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Editor's Choice

ORIGINAL ARTICLE

Caution at choosing a particular colony-forming unit from faecal *Escherichia coli*: it may not represent the sample profile

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Significance and Impact of the Study: This study provides relevant data about the high phylogenetic and antimicrobial susceptibility diversity observed in *Escherichia coli* colony-forming units (CFUs) from a bacteriological culture of faeces from healthy calves, foals and lambs. The selection pressure exerted by the herd treatment may directly impact the intestinal microflora of animals that have never been treated. Finally, we emphasize the importance of Clinical Laboratory Standards Institute guidelines and we recommended to analyse at least four *E. coli* CFUs to determine, in particular, the antimicrobial susceptibility profile of faecal isolates, independent of the animal's health status.

Keywords
antibiotic resistance, calves, *Escherichia coli* phylo-type, foals, lambs.

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Abstract

Data about phylogenetic classification of *Escherichia coli* colonizing calves, lambs and foals are routinely neglected and restricted to outdated methodologies, even in the context of antimicrobial susceptibility (AS) testing. Thus, the aim of this study was to determine the phylogenetic diversity and the AS profile of *E. coli* colony-forming units (CFUs) from faecal samples of healthy animals. Five CFUs of *E. coli* were randomly selected from each faecal culture of calves (n = 13), foals (n = 13) and lambs (n = 13), totalizing 195 CFUs phylo-typed by quadruplex PCR. The AS profile of five CFUs from 15 samples (five from each animal species; n = 75 isolates) against nine drugs was determined by agar diffusion test. We found *E. coli* belonging to all phylogroups already described, except D group, with the predominance of B1 (65% CFUs; 126/195) in the three-animal species sampled. Most faecal samples of calves (77%; 10/13) and foals (69%; 9/13) harboured both pathogenic and nonpathogenic *E. coli*. All faecal samples showed CFUs with diverse AS profile, highlighting the ineffectiveness of tetracycline, sulphonamide and ampicillin. As a key point, our data reinforce the importance to select at least four *E. coli* CFUs for AS testing.

Introduction

Escherichia coli isolated from animals, humans and environment have been classified into phylogenetic groups named A, B1, B2, C, D, E, F and clade I (Clermont *et al.*, 2013). Currently, isolates belonging to A/B1/C phylogroups are considered commensal, those classified into B2/D/F and E are considered pathogenic extra-intestinal and enteropathogenic respectively and *E. coli* clade I, which resulted from extent genetic, have no well-defined pathogenic potential (Walk *et al.*, 2009). The presence of several mobile elements related to antibiotic resistance, the facilitated exchange of genetic material with other *Enterobacteriaceae*, and the high diversity of hosts and their coexistence in nature (Khachatryan *et al.*, 2006) make it easier for commensal *E. coli* become pathogenic by acquisition of virulence factors and antibiotic resistance genes.

Table 1 Phylogenetic groups found in 195 *Escherichia coli* colony-forming units (CFUs) from 39 isolates from faeces of healthy foals, lambs and calves

	Strain identification												
	1	2	3	4	5	6	7	8	9	10	11	12	13
CFU foals													
A	C	E	B1	B1	B1	B1	B1	E	C	B1	E	E	B1
B	C	Unk	B1	B1	Unk	B1	B1	A	C	B1	E	E	B1
C	C	B1	E	B1	B1	B1	B1	E	C	B1	B1	E	B1
D	C	B1	B1	F	B1	B1	B1	A	C	B1	B1	E	B1
E	C	C	B1	B1	A	E	Unk	E	Unk	B1	E	E	B1
DI	0·2	0·8	0·4	0·4	0·6	0·4	0·4	0·4	0·4	0·2	0·4	0·2	0·2
Lambs													
A	B1	B1	C	C	B1	B1	B1	B1	B1	B1	B1	B1	A
B	B1	B1	C	B1	B1	B1	B1	B1	B1	B1	B1	B1	E
C	B1	B1	C	B1	B1	B1	B1	B1	B1	B1	B1	B1	A
D	B1	B1	C	B1	B1	B1	B1	B1	B1	B1	B1	B1	A
E	B1	B1	C	C	B1	B1	B1	B1	B1	B1	B1	B1	A
DI	0·2	0·2	0·2	0·4	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·4
Calves													
A	Unk	B1	B1	B1	B2	B1	B1	Unk	E	B2	B1	B1	B1
B	E	E	B1	B1	Unk	B1	E	B1	E	B1	B1	B1	B1
C	E	E	B1	B1	B1	B1	B1	B1	E	B1	B1	B1	B1
D	Unk	E	B1	B1	B1	E	B2	Unk	E	Unk	E	B1	B1
E	Unk	B1	B1	B1	Unk	B1	B1	B1	B1	Unk	B1	E	B1
DI	0·4	0·4	0·2	0·2	0·6	0·4	0·6	0·4	0·4	0·6	0·4	0·4	0·2

