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Article

Assessment of antibiotic resistance in fecal samples from calves with diarrhea in the Cajamarca region, Peru

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Abstract:

Diarrhea is associated with infectious bacteria that cause mortality in calves, such as *Escherichia coli*, representing a problem for milk and meat producers globally, causing large economic losses. This study assessed the resistance to *E. coli* strains isolated from diarrheal feces of newborn calves from the Cajamarca region. Fifty two (52) fecal samples from calves from five provinces of the Cajamarca region were collected for the isolation of *E. coli* on MacConkey agar with sorbitol. The molecular identification of *E. coli* was performed by amplification of the *uidA* gene by conventional PCR and then antibiotic susceptibility/resistance was assessed using the Kirby-Bauer methodology and antibiotic discs with neomycin, tetracycline, sulfamethoxazole-trimethoprim and enrofloxacin. The results were that 96.15 % of *E. coli* strains were resistant to tetracycline, 51.92 % to sulfamethoprim, 26.92 % to neomycin and 9.61 % to enrofloxacin. It was also demonstrated that 30.76 % had resistance to two drugs, 19.23 % to three drugs and 5.76 % to four drugs; a significant difference was found in resistance to tetracycline (P<0.0001). It is concluded that newborn calves from the Cajamarca region that presented

diarrhea are carriers of antibiotic-resistant *E. coli*, representing a problem for cattle farmers, since these strains can cause the death of animals and contribute to the spread of antibiotic resistance.

Keywords: Resistance, Antibiotics, *E. coli*, Calves, Diarrhea.

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Introduction

Diarrhea in calves has been linked to infectious pathogens, representing a challenge for those engaged in milk and meat production globally[1]. The most important infectious bacteria that cause mortality associated with diarrhea in calves are *E. coli* and *Salmonella*[2], generating large economic losses if the disease is not treated in time with appropriate antimicrobials and supportive therapy[2,3]. Antibiotics have been used in animals for the treatment of diseases, prevention and control of common infections[4,5], however, the inappropriate and excessive use of antibiotics contributes to antimicrobial resistance, threatening the health of animals and humans[6]. In relation to animals that are destined for slaughter and finally for human consumption, they act as reservoirs of antimicrobial-resistant strains[7].

For example, it has been reported that, in Egyptian dairy farms, strains of *E. coli* have been isolated from diarrheal feces of calves, which were resistant to ampicillin[8]. Calves frequently eliminate microorganisms through their feces, generating the spread of bacteria in the farm environment, which could cause infections in other animals. *E. coli* isolates have been obtained from feces of dairy calves, which present resistance to multiple antibiotics from the fluoroquinolone group and the *iuc*D gene was determined as the most prevalent[9]. Likewise, other studies mention the aerobactin operon (*iuc*D), which produces four types of siderophores: enterobactin, salmochelin, aerobactin and yersiniabactin. The genes involved in the biosynthesis of siderophores are found in uropathogenic strains (UPEC) and commensal strains; nevertheless, salmochelin, aerobactin and yersiniabactin are located in UPEC-associated pathogenicity islands, but not in commensal strains, suggesting that iron uptake systems were acquired by horizontal gene transfer. Aerobactin is a siderophore present in most UPEC strains, having a great stability in the binding to Fe^{3+} and is one of the responsible for iron sequestration during a urinary tract infection (UTI), the combination of adhesion/iron uptake/toxicity genes shows the high virulence and the potential for damage that the strains have to cause a UTI[9,10].

On the other hand, the French surveillance network for antimicrobial resistance in sick animals indicated that *E. coli* strains carrying most resistances have been isolated from diarrheal feces of neonatal calves[10], with amoxicillin, tetracycline and streptomycin being the main antibiotics to which resistance has been generated[7,11].

