DIRCÉIA APARECIDA DA COSTA CUSTÓDIO

ANTIMICROBIAL RESISTANCE AND PUBLIC AND ANIMAL HEALTH RISKS ASSOCIATED WITH VIRULENT *Escherichia coli* ISOLATED FROM CALVES

LAVRAS – MG

2023

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Thesis presented to the Federal University of Lavras, as part of the requirements of the Postgraduate in Veterinary Sciences, to obtain the title of PhD.

Prof. Dr. Geraldo Márcio da Costa
Advisor
Prof. Dra. Elaine Maria Seles Dorneles
Co-advisor

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Custódio, Dircéia Aparecida da Costa.

Antimicrobial resistance and public and animal health risks associated with virulent *Escherichiacoli* strains isolated from calves / Dircéia Aparecida da Costa Custódio. - 2023.

95 p: il.

Orientador(a): Geraldo Márcio da Costa.

Coorientador(a): Elaine Maria Seles Dorneles.

Tese (doutorado) - Universidade Federal de Lavras, 2023.

Bibliografia.

1. antimicrobial susceptibility. 2. pathogenesis. 3. diarrhea. I. Costa, Geraldo Márcio da. II. Dorneles, Elaine Maria Seles. III. Título.

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RESISTÊNCIA A ANTIMICROBIANOS E RISCOS A SAÚDE PÚBLICA E ANIMAL ASSOCIADOS A *Escherichia coli* VIRULENTAS ISOLADAS DE BEZERROS

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Aprovada em 03 de março de 2023.

Dr. Andrey Pereira Lage - UFMG

Dr. Marcos Bryan Heinemann - USP

Dra. Roberta Hilsdorf Piccoli - UFLA

Dra. Telma Maria Alves - CEVA

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Dr. Geraldo Márcio da Costa

Advisor

LAVRAS – MG

2023

Aos meus filhos Pâmela e Luiz Feliphe, pelo amor incondicional, por estarem sempre ao meu lado.

A minha querida mãe "in memoriam "que sempre acreditou em mim e nunca me deixou desistir.

Dedico

AGRADECIMENTOS

A Deus, cuja presença eu sinto todo os dias, como prova maior da minha existência.

A Universidade Federal de Lavras e ao Departamento de Medicina Veterinária - UFLA, pelo suporte aos longos de todos esses anos durante a minha trajetória.

Ao Programa de Pós-Graduação em Ciências Veterinárias – UFLA, pela oportunidade de realizar este curso.

A Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa e fomento ao projeto.

Ao meu orientador e amigo Dr. Geraldo Márcio, por tantos ensinamentos transmitidos durante todo nosso convívio, parceria e orientação.

À minha querida Co-orientadora Dra. Elaine Dorneles, pela paciência e ajuda incondicional durante todas as etapas deste trabalho, além de toda a confiança depositada em mim e por ser grande exemplo de pessoa e profissional.

Às professoras, Ana Paula Peconick, Christiane Rocha, Glaucia Frasnelli e Roberta Piccoli pelo apoio e incentivo.

Às amigas queridas da pós-graduação, Maysa, Anna Cecília, Rafaella, Amanda, Carine, pelo apoio e experiências trocadas ao longo de todos esses anos, também pelos conselhos, sempre nas horas difíceis estavam ali para me apoiar e nunca me deixaram desistir, amo cada uma de vocês.

Aos amigos Erika e Daniel pelas experiências trocadas e pelo incentivo, vocês estarão sempre comigo.

Às amigas, Juliana, Glei pelo apoio e incentivo.

As amigas Verônica e Yully, mesmo de longe sempre se fizeram presentes.

À Bruna, Giovana e a toda equipe LEM, por toda ajuda na execução de etapas do experimento.

À Cristiane, Marcos Túlio, Maria Eduarda e demais colegas da Pós-graduação, pelas experiências trocadas ao longo do curso.

Às queridas amigas, Adjamara, Rosana e Fátima, que sempre estiveram presentes nos momentos difíceis, sempre acreditaram em mim.

À querida D. Cida, pelas orações.

Aos amigos do DMV, Marcão, Willian, Marquinhos, Rose, Vilma, Adriana, Zélia e tantos outros que torceram por mim.

Ao meu esposo Carlos Alberto, pelo apoio, por me substituir na família nas horas quando eu não pude estar presente.

Aos meus queridos filhos Pâmela e Luiz Feliphe, pelo amor incondicional.

À minha querida mãe Dona Maria "*in memorian*, que sempre foi um exemplo para mim. Tenho certeza de que estará sempre comigo.

Aos meus irmãos Adriano, Adilson, Sônia e minha cunhada Lúcia, pelo incentivo.

"O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível superior – Brasil (CAPES) – Código de Financiamento 001".

Que se um dia eu pensar em desistir, o Senhor segure a minha mão impedindo minha queda. Que se um dia eu não acreditar em mim, que o senhor acredite e me faça enxergar tudo aquilo que não consigo ver. "Que o senhor esteja sempre aqui por mim!

Summary

Abstract

Escherichia coli is a bacterial agent that affects livestock and public health, being one of the most prevalent causes of diarrhea among human beings and several animal species. In bovine and buffalo calves, the disease causes serious injuries to the gastrointestinal tract of animals, impairing the absorption of nutrients, in addition to a systemic condition due to dehydration, loss of electrolytes, prostration and anorexia, which can lead to the death of affected animals. In addition to their importance for animal health, some *E. coli* pathotypes can also be transmitted to humans via the food chain, resulting in public health problems related systemic infections and increasing the concern to antibiotic resistance. Therefore, the aims of the present study were (i) perform a systematic review to determine the antimicrobial resistance profile of pathogenic *E. coli* isolated from intestinal tract of calves worldwide and (ii) determine the antimicrobial susceptibility profile of pathogenic *E. coli* strains isolated from calves and buffalo calves from 1990 to 2013 in Minas Gerais, Brazil, as well as, the frequency of O157 strains and strains carrying extended-spectrum beta-lactamases (ESBL) and mobile colistin resistance (*mcr*) genes. The systematic review recovered 932 papers and ended up with 56 studies, published between 1982 and 2020, which tested antimicrobial susceptibility among pathogenic *E. coli* mainly to disk diffusion method (82.14%) through cross-sectional studies (58.92%). Overall, high rates of resistance to the main classes of antimicrobials used in the treatment of gastrointestinal infections caused by *E. coli* strains it was observed among the selected studies. Likewise, among the virulent *E. coli* strains isolated from calves and buffalo calves in Minas Gerais, 1990 to 2013, high rates of resistance to penicillin, tetracyclines and folate inhibitors was observed, in addition to an alarming rate of multidrug resistance and strains able to produce ESBL. Altogether, our results point to the need of monitoring antimicrobial resistance among *E. coli* strains from animal origin, which should be developed from the perspective of One Health, through policies of pathogen prevention and control.

Keywords: Pathogenic. Virulence. Multidrug Resistance.

Resumo

Escherichia coli é um agente bacteriano que afeta a pecuária e a saúde pública, sendo uma das causas mais prevalentes de diarreia entre os seres humanos e diversas espécies animais. Em bezerros e bubalinos, a doença causa graves lesões no trato gastrointestinal dos animais, prejudicando a absorção de nutrientes, além de um quadro sistêmico devido à desidratação, perda de eletrólitos, prostração e anorexia, podendo levar à morte dos animais acometidos. Além de sua importância para a saúde animal, alguns patotipos de *E. coli* também podem ser transmitidos aos humanos através da cadeia alimentar, resultando em problemas de saúde pública relacionados a infecções sistêmicas e aumentando a preocupação com a resistência a antibióticos. Portanto, os objetivos do presente estudo foram (*i*) realizar uma revisão sistemática para determinar o perfil de resistência antimicrobiana de *E. coli* patogênica isolada do trato intestinal de bezerros em todo o mundo e (*ii*) determinar o perfil de suscetibilidade antimicrobiana de cepas de *E. coli* patogênicas isoladas de bezerros e bezerros bubalinos de 1990 a 2013 em Minas Gerais, Brasil, bem como a frequência de cepas O157 e cepas portadoras de genes de beta-lactamases de espectro estendido (ESBL) e resistência à colistina móvel (*mcr*). A revisão sistemática recuperou 932 artigos e finalizou com 56 estudos, publicados entre 1982 e 2020, que testaram a suscetibilidade antimicrobiana entre *E. coli* patogênica principalmente ao método de difusão em disco (82.14%) em estudos transversais (58.92%). No geral, altas taxas de resistência às principais classes de antimicrobianos utilizadas no tratamento de infecções gastrointestinais causadas por cepas de *E. coli* foram observadas entre os estudos selecionados. Da mesma forma, entre as cepas virulentas de *E. coli* isoladas de bezerros e búfalos em Minas Gerais, de 1990 a 2013, foram observadas altas taxas de resistência à penicilina, tetraciclinas e inibidores de folato, além de uma taxa alarmante de multirresistência e cepas capazes de produzir ESBL. Em conjunto, nossos resultados apontam para a necessidade de monitoramento da resistência antimicrobiana entre cepas de *E. coli* de origem animal, o que deve ser desenvolvido na perspectiva de Uma Saúde, por meio de políticas de prevenção e controle de patógenos.

Palavras chaves: Patogênica. Virulência. Multirresistência.

General Introduction

Antimicrobial resistance (AMR) is one of the greatest challenges of the modern world and addressing this growing threat requires a multisectorial approach, not only to human health, but also to the animal and environment health (TACCONELLI et al., 2019). The antimicrobial resistance is a natural process, but it can be as accelerated by abuse and misuse of medicines in humans and animals. Animal production systems aligned with the unproper use of antimicrobials have been identified as one of the main responsible for the emergence of AMR bacteria (USE et al., 2017).

Among the enteric diseases in animals, diarrhea caused by *E. coli* is one of the most frequent, associated with different pathotypes causing high losses in animal production systems and different zoonotic potential (ANDRADE et al., 2012; CHO et al., 2014). Plasticity, in line with its ability to adapt to constantly changing environments, allows *E. coli* to acquire a large number of AMR mechanisms.

In this context, some *E. coli* pathotypes are particularly important in the pathogenesis of diarrhea in calves, such as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC) and necrotoxigenic *E. coli* (NTEC) (Coura et al., 2014). In addition to their importance for animal health, some *E. coli* pathotypes, such as STEC and EHEC, can also be transmitted to humans through direct contact with animals or through food products (JOSEPH et al., 2020). Furthermore, it can also cause urinary tract infections (cystitis), neonatal meningitis, or septicemia in human beings (ARSHAD et al., 2015).

Given the current scenario, new approaches for the characterization *E. coli* strains and monitoring of its antimicrobial resistance profiles must be developed from the perspective of One Health, aiming the protection of public health through policies for the prevention and control of this pathogen in animal populations at the interface between humans, animals and the environment. Therefore, the objectives of chapter one is to conduct a systematic review to assess ADR among pathogenic *E. coli* isolated from the intestinal tract of calves worldwide and chapter two was to determine the antimicrobial susceptibility profile of pathogenic strains of *E. coli* isolated from calves and buffalo

calves from 1990 to 2013 in Minas Gerais, Brazil, as well as the frequency of O157 strains and strains carrying beta-lactamase spectrum (ESBL) and mobile colistin resistance genes (*mcr*).

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0095-5108.

CHAPTER 1

Formatted according to the submission guidelines of Microbial Pathogenesis

Systematic review on antimicrobial resistance in pathogenic *Escherichia coli* isolated from calves

Dircéia Aparecida da Costa Custódio[a], Amanda Carvalho Rosado Ferreira[a], Maysa Serpa Gonçalves[a], Anna Cecília Trolesi Reis Borges Costa[a], Carine Rodrigues Pereira[a], Fernanda Morcatti Coura[b], Andrey Pereira Lage[c], Geraldo Márcio da Costa[a], Elaine Maria Seles Dorneles[a*]

[a] Departamento de Medicina Veterinária, Faculdade de Zootecnia e Medicina Veterinária, Universidade Federal de Lavras

[b] Departamento de Ciências Agrárias do Instituto Federal de Minas Gerais - Campus Bambuí, Minas Gerais, Brazil

[c]Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

*Corresponding author: elaine.dorneles@ufla.br. Postal address: Departamento de Medicina Veterinária, Faculdade de Zootecnia e Medicina Veterinária, Universidade Federal de Lavras, Campus Universitário S/N, Caixa Postal 3037, Lavras, MG, Brazil. Postal Code: 37200-900.