Pathogenic phylo-groups are highlighted in grey.
DI, diversity index; Unk, Unknown phylo-group.

Calves are very often infected with enterotoxigenic *E. coli* (ETEC), which cause bovine neonatal diarrhoea (Verdier *et al.,* 2012), and are considered a source of pathogenic *E. coli* for humans (Kolenda *et al.,* 2015). Additionally, shiga toxin-producing *E. coli* (STEC), which has bovine, sheep and other animal species as reservoir, have been also spread by faeces (Cornick *et al.,* 2000; Brandal *et al.,* 2012). However, the role of horses as reservoirs of pathogenic *E. coli* has received minor attention, despite their close contact with humans and the growing consumption of horse meat (Martuzzi *et al.,* 2001). Lastly, there is a lack of information about the general profile of *E. coli* from calves, lambs and foals, especially from healthy animals, never submitted to antibiotic therapy.

Phylogenetic classification of *E. coli* from calves, lambs and foals data are scarce, being the most of them based in the strictly Clermont *et al.* (2000) methodology. Furthermore, the high genetically diversity of *E. coli* strains in a same sample is spectated, however, routinely neglected. Therefore, we have selected five colony-forming units (CFUs) of *E. coli* from pure cultures from 39 animal-faecal-samples (13 foals, 13 calves and 13 lambs) to determine their phylogenetic profile by Clermont *et al.* (2013) methodology and five CFUs from 15 animal-faecal-samples (five foals, five calves and five lambs) to investigate their antimicrobial susceptibility profile. Briefly, we have found that the selection of single *E. coli* CFU may likely mischaracterize the whole sample antimicrobial susceptibility profile, including its phylogenetic classification.

Results and discussion

Using the quadruplex PCR method described by Clermont *et al.* (2013) we found *E. coli* belonging to all phylo-groups already described, except D group (Table 1). Overall, 65% (126/195) CFUs analysed belonged to commensal phylo-group B1, corresponding to the animals' healthy status. As in the world literature (Son *et al.,* 2009; Tenaillon *et al.,* 2010; Bessalah *et al.,* 2016; Coura *et al.,* 2017; Souto *et al.,* 2017) we also found the predominance of B1 phylo-group (40/65 CFUs; 61·5%) in calves, followed by E (13/65; 20%). Similarly, B1 (33/65 CFUs; 50·8%) predominated in foals, followed by E (14/65 CFUs; 21·5%) and C (10/65 CFUs; 15·4%), being a profile previously reported in healthy horses (Smati *et al.,* 2015). According to previous studies, B1 is also the predominant group in healthy ovine from Brazil, followed by A and D groups (Carlos *et al.,* 2010; Martins *et al.,* 2016). Meanwhile, Derakhshandeh *et al.* (2014) have found in

Iran a quite different profile in *E. coli* isolates from herbivorous (the authors do not differentiate results from sheep, goat and cattle), where 41% belonged to D phylo-group, and 29% to A and B2. We believe that these divergent results are probably due to the geographic effects in the *E. coli* populations.

The concomitant isolation of commensal and pathogenic (Table 1, highlighted in grey) *E. coli* was observed in approximately 69% (9/13) and 54% (7/13) of isolates from calves and foals respectively. The high occurrence of E phylo-group found in calves and foals' isolates mean a potential risk to public health, once the pathogenic O157: H7 *E. coli* belongs to this phylo-group and it is associated to fatal infections in humans (Tenaillon *et al.,* 2010). Differently from calves and foals, lambs' isolates presented only one CFU belonging to a pathogenic phylo-group and were almost restricted to phylo-group B1 (53/65; 81·5%).