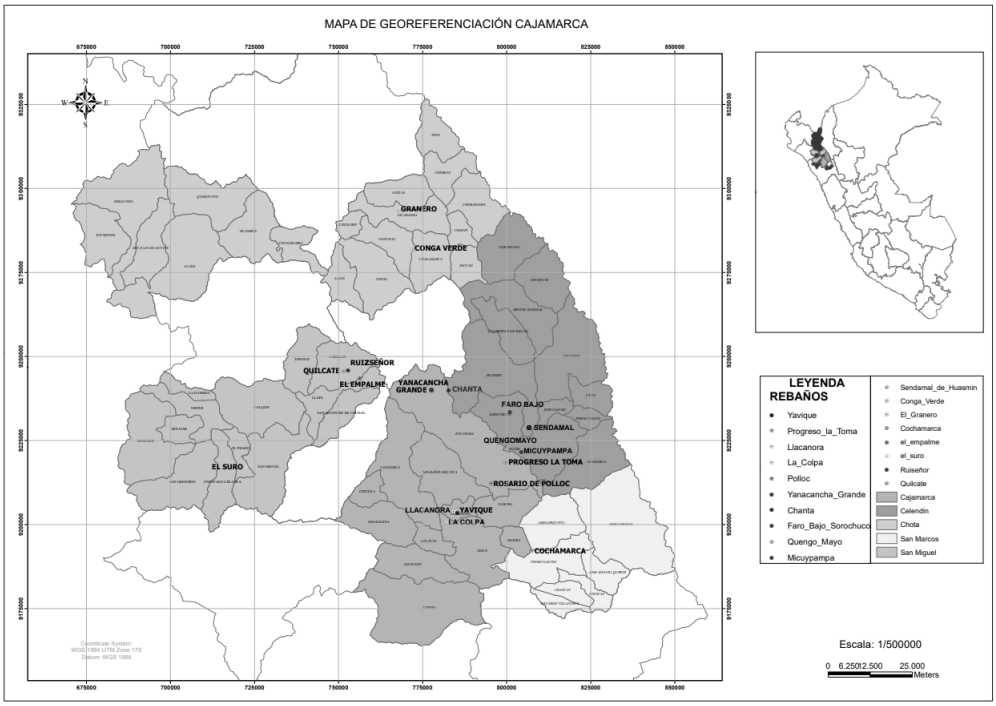
It is necessary to report the resistance that has been generated to multiple types of antibiotics to consider their control and proper use in cattle, because it represents a danger to public health. In addition, there are very few studies of antibiotic resistance in newborn cattle in Peru, with this study being a contribution to have knowledge of the current situation. The objective of the research was to assess the antibiotic resistance of *E. coli* strains isolated from diarrheal feces of newborn calves from five provinces of Cajamarca.

Material and methods

Study location

Work was carried out in 18 dairy farms under a semi-intensive system, which are located in the province of Chota, San Miguel, Celendín, San Marcos and Cajamarca, region of Cajamarca, Peru. A total of 52 samples were collected, which were from calves with diarrhea until the first month of life, of which 35 calves were males and 17 calves were females, in the rainy season (November 2020 – May 2021) (Figure 1). The fecal samples (approximately 3 g) were obtained directly from the rectum through the use of first-use sterile polyethylene bags, these samples were identified and taken in an expanded polystyrene box containing ice to the laboratory of Biotechnology in Animal Health| of the Baños del Inca Agricultural Experimental Station, where the isolation of *E. coli* from all the samples was carried out.

Figure 1: Geographical location of the farms that participated in the study, from the provinces of Chota, San Miguel, Celendin, San Marcos and Cajamarca, region of Cajamarca, Peru



Isolation and identification of *Escherichia coli*

With a sterile bacteriological loop, 300 µl of feces was seeded on MacConkey II agar with sorbitol (Becton, Dickinson and Company® Loveton Circle Sparks, MD 21152, USA), then incubated at 37 °C for 24 h in an oven (Universal oven Memmert UN-110/ Schwabach/Germany), colonies with typical morphology and development, such as bright red colonies, were isolated, which were considered as *E. coli*.

Extraction of deoxyribonucleic acid (DNA) from *Escherichia coli*

Three selected *E. coli* colonies grown on MacConkey II agar with sorbitol were cultured in liquid microbial growth and multiplication medium 2xYT medium (Sigma, REF. Y2377) at 37 °C for 18 h; bacterial growth was determined by concentration of preset values in the spectrophotometer (PCR MAX Lambda 64272, Bibby Scientific Ltd. United Kingdom), the calculation of the Colony Forming Units (CFUs) was performed at 600 nm, determining the growth of viable bacteria in the growth medium. To obtain the *E. coli* DNA template, the Wizard® Genomic DNA purification kit (Promega, REF. A1120) was used, with the manufacturer's indications, the DNA was stored in 1,500 µL polypropylene microtubes (Eppendorf™) in refrigeration, using a Samsung refrigerator,

RT35K5930S8/PE, Samsung, Mexico of 4 °C, which is used for the different processes of the Polymerase Chain Reaction (PCR).

