E. coli* (Table 1). According to CLERMONT et al. (2013), MLST should be performed to classify such strains. All seven phylogenetic groups were detected in *E. coli* isolated from diarrheic feces; although, phylogenetic groups A and F were identified at low frequency. Phylogroups A and C were not found in isolates from normal feces. Phylogroup C was associated with the presence of diarrhea (P=0.05).

The distribution of 134 strains in relation to pathovars and phylogenetic groups from diarrheic and normal feces showed that ETEC pathovars included only phylogroups E (1/2) and F (1/2); NTEC phylogroups B1 (23/37), B2 (6/37), D (3/37), and E (4/37); STEC phylogroups B1 (14/37), B2 (2/37), C (2/37), D (3/37), E (5/37), and F (1/37); all three EPEC strains B1; EHEC phylogroup B1 (3/4); EAEC included phylogroups A (3/40), B1 (30/40), C (4/40), D (1/40), and E (2/40). Strains of "unknown "phylogenetic grouping were identified in NTEC (1/37), STEC (10/37), and EHEC (1/4).

CA was performed using the pathovar and phylogenetic grouping, and a representation of the two dimensions and the value of the third are shown in figure 2. This bidimensional representation explains 94.55% of the total variation, with 47.68% explained by the 1st dimension, 26.63% by the 2nd dimension, and 20.63% by the 3rd. The NTEC was close to phylogroup B2 and, to a lesser extent, to phylogroup D. EPEC and EAEC (EAST-1 positive strains) was proximal to phylogroup B1, and EHEC was relatively coincident with phylogroup A. The ETEC (F5) was close to phylogroup F.

DISCUSSION

Diarrheagenic *E. coli* is an important cause of diarrhea in water buffalos (FAGIOLO et al. 2005; BORRIELO et al., 2012). Only two ETEC (F5[+]) strains were isolated in our study. Previous studies in water buffalos reported infrequent occurrences of ETEC fimbria and thermostable enterotoxin (GALIERO et al., 2005; BORRIELLO et al., 2012) among *E. coli* isolates from those animals. Collectively, these results suggested that ETEC is not an important pathogen for water buffalo calves, whereas it is for bovine calves.

Figure 1 - Virulence factors, pathovars, and number of *E. coli* strains isolated from normal and diarrheic feces of water buffalo calves.
*The finding of a combination of virulence factors that did not allow classification into pathovars was designated as "Others".

In our study, EAST-1was detected at a high frequency and was associated with the presence of diarrhea, indicating that *E. coli* strains harboring the gene for this toxin might play a role in the pathogenesis of diarrhea in water buffalo calves. The EAST-1 is a heat stable toxin, primarily detected in the strains of entero-aggregative *E. coli* (EAEC), and can also occur in other pathovars such as ETEC, EPEC, and EHEC (NAGY & FEKETE 2005). The EAST-1 has been identified in the strains of *E. coli* isolated from swine and cattle suffering from diarrhea and/or edema disease, and in humans with diarrhea (VEILLEUX & DUBREUIL, 2006), but its role in the pathogenesis of diarrhea in calves is unclear (KOLENDA et al., 2015). This is the first study evaluating the importance of this toxin and diarrhea in water buffalo, and our results demonstrated that EAST-1 should be considered as an additional determinant in the pathogenesis of diarrhea in water buffalos.

The low frequency of EPEC in our study is in accordance with other studies on buffalos in Brazil (BERALDO et al., 2014), India (MAHANTI et al., 2013),and central Vietnam (VU-KHAC & CORNICK 2008). The EPEC was also detected at a low frequency in isolates of *E. coli* from bovine fecal samples in Brazil (ANDRADE et al., 2012; COURA et al., 2015a), in Turkey (OK et al., 2009), and in Vietnam (NGUYEN et al.,2011). Although, these studies differ in terms of geographical regions and methodologies, results indicated that the occurrence of EPEC is low.

The overall frequency of STEC was 26.12%. Other studies reported similar results (OLIVEIRA et al., 2007; BERALDO et al., 2014). GALIERO et al. (2005) showed that 6.5% of *E. coli* strains isolated from buffalo calves with enteric and systemic diseases produced Shiga toxin. BORRIELO et al. (2012) investigated the presence of pathogenic *E. coli* in water buffalo calves (younger than 4 weeks

Table 1 - Absolute frequency of phylogenetic groups of *E. coli* isolated form diarrheic and normal water buffalo calf feces.