Regarding the CFU's phylo-typing, the random selection of five CFUs from each *E. coli* growth culture was suitable to determine different levels of phylogenetic diversity among the isolates analysed. The isolates from calves and foals presented a very similar level of phylogenetic diversity, showing DI of 0·4 e 0·38 respectively. These findings corroborate with the knowledge that bacteriological culture of faecal samples from domestic animals normally harbours at least two *E. coli* phylo-groups, which would correspond to DI of 0·4. Although we had not aimed to perform an appropriated experiment to compare the phylo-groups distribution among the species evaluated, it is interesting to note that 'unknown' phylo-type was found in *E coli* isolates from calves and foals and did not in lambs, which were much less diverse in terms of phylo-groups (DI = 0·25).

We have identified an overall antimicrobial sensibility among the *E. coli* isolates analysed, where 100% of the CFU's analysed were sensitive to XNL, GM and CIP. However, foal's isolates showed an increased antibiotic resistance score (7·55) compared to calves (4·44) and lambs (1·77) isolates. Although all animals were healthy and had never received antimicrobial treatment, we believe that herd's treatment history may have a significant influence in AS profile of foals' *E. coli*, which needs to be appropriately addressed in a future study. From the nine antimicrobials tested, we observed moderate sensitivity and resistance especially for TET, SUL and AMP (Tables 2–4). The *E. coli* resistance to TET and SUL observed in isolates from all species is in agreement with several reports (Khachatryan *et al.,* 2006; Turkyilmaz *et al.,* 2013; Bosman *et al.,* 2014; Hanon *et al.* 2015). The

Table 2 Antimicrobial susceptibility profile of 25 colony-forming units (CFUs) of *Escherichia coli* isolated from five faecal samples of healthy calves

Strain/CFU	Phylo-group	AMP	XNL	GM	ENO	CIP	TET	SUL	STX	FFC
1/A	Unk	S	S	S	S	S	S	S	S	S
1/B	E	S	S	S	S	S	I	S	S	S
1/C	E	S	S	S	S	S	I	S	S	S
1/D	Unk	I	S	S	S	S	I	S	S	S
1/E	Unk	S	S	S	S	S	R	S	S	S
7/A	B1	I	S	S	S	S	I	I	S	S
7/B	E	S	S	S	S	S	I	R	S	S
7/C	B1	S	S	S	S	S	I	I	S	I
7/D	B2	S	S	S	S	S	I	R	S	S
7/E	B1	I	S	S	S	S	R	I	S	S
9/A	E	S	S	S	S	S	I	S	S	S
9/B	E	I	S	S	S	S	R	S	S	I
9/C	E	I	S	S	S	S	I	S	S	S
9/D	E	I	S	S	S	S	R	R	S	S
9/E	B1	I	S	S	S	S	R	R	S	I
10/A	B2	I	S	S	S	S	I	S	S	S
10/B	B1	S	S	S	S	S	I	I	S	S
10/C	B1	I	S	S	S	S	I	S	S	S
10/D	Unk	I	S	S	S	S	I	S	S	S
10/E	Unk	I	S	S	S	S	I	S	S	S
13/A	B1	I	S	S	S	S	I	S	S	S
13/B	B1	S	S	S	S	S	R	S	S	S
13/C	B1	S	S	S	S	S	I	S	S	S
13/D	B1	S	S	S	S	S	S	S	S	S
13/E	B1	S	S	S	S	S	S	S	S	S

Unk, unknown; Phylo-group, phylogenetic group; AMP, ampicillin; XNL, ceftiofur; GM, gentamicin; ENO, enrofloxacin; CIP, ciprofloxacin; TET, tetracycline; SUL, sulphonamide; STX, trimethoprim-sulphamethoxazole; FFC, florfenicol; R, resistant; I, intermediate, S, sensitive.

moderate sensitivity against AMP may represent the disseminated resistance to ß-lactam antibiotics, which have been commonly used in both veterinary and human medicine for decades (Stedt *et al.*, 2014).