Molecular identification of *Escherichia coli*

The molecular identification of *E. coli* was performed by PCR using "primers" (F5'-TCAGCGCGAAGTCTTTATACC-3', R5'-CGTCGGTAATCACCATTCCC-3'), for the amplification of the *uidA* gene (248 bp)[11,12,13]. In the PCR reaction, 1 µl (10 mM) of F and R of each primer, 7.7 µl of molecular grade water and 12.5 µl of G2 Green Master Mix (Promega, Madison, USA) were used, 2.8 µl of DNA at a concentration of 50 µg/ml was used as template. The thermal profile of the PCR reaction was: denaturation 94 °C/2 min, 25 cycles denaturation 94 °C/30 sec, hybridization 55 °C/30 sec, extension 72 °C/45 sec; final extension 72 °C/2 min. The reference strain JM 109 of *Escherichia coli* (Promega, REF L2004) was used as a positive control. Fragments of amplified DNA (248 bp) to identify *E. coli* strains were separated by their molecular weight by electrophoresis – agarose 1 %. The fragments were analyzed by agarose gel staining with Sybr Green (Thermo Fisher) and observed on a Labnet U1001 UV transilluminator, 302 nm, Taiwan.

Antimicrobial susceptibility test

For the evaluation of susceptibility/resistance of *E. coli* strains to antibiotics, the Kirby-Bauer methodology was used, where parameters established for bacteria isolated from animals by the Clinical and Laboratory Standards Institute (CLSI) were taken as a reference[14] (Table 1). Before carrying out the susceptibility test, specific Mueller Hinton agar (Merck KGaA 64271, Darmstact Germany) was prepared to determine the sensitivity of clinically important pathogens according to the manufacturer's instructions, and sterilized in an autoclave (Automatic Digital Autoclave AVDA050 Liters, Biogenics Lab, Peru); then it was poured into Petri dishes of 35 mm diameter / 10 mm high and two or three isolated colonies were seeded with a bacteriological loop, incubated using an incubator (Memmert CO_2 ICO50 GmbH + Co. KG) under aerobic conditions for 18 h at 37 °C. Antimicrobial susceptibility of all isolated colonies was determined against neomycin-N 30 µg, tetracycline-TE 30 µg, sulfamethoxazole-trimethoprim-SXT 25 µg and enrofloxacin-ENR 5 µg (Discs - Thermo Scientific™); the sensitivity of the isolated strains was classified as sensitive, intermediate or resistant by measuring the halo of inhibition according to the parameters established by the CLSI[14].

Table 1: Interpretation of antibiotic sensitivity tests, by disc diffusion method (diameter of inhibition zone)

Antibiotic	Disc concentration (µg)	Sensitive	Intermediate resistance	Resistant
Tetracycline	30	≥19	15-18	≤14
Sulfamethoprim	25	≥16	11-15	≤10
Neomycin	30	≥17	13-16	≤12
Enrofloxacin	5	≥23	17-22	≤16

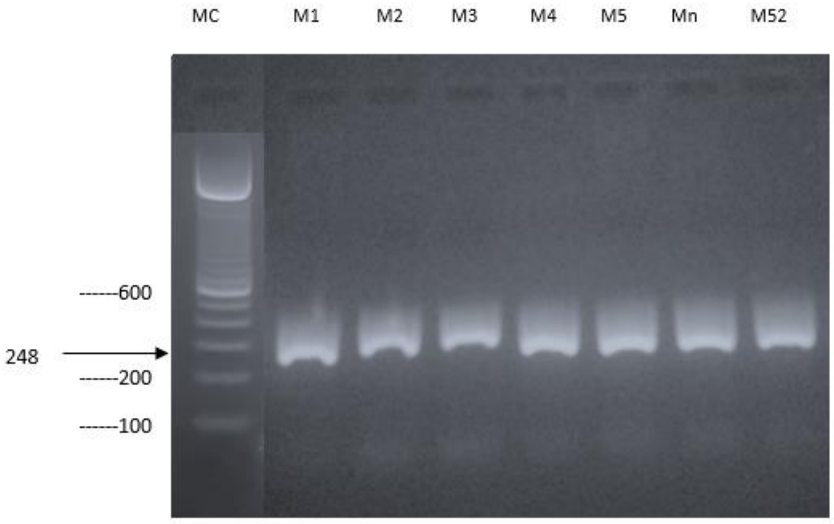
Statistical analysis

Results were analyzed using the Graph Pad Prism 9.3.1 software (Prism Software, Irvine, CA, USA). The normality of the data was determined by "Kolmogorov-Smirnoff". The analysis of variance (ANOVA), followed by Tukey's multiple comparisons analysis for evaluations between antibiotics (parameters related to sensitivity, resistance). The information obtained was considered statistically significant at a $P<0.05$.