Fecal characteristics	---------------Phylogenetic group---------------						
	A	B1	B2	C	D	E	F	Unknown
Diarrheic	3	50	2	8	4	6	1	10
Normal	0	29	6	0	3	7	1	4
Total	3	79	8	8	7	13	2	14

of age) with diarrhea, and STEC was detected in 6.8% of the strains. This variation in frequency may occur due to the number of animals studied, the age of animals, and the different regions involved, but collectively, results demonstrated that STEC is an important pathogen for buffalo calves. Furthermore, ruminants are an important reservoir of the human pathogens STEC/EHEC, and the presence of these pathogens in water buffalo feces may represent a potential risk to human health, especially through consumption of buffalo raw milk.

The presence of the gene for Saa detected in STEC isolates from water buffalos is in agreement with a report by OLIVEIRA et al. (2007). PATON et al. (2001) first described the gene for Saa in *locus of enterocyte effacement*-negative STEC, and Jenkins et al. (JENKINS et al., 2003) suggested that Saa might have a role in attachment to the bovine gut. Enterohemolysin (ehxA) was not detected in association with Saa, but instead was reported in *eae*- and *stx*-positive strains. Similar results were presented by BORRIELO et al. (2012) and BERALDO et al.(2014). In contrast, VU-KHAC and CORNICK (2008) and OLIVEIRA et al. (2007) detected STEC isolated from buffalos carrying stx in association with enterohemolysin and saa. The Hemolysin is produced by several pathogenic types of *E. coli,* which cause intestinal infections, especially NTEC, ETEC, and STEC strains. There is evidence of association of Shiga toxins and hemolysin production, indicating a possible role of hemolysin in bacterial virulence (SCHMIDT et al*.,* 1995; MAINIL 2013). Our results indicated that STEC isolated from buffalos carry *saa*, while EHEC harbor the enterohemolysin gene, *ehxA*, together with *stx* and *eae* genes.
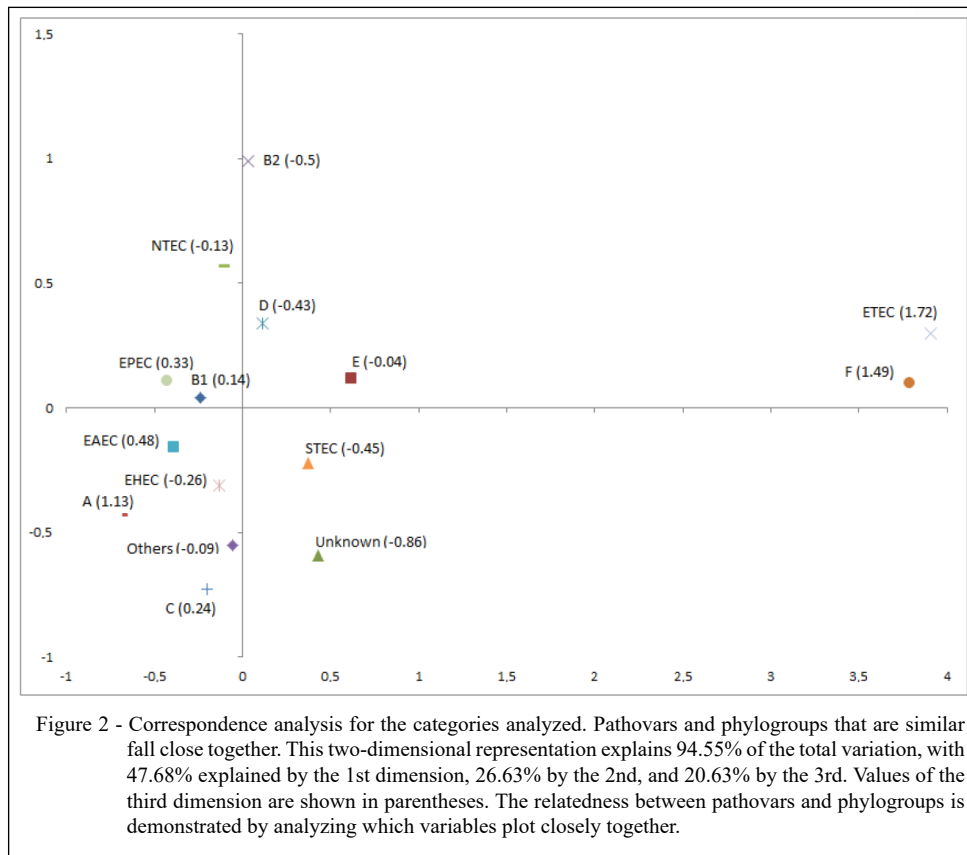
Few studies on NTEC isolated from buffalo calves have been conducted. In our study, NTEC was detected at high frequency, in both diarrheic and non-diarrheic animals. Other studies also reported high incidence of NTEC (GALIERO et al., 2005; BORRIELLO et al., 2012) in relation to

other pathovars. These results indicated that NTEC is a potential pathogenic pathovar for water buffalos.