Highlights

High levels of resistance to several classes of antimicrobials among gastrointestinal infections from calves

Diversity of antimicrobials and potential risks to animal and public health.

E. coli pathotypes involved in gastrointestinal infections in calves.

Abstract

The present study aimed to perform a systematic review to determine the antimicrobial resistance profile of pathogenic *Escherichia coli* strains isolated from intestinal tract of calves worldwide. For this, six databases were searched (CABI, Cochrane, Pubmed, Scielo, Scopus and Web of Science), without restriction when the studies were published. The search recovered 932 papers and ended up with 56 studies, published between 1982 and 2020, after selection based on title, abstract and full text. The technique most used to determine susceptibility to antimicrobials was the disk diffusion test [82.14% (46/56)], followed by MIC [17.85% (10/56)]. Only two studies [3.57% (2/56)] performed both tests (disk diffusion and MIC). For the disk diffusion tests, seventy-nine different antimicrobial drugs of seventeen classes were tested. Regarding, broth diffusion tests to asses minimal inhibitory concentration (MIC), fifteen different classes were tested with a total of sixty-one antimicrobial drugs. Cephalosporins was the most tested antimicrobial class, both in disk diffusion and MIC methods. Antimicrobials classes with highest resistance levels were observed for tetracyclines, penicillin's, folate inhibitors, aminoglycosides, phenicol's and quinolones. Due the heterogeneity and low quality of the studies, mainly regarding antimicrobial susceptibility test methodology, it was not possible to perform metanalyses. These findings indicate the importance to carry out studies based on well-designed analyzes in order to understand the real emergence and spread of pathogenic and resistant strains.

Keywords: Epidemiology. *Enterobacteriaceae. B*ovine. Antimicrobial Susceptibility. Enteropathogenic.

1. Introduction

Diarrheagenic *Escherichia coli* are responsible for important economic losses in cattle, causing reduced animal weight gain, animal mortality and high drug costs [1]. In this context, some *E. coli* pathotypes are particularly important in the pathogenesis of diarrhea in calves, such as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC) and necrotoxigenic *E. coli* (NTEC) [2]. The classification of *E. coli* in pathotypes is based on their attributes of virulence, pathogenesis and clinical signs present in the host, having different potential to cause disease [3-5].

In addition to its animal health significance, some *E. coli* pathotypes, such as STEC and EHEC, can also be transmitted through food products to humans, being considered a public health issue [6]. Cattle are the main reservoirs for these pathotypes, since they can shed the pathogen in their feces, leading to contamination of the environment, food and water [2]. Data from the CDC [7] showed a significant number of 6,034 infections by Shiga toxin-producing *E. coli* (STEC), including 2,363 infections by O157, evidencing the alarming public health concern that this pathotype represent. Furthermore, some patients with STEC/EHEC infection develop hemolytic uremic syndrome (HUS), a serious complication associated, especially with serotype O157, characterized by renal failure, hemolytic anemia and thrombocytopenia that can be fatal [8, 9].

Other important human health concern associated with *E. coli* infections from animal origin are the worrisome and increasing antimicrobial resistance (AMR), one of the greatest challenges of the 21st century [10, 11]. The dissemination of AMR is a global problem and a One Health priority, as new forms of resistance can emerge and spread rapidly across continents through people, animals, and environments [11, 12]. In addition, according to World Health Organization (WHO) most of antimicrobial drugs are inappropriately prescribed, and most countries do not implement basic policies to promote the rational use of medicines in animal and human health [13].

Given that, the present study aimed to carry out a systematic review to assess the AMR among pathogenic *E. coli* isolated from intestinal tract of calves worldwide, in order to support decisions on public policies for animal and human health and to diagnose the current scenario of drug resistance in this important pathogen.

2. Material and methods

In the present review, the guidelines of the PRISMA (Preferred Reported Items for Systematic Reviews and Meta-Analyses) were adopted (Appendix S1) [14].

2.1 Search strategy

The search was carried out on May 06th 2020 in the following databases: CABI, Cochrane, PubMed, Scielo, Scopus and Web of Science. It was carried out based on the following keywords searched within all the sections from papers (title, abstract and full text): (bovine* OR cattle OR calve* OR calf OR heifer* OR cow* OR herd* OR farm*) AND (pathogenic* OR pathotypes* OR virulence genes* OR virulence factors* OR virulence*) AND (*Escherichia coli*) AND (antimicrobial OR antibiotic OR resistan* OR susceptibility OR minimal inhibitory concentration OR MIC OR disk diffusion OR resistance gene* OR antimicrobial resistance genes OR drug resistan*) AND (intestinal tract OR diarrhea) without restrictions regarding the time when the studies were published. Details on search terms used are described in Appendix S2.

The records retrieved were imported into EndNote X7.8 (Thomson Reuters, USA) and the duplicates were removed.

2.2 Selection strategy

In the initial stage of selection, the studies were selected based on their titles by two reviewers (DACC and ACRF). Right after, the two reviewers (DACC and ACRF) independently evaluated each abstract. Then, the full text of the articles selected based on the abstract were screened in terms of its

relevance and through inclusion/exclusion criteria. When the two reviewers disagreed in any stage, a third reviewer (EMSD) was responsible for the final decision.

2.3 Inclusion and exclusion criteria

Selected articles should be focused on assessment of antimicrobial susceptibility by means of in vitro tests of pathogenic *E. coli* isolated from intestinal tract or feces of calves. Articles written in languages other than English, Spanish, French and Portuguese, as well as those with full text not available or that were no original research papers (proceedings, thesis, abstract, book chapter and reviews) were excluded. Full inclusion and exclusion criteria were described in Appendix S3.

2.4 Quality assessment

Evaluation of the quality of the papers included by eligibility was carried out by two authors (DACC and EMSD) based on the following criteria: (*i*) antimicrobial susceptibility test used (disk diffusion or minimal inhibitory concentration - MIC); (*ii*) the use of reference standards for performance and interpretation of antimicrobial susceptibility tests [15-17] (*iii*) information on the concentration of tested antimicrobials (disk concentration or MIC range); (*vi*) information on the breakpoint or halo diameter for the classification of the strains as resistant or susceptible; (*v*) use of quality control strains in the assays. All criteria were evaluated qualitatively and quantitatively, with the same weight.

2.5 Data extraction

Data extraction was performed by one of the reviewers (DACC) and then checked for accuracy by another reviewer (EMSD). Extracted data included: first author, geographic location where the study was performed, year of bacterial isolation, target population, type of study, type of livestock production (when available), type of clinical sample, number of clinical samples (when available), number of animals (when available), age of animals (when available), number/frequency of positive animals (when available), frequency of diarrhea (when available), number of bacterial isolates, number of pathogenic isolates, diagnostic method used (culture and isolation, biochemical

test, PCR), genotypes of resistance (when available), antimicrobial susceptibility test(s) used (method, standard reference, quality control, antimicrobial concentration, etc.) and pathogenicity assessment method used (phenotypic or genotypic).

2.6 Statistical analysis

Data extracted from the selected papers was imported into R statistical software version 4.2.1 [18] and a descriptive analysis was performed. The figures were performed using ggplot2 [19] and cyclize packages [20]. Numerical variables were analyzed by calculating the mean, standard deviation, median and interquartile range (IQR), whereas categorical variables were examined by frequency distributions.

3. Results

3.1 General characteristics of studies included by eligibility

The initial search identified 932 articles. After removing 199 duplicates, 733 papers remained. Of these, 619 articles were excluded after screening of their title and abstract, lasting 114 articles, from which 13 records were not retrieved. Therefore, a total of 101 articles were screened by full text, from which 45 were excluded based on reading the full text, leaving 56 articles that were included in the study by eligibility and subject to evaluation of quality criteria (Fig.1 and Appendix S4).

The temporal and geographical distribution of the articles selected in the present study is shown in Figure 2 (A and B). Most of papers included by eligibility were published in 2019 [17.85% (10/56)], followed by 8.97% (5/56) in 2017, 7.14% (4/56) in 2006, 2012, 2014 and 2015, 5.35% (3/56) in 2011, and 3.57% (2/56) in 2005, 2008 and 2018. In some years (1982, 1988, 1989, 1996, 1999, 2000, 2001, 2002, 2004, 2010, 2013, 2016 and 2020) only one study (1.78%) on antimicrobial resistant *E. coli* from calves was selected.

Regarding the geographical distribution of the papers, most of the studies were published in India [16.07% (9/56)], followed by Egypt [10.71% (6/56)], USA and Spain [8.92% (5/56) each],

Brazil [7.14% (4/56)], China, France, Italy and South Africa [5.35% (3/56) each], and Argentina, Iran and Turkey [3.57% (2/56) each]. Countries with only one study published were [1.78% (1/56)] Bangladesh, Belgium, Canada, Chile, Pakistan, Sweden, Tanzania and Uruguay. One [1.78% (1/56)] of the selected studies did not inform where the study was carried out (Khalifa et al., 2019) (Fig. 2A).

Among the selected papers, 83.92% (47/56) of the studies isolated pathogenic *E. coli* strains from intestinal tract of calves and 16.07% (9/56) of buffalo calves. For sampling, 78.57% (44/56) performed the isolations from stool samples, 14.28% (8/56) from rectal swab and 1.78% (1/56) from intestinal content, whereas three studies (5.36%) did not report the clinical sample used although they state that the strains were isolated from diarrheic calves. Regarding the type of livestock production, 41.07% (23/56) of the studies were conducted in dairy farms, 3.35% (3/56) in dairy/beef farms and 53.57% (30/56) did not inform the type of production sampled. Most of the papers adopted the cross-sectional study design [58.92% (33/56)], while 5.35% (3/56) were case-control studies and 35.71% (20/58) had no study design.

The frequency of diarrhea among the sampled animals ranged from 0 to 100%, however 14.28% (8/56) of the studies had no information on diarrhea. The number of clinical samples tested per study ranged from 4 to 824, with mean 212.55 (±199.94) and median 118.50 (IQR 247) (14 articles did not inform the number of tested samples). The number of tested animals varied from 16 to 600, with mean 165.19 (± 157.31) and median 107.5 (IQR 114.14). This information was not available in 42.86% (24/56) of the selected papers. Among the selected papers, the number of isolates ranged from 1 to 700, with mean 133.04 (± 152.25) and median 87 (IQR 175.25), nevertheless, the number of isolates considered pathogenic (positive for at least one of the tested virulence factors – based on phenotypic or genotypic tests) ranged from 1 to 419, with mean 45.10 (± 66.43) and median 18 (IQR 55). *E. coli* isolates were confirmed by a species-specific PCR (Polymerase Chain Reaction) in 26.78% (15/56) of the studies, while the other used only biochemical tests for species identification.

The main characteristics of the studies are summarized in Fig. 3 and will be further detailed in the following sections.