Results

From the total samples, a total of 52 with positive growth of *E. coli* on MacConkey II Sorbitol agar were selected. The molecular identification of *E. coli* present in feces of calves with diarrhea, the *uidA* gene encoding the β-glucoronidase enzyme[12,13,14] was amplified. The processing of the PCR products was analyzed by 1 % agarose gel electrophoresis, a simple and effective method to separate, identify and purify DNA fragments with a molecular size of 0.5 to 25 kb. Electrophoretic bands of the expected size were observed: 248 bp positive for the amplified region of the *uidA* gene. It was detected in the 52 samples analyzed, evidencing the identification of *E. coli*, since this gene is specific to the bacterium (Figure 2), of which 63.30 % (n= 35) corresponded to male calves and 32.69 % (n= 17) came from female calves.

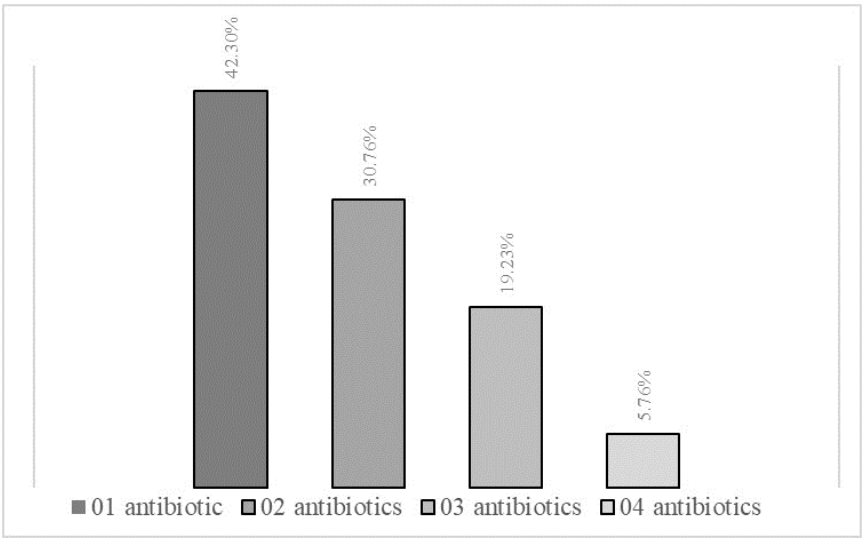
Figure 2: Amplification of the *uidA* gene from calf fecal samples by (1 %) agarose gel electrophoresis



Lane 1 100 bp marker. Positive samples in all lanes n= 52.

The presentation of susceptibility/resistance to drugs of diarrheal fecal samples from calves was analyzed, where different percentages of resistance could be observed; most strains of *E. coli* showed resistance to tetracycline (96.15 %, 50/52), likewise, more than 50 % of the samples were resistant to sulfamethoxazole-trimethoprim (51.92 %, 27/52), followed by a significant percentage of resistance to neomycin (26.92 %, 14/52), in addition, less resistance to enrofloxacin was observed (9.61 %, 5/52) (Figure 3).

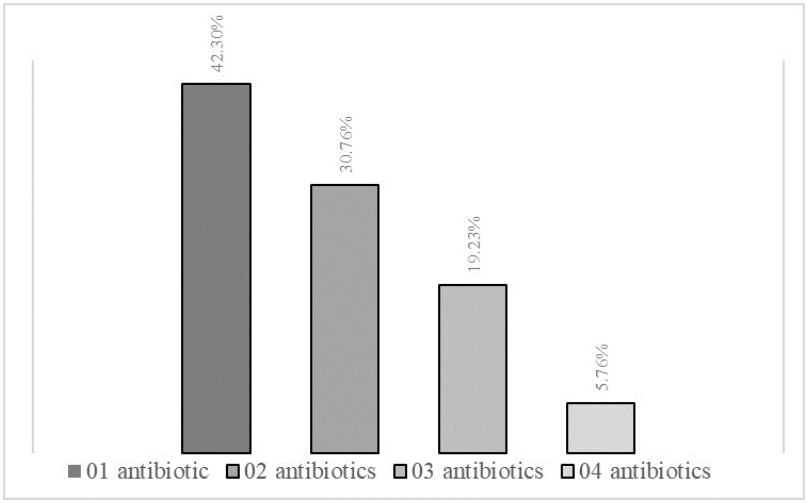
Figure 3: Percentage of *E. coli* strains with characteristics of resistance to four drugs