According to the guidelines of the CLSI (2015), the agar diffusion test should be done using at least four isolated CFUs with the same morphological characteristics from a growth culture. Here we demonstrate the importance of this recommendation, once all isolates presented CFUs with variable antimicrobial susceptibility profile against at least one drug tested. Indeed, the high level of antibiotic resistance observed here for foals' isolates may be the result of the large use of antimicrobials in the stud farms, as the intermediate level of antibiotic resistance may correspond to their frequent use in bovine herds and finally, the very low antibiotic resistance score observed in lambs' *E coli* is in accordance with the unusual use of antibiotic therapy reported by the sheep owners.

Materials and methods

Sampling

Faecal samples were collected directly from the rectal ampulla of 13 calves (from the same herd) aged 30- to 60-weeks (May to July 2016), 13 thoroughbred foals (from different farms) aged 3- to 5-weeks (September to November 2015) and 13 mixed breed lambs (from the same herd) aged 2- to 3-months (November to December 2016) located in different farms in the Rio Grande do Sul (RS) state, Brazil. All animals were clinically examined by a veterinarian to ensure their health status. According to the owners, the animals sampled had never been submitted to antibiotic therapy previously. Nonetheless, in relation to the sampling farms status, foals' owners reported that antimicrobials were widely used in the stud farms to treat several disorders, especially respiratory disease, as rhodococcosis. Furthermore, the bovine herd was periodically submitted to antibiotic therapy mainly to treat diarrhoea. In contrast, the bovine herd had received therapy usually for prevention of parasitic diseases, being the antibiotic therapy rarely utilized according to the farmer. The farmers and veterinarians collected all the samples used in the present study to routine parasitological tests, including microbiological analysis.

E. coli isolation and selection of colony-forming units

An aliquot of 1 g of each faecal sample was dissolved and homogenized in 9 ml of sterile 0·9% saline solution. Fifty

Table 3 Antimicrobial susceptibility profile of 25 colony-forming units (CFUs) of *Escherichia coli* isolated from five faecal samples of healthy foals

Strain/CFU	Phylo-group	AMP	XNL	GM	ENO	CIP	TET	SUL	STX	FFC
1A	C	I	S	S	S	S	S	S	S	I
1B	C	I	S	S	S	S	S	S	S	S
1C	C	I	S	S	S	S	S	I	S	I
1D	C	I	S	S	S	S	S	S	S	S
1E	C	S	S	S	S	S	S	S	S	I
2A	E	S	S	S	S	S	I	S	S	I
2B	Unk	S	S	S	S	S	R	S	S	I
2C	B1	S	S	S	S	S	R	S	S	S
2D	B1	S	S	S	S	S	R	S	S	I
2E	C	S	S	S	S	S	I	S	S	S
5A	B1	I	S	S	S	S	R	S	S	I
5B	Unk	I	S	S	S	S	I	S	S	S
5C	B1	I	S	S	S	S	I	S	S	S
5D	B1	I	S	S	S	S	I	S	S	S
5E	A	I	S	S	S	S	R	S	S	I
6A	B1	S	S	S	S	S	R	R	S	S
6B	B1	S	S	S	S	S	S	S	S	S
6C	B1	S	S	S	S	S	R	R	S	S
6D	B1	S	S	S	S	S	S	S	S	S
6E	E	S	S	S	S	S	I	S	S	S
12A	E	R	S	S	S	S	I	S	R	S
12B	E	I	S	S	S	S	I	S	S	S
12C	E	I	S	S	S	S	R	S	S	S
12D	E	R	S	S	S	S	R	R	R	S
12E	E	I	S	S	S	S	I	S	R	S

See Table 2 for keys.