3.2 Determination of virulence in *E. coli* strains

The virulence of *E. coli* isolates was evaluated by PCR amplification of virulence genes in 82.14% (46/56) of the selected papers (Table 1), whereas 23.21% (13/56) of the studies assessed the pathogenicity of the strains by different phenotypical assays (Table 2). The virulence genes assessed and the frequency of studies in which they were observed are shown in Table 1. The main virulence genes investigated in the articles were: Shiga toxins (*stx, stx1, stx2, stx2a, stxb, stxc, stxd, stx2e* and *stx2g*) [71.74% (33/46)], intimin (*eae* and *eaeA*) [60.86% (28/46)], fimbrial adhesins (*F4, F5, F6, F17, F17c, F17g, F17f, F18* and *F41*) [26.09% (12/46)], thermolabile enterotoxins (*IntII_LT*, *elt*, and *eltA*) [23.91% (11/46)], hemolysins (*hlyA* and *hlyF*) [21.74% (10/46)], thermostable enterotoxins (*st, sta,* and *stb*) [21.74% (10/46), enterohemolysin (*ehxA*, *ehlyA*, and *ehly*) [17.39% (8/46)], necrotizing factor (*cnf1* and *cnf2*) [15.22% (7/46)], cytolethal distending toxins (*cdt, cdtb*, and *cdtIII*) [8.70% (4/46)] and verocytotoxin (*vt, vt2e, vtx*, and *vtx2*) [4.35% (2/46)].

Regarding serotyping, 62.5% (35/56) of the studies determined serogroups, 80.0% (28/35) performed serotyping by serum agglutination tests and 20% (7/35) identified serogroups by PCR, while 37.5% (21 /56) did not inform the methodology used.

3.3 Main characteristics of the antimicrobial susceptibility tests used

Among all the studies selected, the technique most used to determine susceptibility to antimicrobials was the disk diffusion test [82.14% (46/56)], followed by MIC [17.85% (10/56)]. Only two studies [3.57% (2/56)] performed both tests (disk diffusion and MIC). The E-test was not used by any of the selected studies.

Regarding procedure, 57.14% (32/56) of the studies followed the methodology and interpretation parameters described by the CLSI or Eucast, 23.2 % (13/56) followed the parameters proposed by Bauer et al. (1966), whereas 12.58% (7/56) followed other references and four studies [7.14% (4/56)] did not inform the adopted reference. Most of the studies did not present quality controls (QC) [69.64% (39/56)], which was informed only by 30.35% (17/56) of studies. Regarding

concentration of antimicrobials, most studies reported the concentration used [64.28% (36/56)] and 35.71% (20/56) did not provide this information (Appendix S5).

3.4 Antimicrobials classes and drugs used in disk diffusion tests

Among the studies selected in this systematic review, forty-six (82.14%) used the disk diffusion method to assess antimicrobial susceptibility of *E. coli* strains. The percentages of studies that tested and observed resistance to different antimicrobial classes and drugs, as well as the concentrations used, are shown in Table 3.

A total of seventeen classes were tested, totalizing seventy-nine different antimicrobials drugs. The class with more representatives was cephalosporins, with sixteen different antimicrobials tested drugs [20.25% (16/79)], followed by penicillins [18.99% (15/79)], aminoglycosides and folate inhibitors [10.13% (8/79) each], fluoroquinolones [7.59% (6/79)], quinolones [6.33% (5/79)], macrolides [5.06% (4/79)], tetracyclines and lincosamides [3.80% (3/79) each], carbapenems, phenicols, and polymyxins [2.53% (2/79)], and aminocoumarins, fosfomycins, macrocyclic, monobactam, and nitrofurans [1.27% (1/79)].

Among the cephalosporins, the most tested antimicrobial within the class was cefotaxime, in 23.91% of the studies (11/46), followed by cephalothin [17.39% (8/46)]; ceftazidime [15.21% (7/46)]; cefepime, ceftiofur and cefuroxime [23.91% (11/46) each]; ceftriaxone [8.69% (4/46)]; cefalexin, cefoxitin and cefaclor [6.52% (3/46)]; each]; and cefaloridine, cefazolin, cefetrizole, cefixime, cefoperazone, cephalonium [2.17% (1/46) each].

Regarding penicillins, ampicillin was the main antimicrobial tested, present in 60.86% (28/46) studies, followed by amoxicillin [26.08% (12/46)], amoxicillin/clavulanic acid [23.91% (11/46)], penicillin G [10.86% (5/46)], cloxacillin, oxacillin and piperacilline/tazobactam [4.34% (2/46) each], and amdinocillin, amoxiclav, ampicillin/sulbactam, mezlocillin, tazobactam, temocillin, ticarcillin, and ticarcillin/clavulanate acid [2.17% (1/46) each].

For the aminoglycosides, gentamicin was the most frequent [58.69% (27/46)], followed by streptomycin [52.17% (24/46], kanamycin [36.95% (17/46)], amikacin [30.43% (14/46)], neomycin [26.08% (12/46)], spectinomycin [6.52% (3/46)], apramycin [4.34% (2/46)], spectinomycin [6.52% (3/46)] and tobramycin [2.17% (1/46)]. Sulfamethoxazole/trimethoprim was the more frequent among folate inhibitors [50.0% (23/46)], followed by cotrimoxazole [13.04% (6/46)], trimethoprim [10.86% (5/46)]; sulfamethoxazole and sulfonamides [8.69% (4/46) each], and sulfadiazine, sulfaprim, and trimethoprim/sulfadiazine [2.17% (1/46) each].

Enrofloxacin was the main antimicrobial tested among fluoroquinolones, present in 43.18% (19/44) studies. Besides enrofloxacin, other five fluoroquinolones was tested, all present only in one study: danofloxacin, flumequine, marbocyl, marbofloxacin, and ofloxacin [2.17% (1/46) each]. Quinolones were represented by five antimicrobials, being nalidixic acid the most tested [34.78% (16/46)], followed by ciprofloxacin [28.26% (13/46)], norfloxacin [23.91% (11/46)], and levofloxacin and pefloxacin [2.17% (1/46) each]. Among the macrolides, erythromycin was the most tested [13.04% (6/46)], while espiramycin, thiomicosin, and tilosin were observed just in one study each [2.17% (1/46)].

Regarding lincosamides, lincomycin was tested in two studies [4.34% (2/46)], and clindamycin and lincospectin were tested in only one each [2.17% (1/46)]. Tetracycline was the main antimicrobial tested among tetracyclines, present in 67.39% (31/46) of the studies, while doxycycline and oxytetracycline was observed in only one [2.17% (1/46)]. Carbapens was represented by imipenem and meropenem [8.69% (4/46) each]. About phenicols, chloramphenicol was tested in 45.65% (21/46) of the studies, while florfenicol was tested in 13.04% (6/46). Representing the polymyxins, colistin was present in 15.21% (7/46) of the studies, while polymyxin B was present in only one [2.17% (1/46)].

Finally, the following classes were represented by only one antimicrobial each: aminocoumarin, with novobiocin tested in one study [2.17% (1/44)]; fosfomycins, with fosfomycin also in one study [2.17% (1/46)]; macrocyclic, represented by rifampicin in two studies [4.34%

(2/46)]; monobactam, with aztreonam, in four studies [8.69% (4/46)]; and nitrofurans, represented by nitrofurantoin in 10.86% (5/46) of the studies.

3.5 Antimicrobials classes and drugs used in minimal inhibitory concentration (MIC)

Only 17.85% (10/56) of the studies used MIC to assess antimicrobial susceptibility of *E. coli* strains and the percentages of the studies that tested each class and antimicrobial drug, as well as the concentration ranges, are shown in Table 4.

Fifteen different classes were tested representing a total of sixty-one antimicrobials drugs. Cephalosporins was the class with more representatives [16.39% (10/61)], followed by penicillins [14.75% (9/61)], aminoglycosides [13.11% (8/61)], quinolones and folate inhibitors [9.84% (6/61)], macrolides [8.20% (5/61)], carbapenems, phenicols, and tetracyclines [4.92% (3/61)], nitrofurans and polymixins [3.28% (2/61)], and lincosamides, manobactam, and glicyclines [1.64% (1/61)]. Among cephalosporins, ceftiofur was the main antimicrobial tested, present in seven studies [70.0% (7/10)], followed by ceftriaxone and cefoxitin, in two studies each [20.0% (2/10)], and cephazolin, cephalotin, cefuroxime, cefotaxime, cefquimone, cefepime, and ceftazidime in one study each [10.0% (1/10)]. Ampicillin was the main antimicrobial teste among penicillins, present in ten studies [100.0% (10/10)]. Besides ampicillin, other eight penicillins were tested in one study each: co-amoxiclav, amoxicillin/clavulanate acid, ampicillin/sulbactam, penicillin, piperacillin, piperacillin/tazobactam, ticarcillin, ticarcillin/clavulanic acid [10.0% (1/10)].

Regarding aminoglycosides, kanamycin was the most tested antimicrobial, present in 100% (10/10) of the studies, followed by gentamycin in 80.0% (8/10), streptomycin in 70.0% (7/10), neomycin in 30.0% (3/10), and apramycin, amikacin, spectinomycin and tobramycin in one study each [10.0% (1/10). Quinolones were represented by nalidixic acid in four 40.0% (4/10), ciprofloxacin in 30.0% (3/10) of the studies, enrofloxacin in 20.0% (2/10), enoxacin, danofloxacin, moxifloxacin and oxolinic acid in 10.0% (1/10). About folate inhibitors,

sulfamethoxazole/trimethoprim, sulphonamide, and trimethoprim were present in three studies each [30.0% (3/10)], while sulfadimethoxine was present in two [20.0% (2/10)], and sulfametoxazol, and sulfisoxazol were present in only one study each 10.0% (1/10). Carbapens were represented by imipenem, ertapenem, and meropenem present in one study each [10.0% (1/10)].

For the phenicols, chloramphenicol and florfenicol were tested in five studies [50.0% (5/10)]. Tetracycline was the main antimicrobial tested among tetracyclines [50.0% (5/10)], while chlortetracycline and oxytetracycline were tested in only one study each [10.0% (1/10)]. Nitrofurantoin and nitrofurazone, belonging to nitrofurans class, were present in one study each [10.0% (1/10)], while polymyxins were represented by polymyxin B and colistin, both present in one study each as well [10.0% (1/10)]. Lincosamides, glicyclines, and monobactam classes were represented by one antimicrobial each: clindamycin [10.0% (1/10)], tigecycline [10.0% (1/10)], and aztreonam [20.0% (2/10)], respectively.

3.6 Resistance genotypes prospected by PCR

Seventeen studies [30.36% (17/56)] assessed antimicrobial resistance genes in virulent *E. coli* strains isolated from intestinal tract of calves. The genes evaluated are associated with resistance against to aminocoumarins, aminoglycosides, carbapenems, cephalosporins, cephamycins, diaminopyrimidines, macrolides, monobactam-cephalosporins, monobactams, phenicols, quinolones, quinolones-macrolides, sulfonamides-macrolides-cephalosporins, sulfonamides, and tetracyclines.

The aminoglycosides were the class with the highest number of different genes associated with resistance researched in the selected articles, with 17 genes; followed by the class of quinolones with twelve genes researched and tetracyclines with eight genes. The classes with less evaluated genes were aminocoumarinium, carbapenems, cephamycin with one or two assessed genes each. Detailed information about the researched genes by antimicrobial class, as well as those identified in the selected articles, are shown in the Table 5.

The number of studies that used phenotypic and molecular methods to assess virulence and resistance in *E. coli* strains isolated from intestinal tract or feces of calves is shown in Figure 4.

4. Discussion

The present review aimed to provide reliable data on the situation of the AMR among pathogenic *E. coli* from calves around the world. However, the analysis of the selected studies showed important gaps in the information regarding the methodology used for antimicrobial susceptibility tests, such as the no use of quality control strains in the assays, no information on the tested antimicrobial concentration, as well as poor report on the breakpoints/halo diameter criteria used to classify strains as resistant or susceptible to antimicrobials. The absence of these critical information difficult to guarantee the reliability of the results observed in some studies, in addition to preclude the performance of more robust analysis (meta-analysis) on these data and thereby the drawing of strong inferences, since key data is missing.