The presentation of antibiotic resistance was also determined in terms of the variation of the strains isolated in each calf, most had resistance to one (42.30 %, 22/52) and two (30.76 %, 16/52) drugs, the problem worsens in a significant number of calves, presenting multiple resistance in the isolated strains of *E. coli*, observing resistance to three

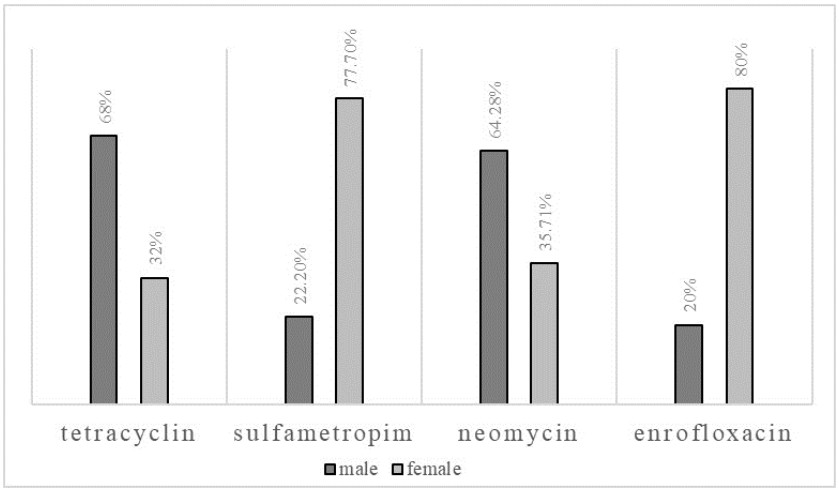
(19.23 %, 10/52) and four (5.76 %, 3/52) drugs, with the resistance to tetracycline being the most common in all; in addition, resistance to enrofloxacin was the one that occurred in the lowest proportion in the isolated strains (Figure 4).

Figure 4: Percentage of multiple resistance of drugs used in the control of diarrhea in calves



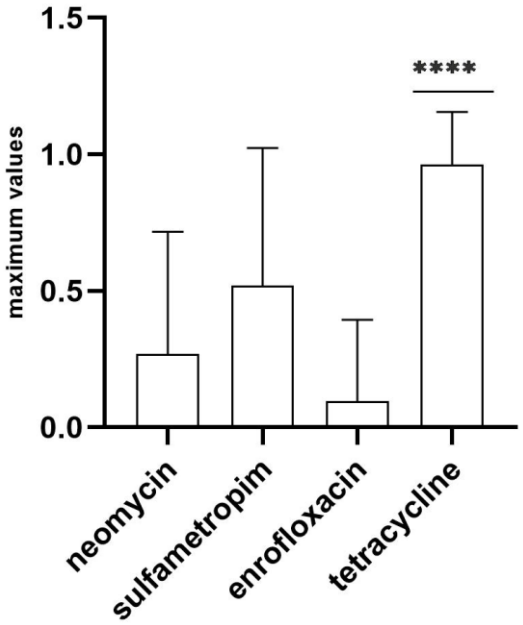
It was observed that both males and females presented resistance, with higher percentages of resistance observed in males for tetracycline (68 %) and neomycin (64.28 %) respectively; but curiously, regarding females, it could be observed that resistance to the drugs sulfamethoprim (77.70 %), enrofloxacin (80 %) was higher compared to males (Figure 5).

Figure 5: Percentage of drugs most commonly used in the control of colibacillosis in calves



The statistical analysis revealed a significant difference (P<0.0001) regarding the presentation of resistance of *E. coli* strains from samples of calves with diarrhea to tetracycline (Figure 6).

Figure 6: Statistical difference in resistance of *E. coli* strains to tetracycline



(*P*<0.0001). The data are expressed in absolute values and percentage (%).