Table 4 Antimicrobial susceptibility profile of 25 colony-forming units (CFUs) of *Escherichia coli* isolated from five faecal samples of healthy lambs

Strain/CFU	Phylo-group	AMP	XNL	GM	ENO	CIP	TET	SUL	STX	FFC
1A	B1	S	S	S	S	S	S	S	S	S
1B	B1	S	S	S	S	S	S	S	S	S
1C	B1	I	S	S	S	S	S	S	S	S
1D	B1	S	S	S	S	S	S	S	S	S
1E	B1	S	S	S	S	S	S	S	S	S
3A	C	S	S	S	S	S	S	S	S	S
3B	C	S	S	S	S	S	S	S	S	S
3C	C	S	S	S	S	S	S	S	S	S
3D	C	I	S	S	S	S	S	S	S	S
3E	C	S	S	S	S	S	S	S	S	S
4A	C	S	S	S	S	S	S	S	S	S
4B	B1	S	S	S	S	S	S	S	S	S
4C	B1	I	S	S	S	S	S	S	S	S
4D	B1	S	S	S	S	S	S	S	S	S
4E	C	S	S	S	S	S	S	S	S	S
12A	B1	S	S	S	S	S	S	S	S	S
12B	B1	R	S	S	S	S	I	S	S	S
12C	B1	S	S	S	S	S	S	S	S	S
12D	B1	I	S	S	I	S	R	S	S	S
12E	B1	R	S	S	S	S	I	S	S	S
13A	A	S	S	S	S	S	I	S	S	S
13B	E	S	S	S	S	S	R	S	S	S
13C	A	S	S	S	S	S	I	S	S	S
13D	A	I	S	S	S	S	I	S	S	S
13E	A	S	S	S	S	S	I	S	S	S

See Table 2 for keys.

microlitres were platted on MacConkey Agar and incubated in aerobiosis for 24 h at 37°C. Five lactose-positive CFUs were randomly selected for isolation and identification of *E. coli* by biochemical series interpreted according to the bacterial identification keys available in MacFaddin (2000). Following, the isolates were kept in freezing media at −20°C until use.

Phylo-typing and calculation of diversity index

Five *E. coli* CFUs of each culture of faecal samples (13 from each species) were used for DNA extraction by the boiling method. DNA quality and quantity were assessed by electrophoresis gel analysis. All 195 DNA templates (from 5 CFUs of 39 isolates) were used in a quadruplex PCR performed by the amplification of *arpA*, *chuA*, *yjaA* genes and DNA fragment TspE4.C2 (Clermont *et al.*, 2013). Each CFU was classified into one of the phylogenetic groups (A, B1, B2, C, D, E, F and clade I or as 'unknown') as proposed by Clermont *et al.* (2013). Based on the phylo-typing we have calculated the diversity index (DI) for each isolate by dividing the number of phylogenetic groups found (x) by five (the number of CFU evaluated from each isolate), being the absence of diversity equal 0·2. It is important to highlight that we have considered 'isolate' the *E. coli* cultivated from each faecal sample, totalizing 69 isolates analysed.

Antimicrobial susceptibility and calculation of antibiotic resistance score

Five *E. coli* CFUs from five animals from each species (totalizing 25 CFUs from each animal species) were used for antimicrobial susceptibility test by the disc-diffusion technique in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2014, 2015). A total of 75 CFUs were tested for the following antimicrobials (Laborclin®, Pinhais, PR, Brazil): β-Lactams-ampicillin (AMP, 10 μg), and ceftiofur (XNL, 30 μg); Aminoglycosides-gentamicin (GM, 10 μg); Quinolones-enrofloxacin (ENO, 5 μg) and ciprofloxacin (CIP, 5 μg); Tetracyclines-tetracycline (TET, 30 μg); Sulphonamides-sulphonamide (SUL, 300 μg) and trimethoprim-sulfamethoxazole (STX, 25 μg); Phenicols-florfenicol (FFC, 30 μg). *E. coli* ATCC 25922 was used as the reference strain for quality control. The CFUs were classified as sensitive (S), resistant (R) and intermediate (I) against each antimicrobial tested. The antibiotic resistance score was calculated as $(R/nA) \times 100$, where R is the summation of the number of resistant strains to each antimicrobial tested, n is the

number of CFUs (= 25, from 5 strains) tested and *A* is the number of antibiotics tested (Skurnik *et al.,* 2006).

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Conflict of Interest

The authors declare that they have no conflicts of interests to disclose.

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