However, despite the lack of important data in some papers included, this systematic review judiciously analyzed the selected studies using several criteria of eligibility, which allowed to generate information of great relevance to the proposed subject, even that the assessment on the exact frequency of isolates resistant or susceptible to antimicrobials could not be performed. In general, the results of the selected studies demonstrated high resistance rates to the main classes of antimicrobials recommended for the treatment of gastrointestinal infections in calves, such as tetracyclines, penicillin, folate inhibitors (sulfamethoxazole-trimethoprim), aminoglycosides, phenicol and quinolones. Not coincidentally, these antimicrobial classes, especially tetracyclines, penicillin, folate inhibitors, macrolides and aminoglycosides, are among the most used in food producing animals in United States [21] and European Union [22]. Therefore, it is undeniable that the alarming rates of AMR observed in the present study, as well as by elsewhere [23, 24], among pathogens from animal origin are strongly related to the use of drugs of medical importance in food producing animals.

Furthermore, this intense use antimicrobials in animals intended for human consumption can also lead another concerning associated to their residues or metabolites in meat, milk and eggs [25, 26]

In fact, among the seventeen antimicrobial classes tested by disk-diffusion method, including 79 different bases, only for six drugs resistance was not observed, while for 11 information on AMR was not available. Likewise, among the studies that performed MIC, 15 different classes were tested, representing a total of 61 different antimicrobial drugs, from which only 7 did not exhibit AMR, while for 16 information was not available. These findings, especially considering the great diversity of drugs and concentrations assessed, emphasize the disturbing situation of the AMR among zoonotic pathogens from animal origin, evidencing their potential risks for animal and public health. Disk diffusion was the most used technique among the selected studies, probably due to the fact that this technique is less expensive and laborious compared to the MIC method. However, a negative point of this method is that it only provides qualitative information (resistant, intermediate and susceptible), whereas by using the MIC, it possible to obtain qualitative and quantitative results (being possible to determine the lowest concentration of the antimicrobial that will be able to inhibit bacterial growth).

Furthermore, our findings also suggest a difficulty in treating these infections caused by *E. coli*, which can be even worse taking into account that all studies included in the systematic review tested only strains that exhibited at least one virulence factor (not commensal bacteria which does not need to be treated with antimicrobials). Several different *E. coli* pathotypes with different potential to cause disease in animals and humans were investigated among selected studies, being STEC (Shiga toxin) the most searched, probably because of cattle is the main reservoirs for this pathotype and due its clinical importance in humans [2, 27]. In addition to the consequences on public health, several *E. coli* pathotypes identified in the selected papers are also involved in gastrointestinal infections in calves, being important causes of diarrhea and economical losses for animal production worldwide [5].

Regarding the spatial and temporal distribution of the papers included in the systematic review, they were published in the last 23 years, being India the country with the highest number of publications, followed by Egypt, USA, Spain and Brazil. India has the largest cattle herd in the world and is considered the epicenter of the global antimicrobial resistance crisis, with unprecedented consumption and inadequate production of antimicrobials, which can explain the first position among the selected papers [27, 28]. The spatial distribution of the studies point to a global interest in AMR in pathogenic *E. coli* from calves, revealing a important participation of countries that are central players in livestock production, as importers or exporters (Figure 2B). On the other hand, the temporal distribution of the selected papers shows a more recent concentration of studies on AMR among *E. coli*, which can be justified by the also recent global increase in AMR among bacteria of medical importance [29] In fact, the intensification of animal production, the large food trade in general among countries can contribute to the spread of various forms of resistance [30]. In this sense, our results also showed that the different antimicrobial resistance genes (ARG) were identified among the pathogenic *E. coli* strains from calves in the selected studies, which is an important issue, considering the ability of *E. coli* to exchange genetic material with numerous other bacteria, including microorganisms from normal microbiota [31]. In this context, ARG can be transmitted to humans or other animals (wildlife and domestic) through the contamination of different environments, representing a One Health risk [13].

A limitation of this study was the inability to carry out an assessment of the exact frequency of antimicrobial resistant or susceptible isolates (meta-analysis), due to heterogeneity among studies, in addition to the lack or poor description of crucial information regard AMR, which prevented a more robust analysis of the results. These findings highlight the importance of adopting a judicious methodology in scientific research, in order to guarantee the reliability of the study and the full use of the data generated.

5. Conclusion

This systematic review observed great heterogeneity in the criteria used by the studies to assess the AMR on pathogenic *E. coli* isolated from calves worldwide, revealing a low methodological quality in most of the selected papers. Nonetheless, despite that our results showed a high prevalence of AMR among the main classes used in the treatment of gastrointestinal infections caused by *E. coli*, especially tetracyclines, penicillin, folate inhibitors, macrolides and aminoglycosides, besides a great pathogenic potential of the strains analyzed considering the virulence profiles and ARG observed.

Conflict of Interest Statement

The authors declare no conflict of interest

Acknowledgements

The EMSD laboratory was supported by Fundação de Amparo à Pesquisa de Minas Gerais (Fapemig), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes). DACC, ACRP and CRP are thankful to Capes for their fellowships. EMSD is also grateful to CNPq for her fellowship.

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Figures

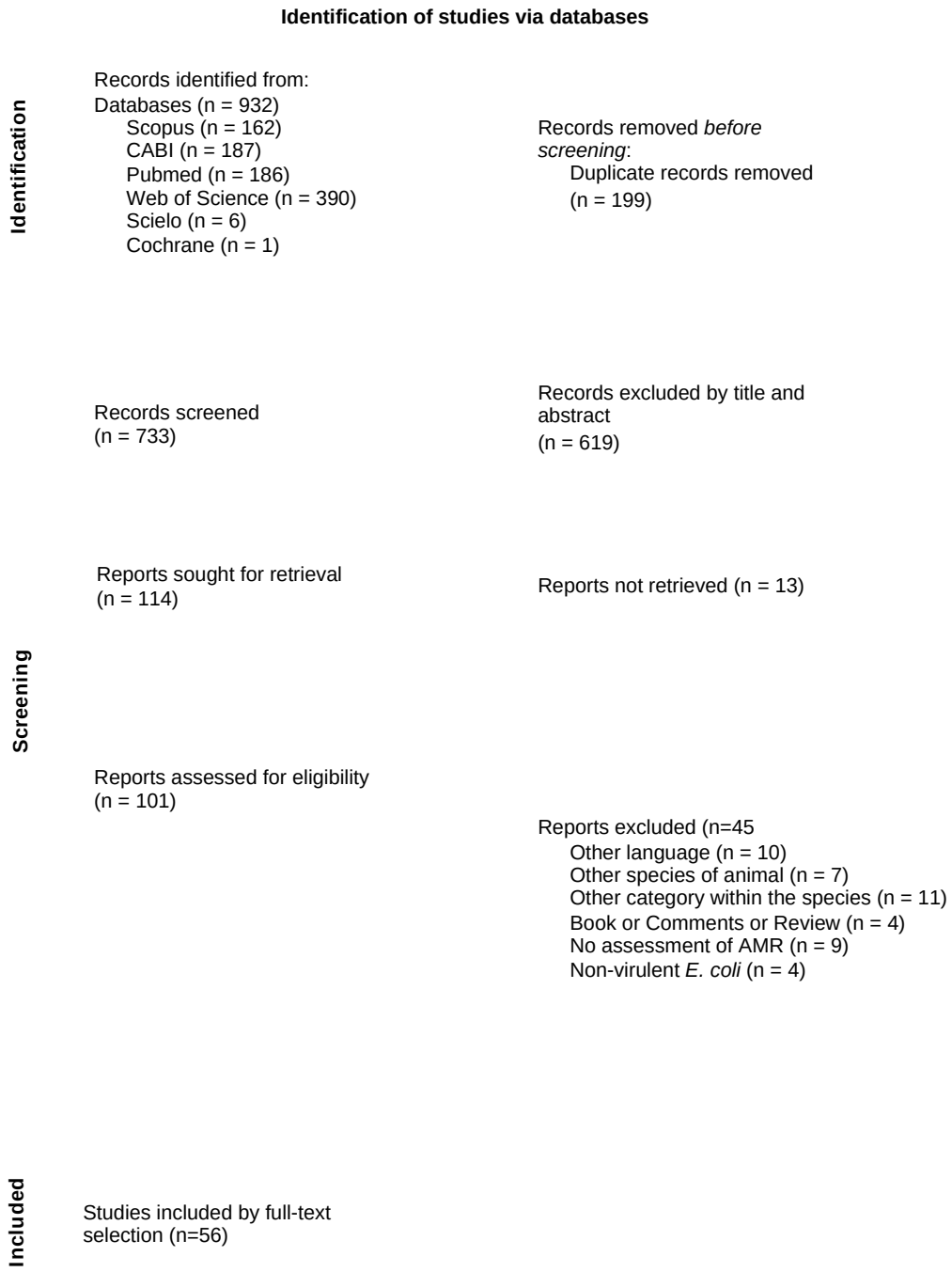

Fig. 1 – PRISMA flow diagram of selected studies by this systematic review on *Escherichia coli* isolated from intestinal tract of calves and buffalo calves, published between 1982 and 2020.

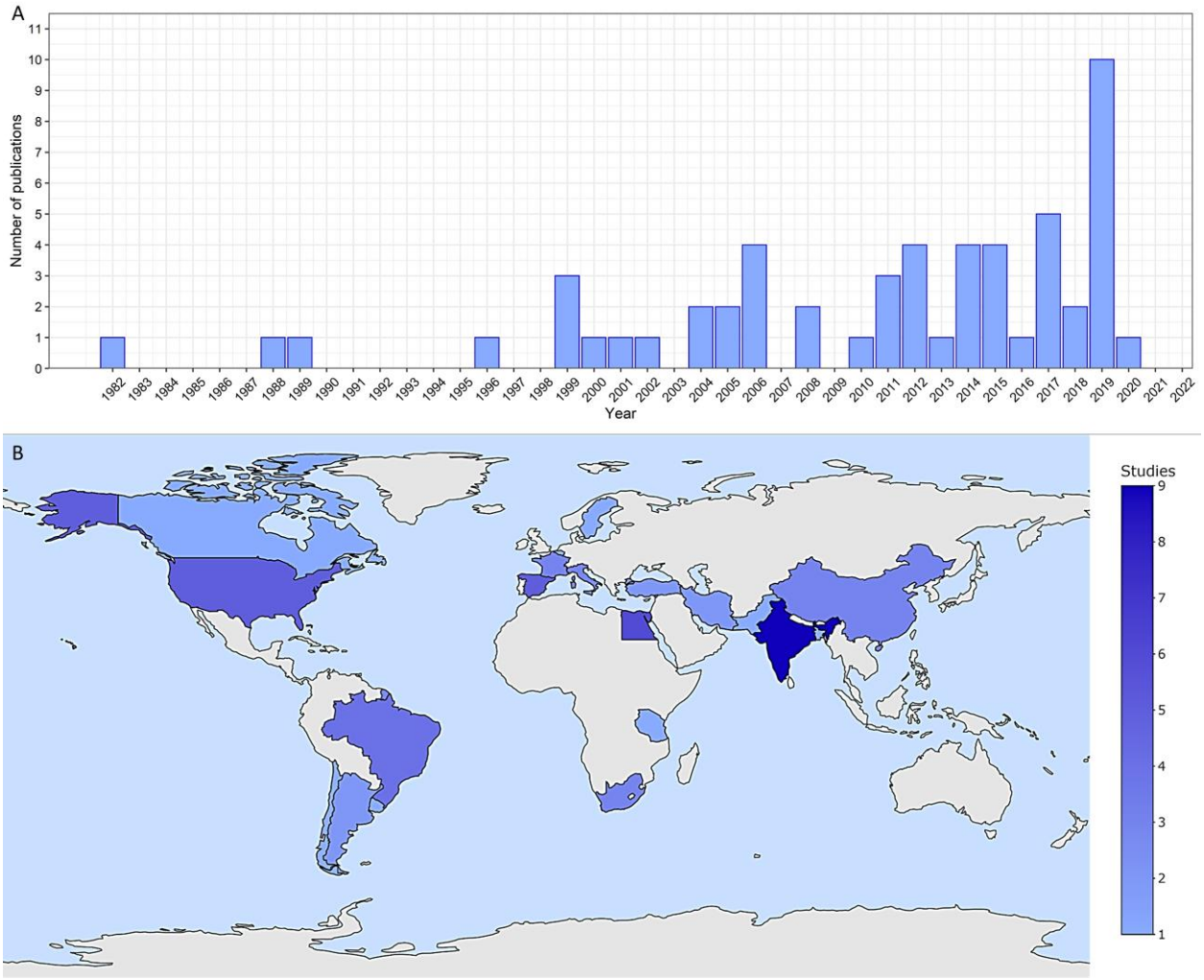

Fig. 2 – Temporal and geographical distribution of the selected articles. (A) Distribution of the articles included by eligibility according to the year of publication. (B) Distribution of the articles included by eligibility according to the country where the study was performed.