Discussion

E. coli is one of the main bacterial agents that cause urinary infections in animals, septicemia and diarrhea in farm animals; the phenomenon of resistance expressed by *E. coli* strains to the drugs used in their control shows therapeutic failure, in addition, many cases of multiple resistance are being observed, which increases worldwide, with the dissemination of resistance becoming a public health problem[15,16].

In this research, it was possible to determine different characteristics of resistance to antibiotics and with different percentages, which has allowed determining that the therapeutic failure to antibiotics expressed by the bacterium in calves raised in dairy cattle farms in the region of Cajamarca, Peru, is widespread, being able to identify that all isolated strains of *E. coli* show resistance; thus, it was possible to observe a higher percentage of resistance to tetracycline and with a significant percentage of multiple resistance with a higher prevalence to two drugs, followed by three and four antibiotics evaluated.

The data obtained on antibiotic resistance in the Cajamarca region allows to mention that it is due to the lack of sanitary control records in the herds, making it difficult to trace the drugs used and, according to the version of the owners, some of these animals when presenting the signs of the disease are treated with antibiotics and others are not, however, all presented resistance to at least one of the antibiotics analyzed, which allows to mention that resistance could be due to the misuse of antibiotics by farmers, being used

frequently[17], and it is advisable to monitor the calf by electrolyte reconstitution and, if possible, not to administer antibiotics due to the presentation of resistance to these drugs[18].

Another possible cause of this widespread resistance to antibiotics could be that the majority of treated calves and mainly in untreated calves, possibly this phenomenon is due to the fact that milk and colostrum from cows that have been treated with an antibiotic facilitates the presence of antibiotic residues in milk, increasing the selection pressure of *E. coli* strains, with which resistant strains are selected, a practice well established in the regional livestock farming of Cajamarca[19,20,21], in addition, it can be assumed that there is diffusion of genes between commensal and pathogenic strains of antibiotic resistance among animals and herds, increasing resistance levels[22,23,24].

The pattern of resistance observed in isolated strains of *E. coli* has an order from highest to lowest prevalence tetracycline, sulfamethoprim, neomycin and enrofloxacin in a smaller proportion, common results that were also obtained by other researchers who report a pattern of resistance to tetracyclines, sulfonamides, penicillins and fluoroquinolones[25], cephalothin, tetracycline, trimethoprim-sulfadiazine, ampicillin[26], the most common multiresistance pattern was ampicillin-kanamycin-streptomycin-sulfamethoxazole-tetracycline[27].

The most frequent resistance phenotype of *E. coli* strains was to tetracycline 96.15 % (50/52), this is possibly due to the fact that *E. coli* strains carry tetracycline-resistant phenotypes due to the inappropriate use of the drug by producers, which has generated greater selection pressure in strains carrying genes that confer resistance to tetracycline, contributing to the transfer of antimicrobial resistance genes through strain diffusion[28]; in addition, these strains are possible sources that spread resistance to the environment when manure is spread in grazing areas as fertilizer[29].

The presence of resistance of local strains of *E. coli* to tetracycline is widespread based on its broad-spectrum use in animal health, as a reservoir of gram-negative bacteria with genes of resistance to tetracycline as a source of infection and with a higher prevalence in *E. coli* causing diarrhea in calves, a frequent problem that has also been reported in other studies with similar results[30,31,32].

It is very important to determine the genes that are involved in these resistance processes related to the use of these drugs, such as adhesion, iron transporters genes[33,34], as is the case of the *iuc*D gene[9,35], in addition, the processes of horizontal gene transfer, selection pressure, caused by the indiscriminate use of antibiotics[36,37], all of the above is necessary knowledge to evaluate the processes of treatment and control of diarrhea in calves in the region of Cajamarca.

Conclusions and implications

The strains of *E. coli* causing neonatal diarrhea in calves in the Cajamarca region present a prevalence of multiple resistance to the drugs used by farmers in their control, observing a profile of resistance to tetracycline, sulfamethoprim, neomycin and enrofloxacin (TSNE), as a result of the misuse of drugs which increases the selection pressure on strains that promote the expression of genes of virulence and resistance to antibiotics, becoming foci of transmission of resistance to both animals and humans due to the possibility of horizontal transmission between microorganisms. Considering that a definitive diagnosis should be made, determining the etiological agent and susceptibility to antibiotics, then correctly apply the selected antibiotic in correct dose and frequency. In addition, based on the results obtained, it is necessary to determine the resistance genes involved in multiple resistance to antibiotics.

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