The B1 was the most common phylogroup detected, and phylogroup C was associated with the presence of diarrhea. Our results are in accordance with those of others, demonstrating a high frequency of B1 *E. coli* strains in herbivorous animals (HIGGINS et al., 2007; BALDY-CHUDZIK; MACKIEWICZ; STOSIK, 2008; TENAILLON et al., 2010). CLERMONT et al. (2011) showed that intra-intestinal infections are caused mostly by A/B1/E, while phylogroup C, a phylogroup closely related to phylogroup B1, included extra-intestinal and intestinal pathogenic *E. coli.* Our results demonstrated that B1 is the main *E. coli* phylogroup isolated from water buffalo calves, followed by phylogroup E, and that phylogroup C is an intestinal pathogenic *E. coli* in water buffalo.

Using CA, the total variance explained by the analysis was 94.55%, allowing the analysis to be used to study the relationship between *E. coli* pathovars and phylogroups. The analysis indicated that NTEC from water buffalo calves are close related to phylogroup B2 and, to a lesser extent, phylogroup D. NTEC (CNF2 positive strains) are able to cause diarrhea and invade the blood stream, causing bacteremia and septicemia in colostrum deprived newborn calves (VAN BOST; ROELS; MAINIL, 2001), and its importance in causing diarrhea in calves has increased over the years (KOLENDA; BURDUKIEWICZ; SCHIERACK, 2015). Extraintestinal *E. coli* (ExPEC) strains are clustered mostly in groups B2 and D (ESCOBAR-PÁRAMO et al., 2004). *E. coli* strains isolated from septicemic human patients belong mainly to groups B2 and D (ČUROVÁ et al., 2014), and CNF1 strains of phylogroup B2 are associated with diarrhea and mortality in puppies (TURCHETTO et al., 2015).

EPEC and EAEC isolated from water buffalo calves are mainly B1, and EHEC is mostly represented by phylogroup A. These findings are similar to results identified in other studies (BALDY-CHUDZIK; MACKIEWICZ; STOSIK, 2008; TRAMUTA; ROBINO; NEBBIA, 2008). The ETEC is mainly phylogroup F. Studies showed no particular phylogenetic clustering of ETEC strains and toxin-producing and/or entero-invasive pathovars, such as ETEC and EHEC, which are usually reported in groups A, B1, C, or E (ESCOBAR-PÁRAMO et al., 2004). Our results involving ETEC are difficult to compare, since only two isolates of ETEC were detected, and both were negative for the toxin STa.

Figure 2 - Correspondence analysis for the categories analyzed. Pathovars and phylogroups that are similar fall close together. This two-dimensional representation explains 94.55% of the total variation, with 47.68% explained by the 1st dimension, 26.63% by the 2nd, and 20.63% by the 3rd. Values of the third dimension are shown in parentheses. The relatedness between pathovars and phylogroups is demonstrated by analyzing which variables plot closely together.

The detection of "unknown" phylogroup *E. coli* strains is not an unexpected result, since studies of *E. coli* phylogroups in water buffalo are rare. According to CLERMONT et al. (2013), *E. coli* strains that cannot be assigned to a phylogroup can occur due to the extremely rare occurrence of the phylogroup, the result of large-scale recombination involving two different phylogroups, or highly variable genome content of *E. coli* driven by gain and loss of genes.

The current study is important to advance our understanding of the epidemiology of pathogenic *E. coli* in water buffalos and the importance of diarrhea in these young animals, results that will likely have an impact on future control and prophylactic measures.

CONCLUSION

Pathovars EAEC (EAST-1$^+$) and, to a lesser extent, NTEC (CNF2$^+$) and STEC, seem to be important agents of diarrhea. In contrast, pathovars ETEC (F5$^+$) and EPEC are not as important as the other pathovars. The Phylogroup B1 predominates in pathogenic *E. coli* isolated from water buffalo, and phylogroup C constitutes an enteric pathogenic *E. coli* in water buffalo calves. Identification of virulence factors, and performing microbial phylogenetic analyses, are useful molecular epidemiological tools when studying the occurrence of pathogenic *E. coli* and provided additional knowledge in understanding the pathogenesis of *E. coli* strains and their relationships with commensalism, host, and disease.

ETHICAL STANDARDS

All procedures and animal handling followed ethical principles in animal experimentation, adopted by the Ethics

Committee in Animal Experimentation of Universidade Federal de Minas Gerais (UFMG)/Comissão de Ética no Uso de Animais (CEUA), under Protocol n° 133/2012.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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