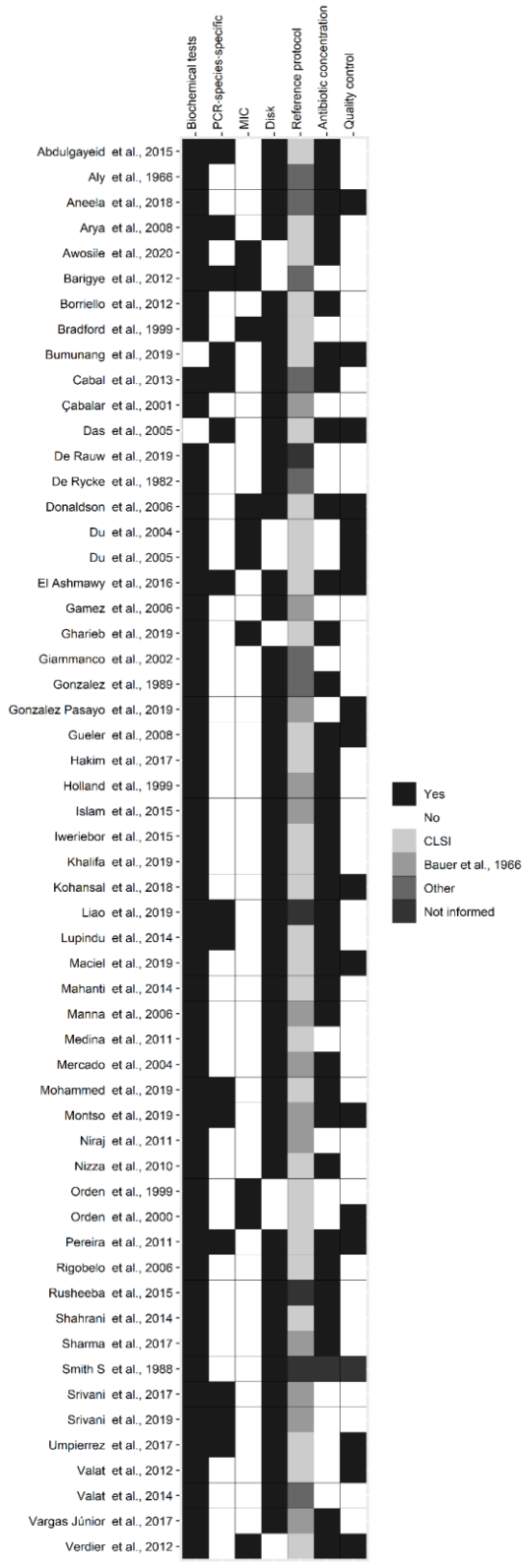

Fig 3. Analysis of the main characteristics of the studies selected by this systematic review on *Escherichia coli* isolated from intestinal tract of calves and buffalo calves, published between 1982 and 2020.

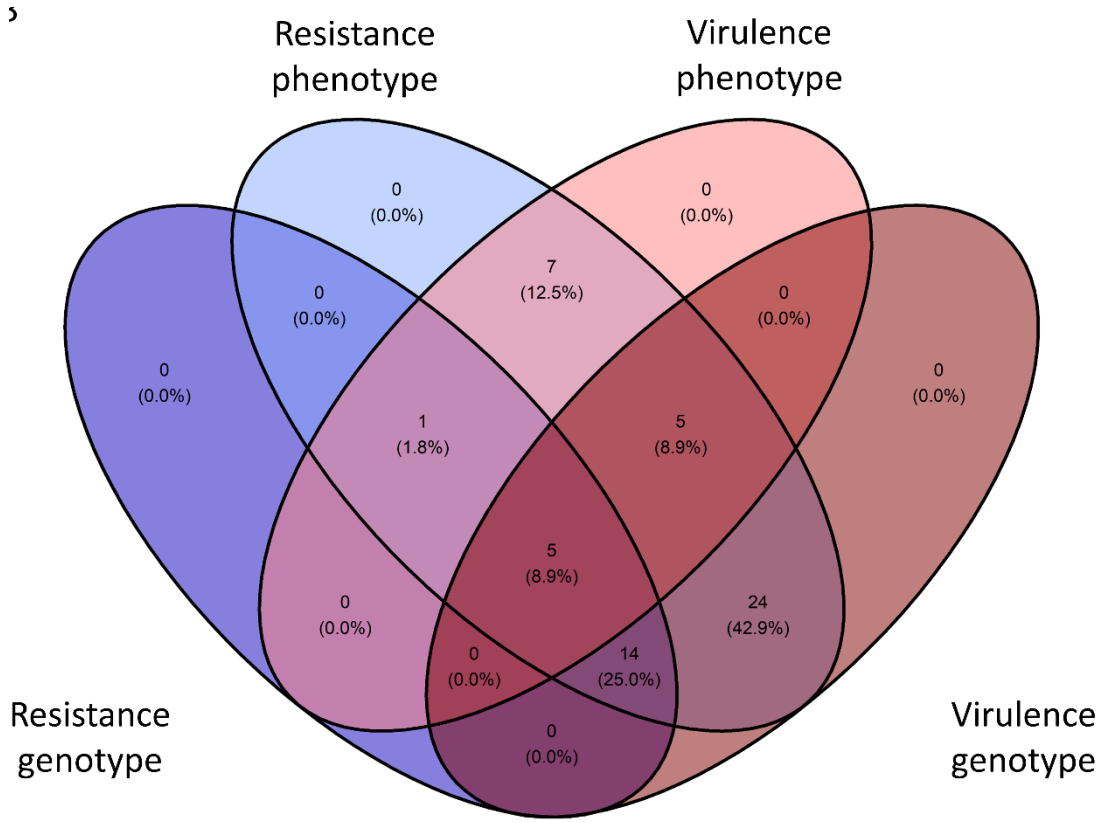

Fig 4. Distribution of studies according to performance of resistance and virulence genotyping and phenotyping tests, selected by this systematic review on *Escherichia coli* isolated from intestinal tract of calves and buffalo calves, published between 1982 and 2020.

Tables

Table 1 – Frequency of prospection and identification of virulence mechanisms in studies selected by this systematic review on *Escherichia coli* isolated from intestinal tract of calves and buffalo calves, published between 1982 and 2020.

Gene/target	Virulence mechanism	N of studies (%)	Identified
Afa	Afimbrial adhesin	1/46 (2.17)	Yes
Air	Autotransporter adhesin	1/46 (2.17)	Yes
aggR	Adherence transcriptional regulator	1/46 (2.17)	Yes
astA	Thermostable cytotonic enterotoxin	5/46 (10.87)	Yes
bcsA	Bacterial cellulose synthesis	1/46 (2.17)	Yes
bfpA	Main subunit of the bfp fimbria, which enables bacterial aggregation	6/46 (13.04)	Yes
Cma	Encodes bacteriocins/microcins	2/46 (4.35)	Yes
Cnf	Cytotoxic necrotizing factor	2/46 (4.35)	Yes
cnf1	Cytotoxic necrotizing factor	5/46 (10.87)	Yes
cnf2	Cytotoxic necrotizing factor	6/46 (13.04)	Yes
Cia	Protein secretion (invasion antigen)	1/46 (2.17)	Yes
clp-g	Protein secretion (Type VI secretion system)	1/46 (2.17)	Yes
cs31A	Capsule-like antigen	1/46 (2.17)	Yes
cvaA	secretion protein (colicin V)	1/46 (2.17)	Yes
Crl	Fimbria curli regulator	1/46 (2.17)	Yes
csgA	Temperature regulated curli filament	2/46 (4.35)	Yes
csgD	Temperature regulated curli filament	1/46 (2.17)	Yes
Ccdt	Cytolethal distending toxins	1/46 (2.17)	Yes
cdtB	Cytolethal distending toxins	1/46 (2.17)	NI
cdtIII	Cytolethal distending toxins	2/46 (4.35)	Yes
eaeA	Adhesion factor plasmid	1/46 (2.17)	Yes
etsC	Response regulator	1/46 (2.17)	Yes
eitB	Virulence transcriptional regulator	1/46 (2.17)	Yes
eilA	Virulence transcriptional regulator	1/46 (2.17)	Yes
east1	Aggregative adherence fimbriae	1/46 (2.17)	Yes

Est	Carboxyl hydrolases/esterase	3/46 (6.52)	Yes
estA	Carboxyl hydrolases/esterase	2/46 (4.35)	Yes
Let	Heat-labile enterotoxin	3/46 (6.52)	Yes
eltA	Heat-labile enterotoxin	1/46 (2.17)	Yes
Efa	Factor for adherence	1/46 (2.17)	Yes
espA	Type III secretion system translocator protein	1/46 (2.17)	Yes
espB	Type III secretion system translocator protein	1/46 (2.17)	Yes
espF	Type III secretion system effector E	1/46 (2.17)	Yes
espP	Serine protease. Cleaves coagulation factor V	2/46 (4.35)	Yes
ehxA	Enterohemolysin	3/46 (6.52)	Yes
Ehly	Enterohemolysin	2/46 (4.35)	Yes
ehlyA	Enterohemolysin	1/46 (2.17)	Yes
Eae	Intimin-like adhesin	27/46 (58.70)	Yes
eaeA	Intimin-like adhesin	5/46 (10.87)	Yes
etpD	type II secretion system secretin	1/46 (2.17)	Yes
ehaAα	Autotransporter adhesin	1/46 (2.17)	Yes
ehaAβ	Autotransporter adhesin	1/46 (2.17)	Yes
Escv	Secretion system genes	2/46 (2.17)	Yes
fyuA	Siderophore receptor	1/46 (2.17)	Yes
F4 (K88)	Fimbrial adhesive	3/46 (2.17)	Yes
F5 (K99)	Fimbrial adhesive	12/46 (26.09)	Yes
F6(987P)	Fimbrial adhesive	3/46 (6.52)	Yes
F17	Fimbrial adhesive	1/46 (2.17)	Yes
F17c	Fimbrial adhesive	2/46 (4.35)	Yes
F17g	Fimbrial adhesive	1/46 (2.17)	Yes
F18	Fimbrial adhesive	4/46 (8.70)	Yes
F41	Fimbrial adhesive	9/46 (19.56)	Yes
fimH	Protein precursor	3/46 (6.52)	Yes
Flu	Cell self-aggregation	1/46 (2.17)	Yes
fiCH4	Flagellin	1/46 (2.17)	Yes
Gad	Acid resistance	2/46 (4.35)	Yes
hlyA	Hemolysin	8/46 (14.29)	Yes
hglyF	Hemolysin	2/46 (4.35)	Yes

ibeA	Invasion protein	1/46 (2.17)	Yes
Iha	Siderophore receptor/adhesin	2/46 (4.35)	Yes
ireA	Siderophore receptor	2/46 (4.35)	Yes
iroN	Iron acquisition	2/46 (4.35)	Yes
irp-2	Polyketide synthase	1/46 (2.17)	Yes
Iss	Serum survival gene	3/46 (6.52)	Yes
lucC	Siderophore biosynthesis protein	1/46 (2.17)	Yes
lucD	L-lysine 6-monooxygenase	1/46 (2.17)	Yes
lpfA	Long polar fimbriae	1/46 (2.17)	Yes
intII_LT	Heat-labile enterotoxins	8/46 (14.29)	Yes
katP	Catalase/peroxidase	1/46 (2.17)	Yes
kpsMII	Polysialic acid transport protein	1/46 (2.17)	Yes
Malx	Encodes enzyme II of the phosphotransferase system	1/46 (2.17)	Yes
mchF	Microcin transport protein	1/46 (2.17)	Yes
mcmA	Protein microcin-bacteriocin	1/46 (2.17)	Yes
mchB	Protein microcin-bacteriocin	1/46 (2.17)	Yes
mchC	Protein microcin-bacteriocin	1/46 (2.17)	Yes
mchF	Protein microcin-bacteriocin	1/46 (2.17)	Yes
mleA	Type III secretion system effector	1/46 (2.17)	Yes
nleB	Type III secretion system effector	1/46 (2.17)	Yes
nleC	Type III secretion system effector	1/46 (2.17)	Yes
ompA	Membrane protein (iron resistance)	1/46 (2.17)	Yes
ompTp	Membrane protein(proteases)	1/46 (2.17)	Yes
Plnv	Integral membrane protein	1/46 (2.17)	Yes
papC	Protein fimbrial	1/46 (2.17)	Yes
papG	Adhesin fimbrial	1/46 (2.17)	Yes
papG (Allelle I)	P-Fimbrial outer membrane protein	1/46 (2.17)	Yes
papG (Allelle II	P-Fimbrial outer membrane protein	1/46 (2.17)	Yes
papG (Allelle III)	P-Fimbrial outer membrane protein	1/46 (2.17)	Yes
papAH	P-Fimbrial outer membrane protein	1/46 (2.17)	Yes
rbfp O157/fliCH7	Somatic and flagellar antigen	1/46 (2.17)	Yes
rpoS	Regulator sigma factor	1/46 (2.17)	Yes
sitA	Iron/manganese ABC transporter substrate-binding protein	1/46 (2.17)	Yes

St	Thermostable enterotoxins	1/46 (2.17)	Yes
Sta	Heat-stable enterotoxin.	10/46 (2.17)	Yes
Stb	Heat-stable enterotoxin.	4/46 (8.70)	Yes
Stx	Shiga toxin	2/46 (4.35)	Yes
stx1	Shiga toxin	31/46 (67.39)	Yes
stx2	Shiga toxin	26/46 (56.52)	Yes
stx1/stx2	Shiga toxin	1/46 (2.17)	Yes
stx2a	Shiga toxin	1/46 (2.17)	NI
stx2b	Shiga toxin	1/46 (2.17)	NI
stx2c	Shiga toxin	3/46 (6.52)	Yes
stx2d	Shiga toxin	1/46 (2.17)	NI
stx2e	Shiga toxin	3/46 (6.52)	Yes
stx2f	Shiga toxin	1/46 (2.17)	NI
stx2g	Shiga toxin	1/46 (2.17)	NI
sfa/focDE	Gene related to biofilm formation	1/46 (2.17)	Yes
Tccp	Cytoskeleton coupling protein	1/46 (2.17)	Yes
Tsh	Temperature sensitive hemagglutinin	1/46 (2.17)	Yes
Tir	Intimin receptor Tir	1/46 (2.17)	Yes
traT	Lipoprotein	1/46 (2.17)	Yes
toxB	Cytotoxin	1/46 (2.17)	Yes
Vt	Vero cytotoxin (verotoxicin in cells vero)	1/46 (2.17)	NI
vt2e	Vero cytotoxin (verotoxicin in cells vero)	1/46 (2.17)	NI
Vtx	Vero cytotoxin (verotoxicin in cells vero)	1/46 (2.17)	Yes
vtx2	Vero cytotoxin (verotoxicin in cells vero)	1/46 (2.17)	Yes
Wzxo	Putative O-antigen flippase	1/46 (2.17)	No
uidA	Encodes the beta-glucuronidase enzyme	2/46 (4.35)	Yes

NI = not informed.

Table 2 – Frequency of studies that performed virulence phenotypic assays in studies selected on *Escherichia coli* isolated from intestinal tract or feces of calves, published between 1982 and 2020.

Target	Method (N of studies)	Total N of studies that tested (%)	Identified
Adhesion assay	Inoculation of Hep-2 Cells, tissue culture plates	2/13 (15.38)	Yes
Biofilm	*In vitro* biofilm induction by the polystyrene microtiter method by the Cristal Violet method	1/13 (7.69)	NI
Cytotoxic necrotizing factor	NI	1/13 (7.69)	Yes
Fimbrial adhesive *F4 (K88)*	NI	1/13 (7.69)	Yes
Fimbrial adhesive *F5 (K99)*	Agglutination on plates with antiserum (2) / NI (3)	5/13 (38.46)	Yes
Fimbrial adhesive *F17*	NI	2/13 (15.38)	Yes
Fimbrial adhesive *F41*	NI	2/13 (15.38)	Yes
Fimbrial adhesive *K101*	NI	2/13 (15.38)	Yes
Enterohemolysin	Detection in sheep blood plates	1/13 (7.69)	Yes
Hemolysin	Detection in sheep blood plates	1/13 (7.69)	Yes
α-hemolysin	Detection in sheep blood plates	1/13 (7.69)	No
Heat-labile enterotoxins	*In vitro* inoculation of vero cells (2) / NI (2)	4/13 (30.76)	NI
Shiga-toxin	*In vitro* inoculation of vero cells	2/13 (15.38)	Yes

NI = not informed.

Table 3- Number of studies that tested and observed resistance to several antimicrobials using disk-diffusion technique in *Escherichia coli* isolated from calves. Studies selected by this systematic review, published between 1982 and 2020.

Class	Antimicrobial	Tested concentration (N of studies)	Total N of studies that tested (%)	N of studies that observed resistance (%)
Aminocoumarin	Novobiocin	NI	1/46 (2.27)	NI/1
Aminoglycosides	Amikacin	30 μg (9) / 30 mg (1) / NI (4)	14/46 (30.46)	5/14 (35.71)

	Apramycin	15 µg (2)	2/46 (4.34)	2/2 (100)
	Gentamicin	10 µg (22) / 30 µg (2) / 10 mg (3)	27/46 (58.69)	15/27 (55.55)
	Kanamycin	10 µg (2) / 30 µg (9) / 30 µg NI (6)	17/46 (36.95)	12/17 (70.58)
	Neomycin	20 µg (1) / 25 µg (1) / 30 µg (9) / NI (1)	12/46 (26.08)	8/12 (66.66)
	Spectinomycin	10 µg (1) / 20 µg (1) /100 µg (1)	3/46 (6.52)	3/3 (100)
	Streptomycin	10 µg (11) / 10 mg (4) / NI (9)	24/46 (52.17)	13/24 (54.16)
	Tobramycin	10 µg (1)	1/46 (2.27)	1/1 (100)
Carbapenems	Imipenem	10 µg (2) / NI (2)	4/46 (8.69)	0/4 (0.00)
	Meropenem	10 µg (1) / NI (1)	4/46 (8.69)	2/4 (50.0)
	Cefaloridine	10µg (1)	1/46 (2.27)	1/1 (100)
	Cefazolin	30 µg (1)	1/46 (2.27)	1/1 (100)
	Cefepime	30 µg (NI)	5/46 (10.86)	1/5 (20.0)
	Cefetrizole	30 mg (1)	1/46 (2.27)	NI/1
	Cefixime	5 µg (1)	1/46 (2.27)	1/1 (100)
	Cefoperazone	75 mg (1)	1/46 (2.27)	NI/1
	Cefotaxime	30 µg (7) / 30 mg (1) / NI (3)	11/46 (23.91)	7/11 (58.33)
Cephalosporins	Cefoxitin	30 µg (2)	2/46 (4.34)	0/2 (0.00)
	Ceftazidime	30 µg (4) / NI (3)	7/46 (15.21)	2/7 (28.57)
	Ceftiofur	0.2 µg (1) / 30 µg (4)	5/46 (10.86)	5/5 (100)
	Ceftriaxone	30 µg (2) / NI (2)	4/46 (8.69)	3/4 (75.0)
	Cefuroxime	30 µg (5)	5/46 (10.86)	5/5 (100)
	Cephachlor	30 mg (1) NI / (1)	2/46 (4.34)	2/2 (100)
	Cephalexin	30 µg (2) / NI (1)	3/46 (6.52)	3/3 (100)
	Cephalonium	NI (1)	1/46 (2.27)	1/1 (100)
	Cephalothin	30 µg (7) / NI (1)	8/46 (17.39)	7/8 (87.5)
	Danofloxacin	NI	1/46 (2.27)	NI/1
Fluoroquinolones	Enrofloxacin	5 µg (11) / 10 µg (5) / 5 mg (2) / 10 mg (1)	19/46 (41.30)	12/19 (63.15)
	Flumequine	30 µg (1)	1/46 (2.27)	1/1 (100)
	Marbocyl	10 µg (1)	1/46 (2.27)	1/1 (100)

	Marbofloxacin	10 µg (1)	1/46 (2.27)	1/1 (100)
	Ofloxacin	NI	1/46 (2.27)	1/1 (100)
	Cotrimoxazole	25 µg (4) / NI (2)	6/46 (13.04)	4/6 (66.66)
	Sulfadiazine	300 mg (1)	1/46 (2.27)	NI/1
	Sulfamethoxazole	25 µg / (1) / NI (3)	4/46 (8.69)	4/4 (100)
Folate inhibitor	Sulfamethoxazole-trimethoprim	25 µg (16) / 30 µg (1) / (2) / 25 mg /NI (4)	23/46 (50.00)	16/23 (69.56)
	Sulfaprim	50 µg (1)	1/46 (2.27)	1/1 (100)
	Sulfonamides	30 µg (1) / 300 µg (1) / NI (2)	4/46 (8.69)	1/4 (25.00)
	Trimethoprim	5 µg (2) / 5 mg (1) / NI (2)	5/46 (10.86)	4/5 (80.00)
	Trimethoprim -sulfadiazine	25 µg (1)	1/46 (2.27)	NI/1
Fosfomycins	Fosfomycin	NI	1/46 (2.27)	NI/1
	Clindamycin	2 µg (1)	1/46 (2.27)	NI/1
Lincosamides	Lincomycin	2 µg (2)	2/46 (4.34)	2/2 (100)
	Lincospectin	100 µg (1)	1/46 (2.27)	0/1 (0.00)
Macrocyclic	Rifampicin	5 µg (2)	2/46 (4.34)	1/2 (50.00)
	Erythromycin	15 µg (4) / 25 µg (1) / NI (1)	6/46 (13.04)	4/6 (66.66)
Macrolides	Espiramycin	NI	1/46 (2.27)	1/1 (100)
	Thiomicosin	NI	1/46 (2.27)	1/1 (100)
	Tilosin	NI	1/46 (2.27)	NI/1
Monobactam	Aztreonam	30 µg (1) / NI (3)	4/46 (8.69)	2/4 (50.00)
Nitrofurans	Nitrofurantoin	30 µg (1) / 300 µg (2) / NI (2)	5/46 (10.86)	4/5 (80.00)
	Amdinocillin	NI	1/46 (2.27)	0/1 (0.00)
	Amoxicillin	10 µg (4) / 25 µg (2) / 30 µg (3) / 20 mg (1) / NI (2)	12/46 (26.08)	9/12(75.00)
	Amoxicillin/clavulanic acid	10 µg (1) / 30 µg (2) / 10 mg (4) / NI (5)	11/46 (23.01)	6/11 (54.54)
Penicillin	Amoxiclav	NI	1/46 (2.27)	NI/1
	Ampicillin	10 µg (19) / 10 mg (4) / NI (5)	28/46 (60.86)	19/28 (67.85)
	Ampicillin/sulbactam	10/10 µg (1)	1/46 (2.27)	1/1 (100)
	Cloxacillin	5 µg (1) / NI (1)	2/46 (4.34)	2/2 (100)

	Mezlocillin	75 µg (1)	1/46 (2.27)	1/1 (100)
	Oxacillin	1 µg (2)	2/46 (4.34)	1/2 (50.00)
	Penicillin G	10 µg (4) / 10 mg (1)	5/46 (10.86)	4/5 (80.00)
	Piperacilline-Tazobactan	NI	2/46 (4.34)	1/2 (50.00)
	Tazobactam	NI	1/46 (2.27)	NI/1
	Temocillin	NI	1/46 (2.27)	0/1 (0.00)
	Ticarcillin	75 µg (1)	1/46 (2.27)	1/1 (100)
	Ticarcillin-clavulanate acid	75/10 µg (1)	1/46 (2.27)	1/1 (100)
Phenicols	Chloramphenicol	10 µg (1) / 30 µg (12) / 30 mg (4) / NI (5)	21/46 (26.08)	15/21 (71.42)
	Florfenicol	30 µg (5) / NI (1)	6/46 (13.04)	3/6 (50.0)
Polymyxins	Colistin	10 µg (5) / 1 mg (1) /NI (1)	7/46 (15.21)	6/7 (85.71)
	Polymyxin B	300 U (1)	1/46 (2.27)	1/1(100)
	Ciprofloxacin	5 µg (9) /5 mg (3) / 10 µg (1)	13/46 (28.26)	6/13 (46.15)
	Levofloxacin	5 µg (1)	1/46 (2.27)	0/1 (0.00)
Quinolones/Fluoroquinolones	Nalidixic Acid	10 µg (1) /30 µg (9) / 30 mg (1) / NI (5)	16/46 (34.78)	9/16 (56.25)
	Norfloxacin	5 µg (1) 10 µg (10) / 10 mg (1) / NI (6)	11/46 (23.01)	7/11 (63.63)
	Pefloxacin	5 µg (1)	1/46 (2.27)	1/1 (100)
	Doxycycline	30 µg (1)	1/46 (2.27)	1/1(100)
Tetracyclines	Oxytetracycline	30 µg (1)	1/46 (2.27)	1/1(100)
	Tetracycline	10 µg (1) / 30 µg (20) / 30 mg (4) / NI (7)	31/46 (67.39)	23/31 (74.19)

NI = not informed

Table 4 - Number of studies that tested and observed resistance to several antimicrobials using microdilution technique in *Escherichia coli* isolated from calves. Studies selected by this systematic review, published between 1982 and 2020.

Class	**Antimicrobial**	**MIC range µg/mL (N of studies)**	**Total N of studies that tested (%)**	**N of studies that observed resistance**

Aminoglycosides	Apramycin	1>512 (1)	1/10 (10.0)	NI/1
	Amikacin	2>64 (1)	1/10 (10.0)	NI/1
	Gentamicin	0.25-256 (1) / 0.25-32 (1) / 1-16 (1) / 0.5-64 (1) / NI (4)	8/10 (80.0)	6/8 (85.71)
	Kanamycin	0.25-256 (1) / 4-28 (1) / NI (9)	10/10 (100)	10/10 (100)
	Neomycin	NI (3)	3/10 (30.0)	2/3 (66.66)
	Streptomycin	0.25-256 (1) / 0.5-512 (2) / 2-256 (1) / NI (3)	7/11 (63.63)	2/7 (28.57)
	Spectinomycin	NI (1)	1/10 (10.0)	1/1 (100)
	Tobramycin	1-16 (1)	1/10 (10.0)	0/1 (0.00)
Cephalosporins	Cephazolin	4-64 (1)	1/10 (10.0)	1/1 (100)
	Cephalothin	1-512 (1)	1/10 (10.0)	1/1 (100)
	Cefuroxime	0.125-64 (1)	1/10 (10.0)	1/1 (100)
	Cefotaxime	0.0625-2 (1)	1/10 (10.0)	1/1 (100)
	Cefquimone	0.0625-2 (1)	1/10 (10.0)	1/1 (100)
	Cefepime	1-62 (1)	1/10 (10.0)	1/1 (100)
	Cefoxitin	0.25-32 (2)	1/10 (10.0)	1/2 (50.00)
	Ceftazidime	NI (1)	1/10 (10.0)	0/1 (0.00)
	Ceftiofur	0.12-16 (5) / 0.25-256 (1) / 6-256 (1)	7/10 (70.0)	0/7 (0.00)
	Ceftriaxone	0.25-0.256 (1) / 1-64 (1)	2/10 (20.0)	1/2 (50.00)
Carbapenems	Imipenem	0.256-16 (1) / NI (1)	22/10 (20.0)	1/2 (50.00)
	Ertapenem	0.25-16 (1)	1/10 (10.0)	0/1 (0.00)
	Meropenem	0.25-16 (1)	1/10 (10.0)	1/1 (100)
Phenicol	Florfenicol	2-64 (1) / 4-32 (1) / 8-256 (1) / NI (2)	5/10 (50.0)	4/5 (80.00)
	Chloramphenicol	0-25-256 (1) / 1-128 (1) / 2-64 (1) / NI (2)	5/10 (50.0)	1/5 (20.00)
Quinolones/Fuoroquinolones	Nalidixic Acid	0.25-256 / (1) / 0.5-512 (1) / 1-128 (1) / 4-64 (1)	4/10 (40.0)	2/4 (50.00)
	Ciprofloxacin	0.008-8 (1) / 0.25-256 (1) / 0.25-4 (1)	3/10 (30.0)	1/3 (33.33)
	Enrofloxacin	0.03-4 (1) / 0.0625-64 (1)	2/10 (20.0)	NI/2
	Enoxacin	0.0625-256 (1)	1/10 (10.0)	NI/1
	Danofloxacin	0.0625 (1)	1/10 (10.0)	NI/1
	Moxifloxacin	0.25-8 (1)	1/10 (10.0)	1/1 (100)
	Oxolinic Acid	0.0625 (1)	1/10 (10.0)	NI/1
Lincosamides	Clindamycin	NI (1)	1/10 (10.0)	1/1 (100)
Monobactam	Aztreonam	1-64 (1) / NI (1)	2/10 (20.0)	1/2 (50.00)
Penicillins	Co-amoxiclav	NI	1/10 (10.0)	NI/1
	Amoxicillin/clavulanate acid	0.25-256	1/10 (10.0)	1/1 (100)

	Ampicillin	0.25-256 (1) / 0.5->32 (1). 0.25-32 (1) 1->512 (1) / 128->512 (2) / NI (4)	10/10 (10.0)	7/10 (70.00)
	Ampicillin/sulbactam	2/1 - 32/16 (1)	1/10 (10.0)	1/1 (100)
	Penicillin	NI (1)	1/10 (10.0)	1/1 (100)
	Piperacillin	NI (1)	1/10 (10.0)	NI/1
	Piperacillin-tazobactam	NI (1)	1/10 (10.0)	NI/1
	Ticarcillin	128->1024 (1)	1/10 (10.0)	1/1 (100)
	Ticarcillin/clavulanic acid	NI (1)	1/10 (10.0)	NI/1
	Tilmicosin	NI (1)	11/10 (10.0)	1/11 (100)
	Tylosin	0->512 (1)	1/10 (10.0)	NI/1
Macrolides	Tiamulin	NI (1)	1/10 (10.0)	1/11 (100)
	Tylosin Tartare base	NI (1)	1/10 (10.0)	0/1 (100)
	Tulathromycin	NI (1)	1/10 (10.0)	0/1 (100)
	Sulfadimethoxine	NI (1) 8-/512	2/10 (20.0)	1/2 (50.00)
	Sulfamethoxazole-trimethoprim	0.25-256 (1) /20-320 (1) / NI (1)	3/10 (30.0)	2/3 (66.66)
Folate inhibitor	Sulfametoxazol	NI (1)	1/10 (10.0)	1/1 (100)
	Sulfisoxazol	0.25-256 (1)	1/10 (10.0)	1/1 (100)
	Sulphonamide	8-1024 (1) / 16-2048 (1) / NI (1)	3/10 (30.0)	2/3 (18.18)
	Trimethoprim	0.25-32 (1) / 0.5-32 (1) / ≤0.062->512 (1)	3/10 (30.0)	2/3 (66.66)
	Tetracycline	0.125->512 (2) / 0.25-256 (1) /0.5-64 (1), NI (1)	5/10 (50.0)	3/5 (60.00)
Tetracyclines	Chlortetracycline	NI (1)	1/10 (10.0)	NI/1
	Oxytetracycline	NI (1)	1/10 (10.0)	NI/1
Glicyclines	Tigecycline	0.5-8 (1)	1/10 (10.0)	0/1 (0.00)
Nitrofurans	Nitrofurantoin	0.5-16 (1)	1/10 (10.0)	0/1 (0.00)
	Nitrofurazone	0.5-64 (1)	1/10 (10.0)	NI/1
Polymyxin	Polymyxin B	0.25-16 (1)	1/10 (10.0)	NI/1
	Colistin	2-4 (1)	1/10 (10.0)	0/1 (0.00)

NI = not informed

Table 5 - Frequency of prospection and identification of resistance genes in studies selected by this systematic review on *Escherichia coli* isolated from calves, published between 1982 and 2020.

Antimicrobial class	Gene/target	Resistance mechanism	N of studies that tested (%)	Identified
Aminocoumarin	*mdtABC-TolC*	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	aac (3)-IV	Drug enzymatic inactivation	1/17 (5.88)	Yes
	aac (3)-II	Drug enzymatic inactivation	3/17 (17.64)	Yes
	aac (6)-Ib	Drug enzymatic inactivation	1/17 (5.88)	Yes
	AadA	Drug enzymatic inactivation	2/17 (11.76)	NI
	aadA1	Drug enzymatic inactivation	5/17 (29.41)	Yes
	aadA5	Drug enzymatic inactivation	1/17 (5.88)	Yes
	AadB	Drug enzymatic inactivation	3/17 (17.64)	Yes
	AmpC	Drug enzymatic inactivation	3/17 (17.64)	Yes
Aminoglycosides	*ant(2)-1*	Drug enzymatic inactivation	1/17 (5.88)	Yes
	aph(3")-Ib	Drug enzymatic inactivation	1/17 (5.88)	Yes
	aph(3")-lc	Drug enzymatic inactivation	1/17 (5.88)	Yes
	aph(6)	Drug enzymatic inactivation	1/17 (5.88)	Yes
	BlaCITM	Drug enzymatic inactivation	1/17 (5.88)	Yes
	Kan	Work by binding to the bacterial 30S ribosomal subunit	1/17 (5.88)	Yes
	RmtB	Enzymatic modification of antibiotic target which	1/17 (5.88)	Yes
	StrA	Enzymatic inactivation of antibiotic	3/17 (17.64)	Yes
	StrB	Enzymatic inactivation of antibiotic	3/17 (18.75)	Yes
Carbapenems	*blaOXA-1*	Drug enzymatic inactivation	1/17 (5.88)	Yes
	Intl	Drug enzymatic inactivation	1/17 (5.88)	Yes
	blaCTX-M-14	Drug enzymatic inactivation	1/17 (5.88)	Yes
	BlaCTX	Drug enzymatic inactivation	1/17 (5.88)	Yes
Cephalosporins	*blaCTX-M-1*	Drug enzymatic inactivation	1/17 (5.88)	Yes
	BlaSHV	Drug enzymatic inactivation	2/17 (11.76)	Yes
	ctxM-1	Drug enzymatic inactivation	1/17 (5.88)	Yes
	ctxM-2	Drug enzymatic inactivation	1/17 (5.88)	Yes

Cephamycin	*ctxM-9*	Drug enzymatic inactivation	1/17 (5.88)	Yes
	BlaCMY	Drug enzymatic inactivation	1/17 (5.88)	NI
	blaCMY-2	Drug enzymatic inactivation	1/17 (5.88)	Yes
Diaminopyrimidines	*dfrA-5*	Substitution of antibiotic action target	2/17 (11.76)	Yes
	DrfA	Substitution of antibiotic action target	1/17 (5.88)	Yes
	drfA-1	Substitution of antibiotic action target	6/17(35.29)	Yes
	*drfA-14_*	Substitution of antibiotic action target	1/17 (5.88)	Yes
	drfA-17	Substitution of antibiotic action target	1/17 (5.88)	Yes
Macrolides	*erm(x)*	Enzymatic modification of antibiotic target	1/17 (5.88)	Yes
	ErmB	Enzymatic modification of antibiotic target	1/17(5.88)	Yes
	Maca	Antibiotic resistance via the transport of antibiotics	1/17 (5.88)	Yes
	MacB	Antibiotic resistance via the transport of antibiotics	1/17 (5.88)	Yes
Monobactam, Cephalosporin	*tem-1*	Inactivation drug enzymatic modification	1/17 (5.88))	Yes
	tem-2	Inactivation drug enzymatic modification	1/17 (5.88)	Yes
Monobactams	*BlaTEM*	Drug enzymatic inactivation	4/17 (23.52)	Yes
	blaTEM-1	Drug enzymatic inactivation	1/17 (5.88)	Yes
	blaTEM-1B	Drug enzymatic inactivation	2/17 (11.76)	Yes
Phenicols	*cat-1*	Drug enzymatic inactivation	2/17 (11.76)	Yes
	catA1	Drug enzymatic inactivation	2/17 (11.76)	Yes
	CmlA	Efflux proteins that pump antibiotic	5/17 (29.41)	Yes
	cmlA-1	Efflux proteins that pump antibiotic	1/17 (5.88)	NI
	FloR	Efflux proteins that pump antibiotic	4/17 (23.52)	Yes
Quinolones	*acrEF-TolC*	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	emrAB-OMF	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	GryA	Enzymatic modification of antibiotic target	1/17 (5.88)	Yes
	ParC	Enzymatic modification of antibiotic target which	1/17 (5.88)	Yes
	Oep	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	Onr	Protection of antibiotic action target	2/17 (11.76)	Yes
	QnrA	Protection of antibiotic action target	1/17 (5.88)	Yes

	QnrB	Protection of antibiotic action target	1/17 (5.88)	Yes
	QnrC	Protection of antibiotic action target	1/17 (5.88)	Yes
	QnrD	Protection of antibiotic action target	1/17 (5.88)	Yes
	QnrE	Protection of antibiotic action target	1/17 (5.88)	Yes
	QnrS	Protection of antibiotic action target	1/17 (5.88)	Yes
Quinolones, Macrolide	*mdtEF-TolC*	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
Sulfonamides, Macrolides, Cephalosporin	*tolC-OpmH*	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	mxAB-OprM	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	MexB	Antibiotic resistance via the transport of antibiotics	1/17 (5.88)	Yes
	sul1	Substitution of antibiotic action target	8/17 (47.05)	Yes
Sulfonamides	*sul2*	Substitution of antibiotic action target	4/17 (23.52)	Yes
	sul3	Substitution of antibiotic action target	2/17 (11.76)	Yes
	emrKY-TolC	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	acrAB-TolC	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	tet(A)	Efflux proteins that pump antibiotic	9/17 (52.94)	Yes
Tetracyclines	*tet(B)*	Efflux proteins that pump antibiotic	5/17 (29.41)	Yes
	tet(C)	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	tet(D)	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	tet(M)	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes
	tet(W)	Efflux proteins that pump antibiotic	1/17 (5.88)	Yes

NI = not informed

Supplementary material

Appendix S1: PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Systematic review on antimicrobial resistance in virulent *Escherichia coli* isolated from calves	§1
ABSTRACT			
Abstract	2	provide relevant and structured information on the main findings regarding the systematic review such as objectives, data source, type of studies, eligibility criteria, evaluation methods, results, limitations and conclusions	§1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	§1, 2, 3
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	§4
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	§4
Information sources	6	Specify all databases, registers, websites, organizations, reference lists and other sources searched or consulted to identify studies.Specify the date when each source was last searched or consulted.	§2
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	§3
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened eachrecord and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	§3
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they workedindependently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used inthe process.	§6
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain ineach study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	§6
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describeany assumptions made about any missing or unclear information.	§6
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessedeach study and whether they worked independently, and if applicable, details of automation tools used in the process.	§5
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	§5

Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristicsand comparing against the planned groups for each synthesis (item #5)).	§6
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or dataconversions.	§6
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	§6
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe themodel(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Not performed
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Not performed
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Not performed
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Not performed
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Not performed
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studiesincluded in the review, ideally using a flow diagram.	§1 Figure 1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Not performed
Study characteristics	17	Cite each included study and present its characteristics.	Supplementary Material Table 1
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Not performed
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimates and itsprecision (e.g). confidence/credible interval), ideally using structured tables or plots.	§1 to 18
Results of syntheses	20a	For each synthesis, briefly summarize the characteristics and risk of bias among contributing studies.	Not performed
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision(e.g.) confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	§ 1 to 18
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Not performed
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Not performed
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Not performed
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Not performed
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	§1 to 5
	23b	Discuss any limitations of the evidence included in the review.	§1
	23c	Discuss any limitations of the review processes used.	Not performed
	23d	Discuss implications of the results for practice, policy, and future research.	§ 1 to 5

OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Not performed
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Not performed
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	Not performed
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	§1
Competing interests	26	Declare any competing interests of review authors.	§1
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted fromincluded studies; data used for all analyses; analytic code; any other materials used in the review.	Not performed

From: Page MJ, McKenzie JE, Bossuyt PM, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ* 2021;372: n71.doi:10.1136/bmj.n71

Appendix S2: Combination of terms used at each database investigated within all the sections from papers (title, abstract and full text), as well as the number of articles found in the search performed on May 6th, 2020.

Database	Combination of words	Results
Cabi	((bovine* OR cattle OR calve* OR calf OR heifer* OR cow* OR herd* OR farm*) AND (enteropathogenic OR pathotypes OR "virulence genes" OR "virulence factors" OR virulence) AND (Escherichia AND coli) AND (antimicrobial OR antibiotic OR resistan* OR susceptibility OR "minimal inhibitory concentration" OR MIC OR "disc diffusion" OR "resistance genes" OR ARG OR "drug resistance") AND ("intestinal tract" OR diarrhea))	187 articles
Cochrane	((bovine* OR cattle OR calve* OR calf OR heifer* OR cow* OR herd* OR farm*) AND (enteropathogenic OR pathotypes OR "virulence genes" OR "virulence factors" OR virulence) AND (Escherichia AND coli) AND (antimicrobial OR antibiotic OR resistan* OR susceptibility OR "minimal inhibitory concentration" OR MIC OR "disc diffusion" OR "resistance genes" OR ARG OR "drug resistance") AND ("intestinal tract" OR diarrhea))	1 article
Pubmed	(((((bovine* OR cattle OR calve* OR calf OR heifer* OR cow* OR herd* OR farm*) AND (enteropathogenic OR pathotypes OR "virulence genes" OR "virulence factors" OR virulence) AND (Escherichia AND coli) AND (antimicrobial OR antibiotic OR resistan* OR susceptibility OR "minimal inhibitory concentration" OR MIC OR "disc diffusion" OR "resistance genes" OR ARG OR "drug resistance") AND ("intestinal tract" OR diarrhea)	186 articles
Scielo	(bovine* OR cattle OR calve* OR calf OR heifer* OR cow* OR herd* OR farm*) AND (enteropathogenic OR pathotypes OR "virulence genes" OR "virulence factors" OR virulence) AND (Escherichia AND coli) AND (antimicrobial OR antibiotic OR resistan* OR susceptibility OR "minimal inhibitory concentration" OR MIC OR "disc diffusion" OR "resistance genes" OR ARG OR "drug resistance") AND ("intestinal tract" OR diarrhea)	6 articles
Scopus	TITLE-ABS-(bovine* OR cattle OR calve* OR calf OR heifer* OR cow* OR herd* OR farm*) TITLE-ABS-KEY(enteropathogenic OR pathotypes OR "virulence genes" OR "virulence factors" OR virulence) AND TITLE-ABS-KEY (Escherichia AND coli) TITLE-ABS (antimicrobial OR antibiotic OR resistan* OR susceptibility OR "minimal inhibitory concentration" OR MIC OR "disc diffusion" OR "resistance genes" OR ARG OR "drug resistance") TITLE-ABS-KEY ("intestinal tract" OR diarrhea)	162 articles
Web of Science	TS= ((bovine* OR cattle OR calve* OR calf OR heifer* OR cow* OR herd* OR farm*) AND (enteropathogenic OR pathotypes OR "virulence genes" OR "virulence factors" OR virulence) AND (Escherichia AND coli) AND (antimicrobial OR antibiotic OR resistan* OR susceptibility OR "minimal inhibitory concentration" OR MIC OR "disc diffusion" OR "resistance genes" OR ARG OR "drug resistance") AND ("intestinal tract" OR diarrhea))	390 articles

Appendix S3: Inclusion and exclusion criteria for studies selected by this systematic review on *Escherichia coli* isolated from the intestinal tract of calves and buffalo calves, published between 1982 and 2020

Inclusion criteria	Exclusion criteria
Papers written in English, Spanish, French or Portuguese	Papers written in other languages than English, Spanish, French or Portuguese
Pathogenic *E. coli*	Other microorganisms
Isolated from calves	Other animal species or other animal category other than calves
Virulence profile assessed by phenotypic or genotypic methods	No assessment of virulent factors
Assessment of in vitro antimicrobial susceptibility by phenotypic methods (MIC, disk diffusion or E-test)	No assessment of antimicrobial susceptibility
Original data	Full text not available
	Thesis, abstract, book chapter and reviews

Appendix S4: Detailed information of the 56 studies selected by this systematic review on antimicrobial resistance in pathogenic *Escherichia coli* isolated from calves, published between 1982 and 2020.

First author, Year	Country	Period	Sample	Type study	Population	Age of animals	N of samples	Diarrhea frequency	AMR test	AMR test reference	N of virulent isolates
Abdulgayeid, 2015	Egypt	NI	Rectal swabs	Sectional	Buffalo calves	< 6 months	193	56.99	Disk diffusion	CLSI or Eucast	95
Aly, 1996	Egypt	NI	Feces	Sectional	Buffalo calves	2-4 months	38	100	Disk diffusion	CLSI or Eucast	12
Aneela, 2018	Pakistan	NI	Rectal swabs	Sectional	Calves	3 months	28	100	Disk diffusion	Other	2
Ary, 2008	India	2004-2005	Rectal swabs	Sectional	Calves	< 2 months	46	100	Disk diffusion	CLSI or Eucast	41
Awosile, 2020	Canada	2014-2015	Rectal swabs	No design	Calves	< 2 weeks	4	0.00	MIC	CLSI or Eucast	4
Barigye, 2012	USA	2010	Feces	Sectional	Buffalo calves	< 2 weeks	97	100	MIC	Other	23
Borriello, 2012	Italy	2006-2009	Intestinal content	Sectional	Calves	< 4 weeks	314	100	Disk diffusion	CLSI or Eucast	65
Bradford, 1999	USA	2006-2009	Feces	No design	Calves	NI	NI	100	Disk diffusion /MIC	CLSI or Eucast	10
Bumunang, 2019	South Africa	1996	Feces	Sectional	Calves	NI	600	NI	Disk diffusion	CLSI or Eucast	NI
Cabal, 2013	Spain	2015-2017	Feces	No design	Calves	NI	NI	0.00	Disk diffusion	CLSI or Eucast	68
Çabalar, 2001	Turkia	NI	Rectal swabs	Sectional	Calves	NI	59	15.25	Disk diffusion	Bauer et al. 1966	1
Das, 2005	India	2001-2002	Feces	Sectional	Calves	NI	111	NI	Disk diffusion	CLSI or Eucast	13
De Rauw, 2019	Belgium	1987-2009-2015	NI	No design	Calves	16 days-2.5 months	NI	100	Disk diffusion	NI	9
De Rycke, 1982	France	1980	Feces	Sectional	Calves	NI	NI	NI	Disk diffusion	Other	10
Donaldson, 2006	USA	2003	Feces	Sectional	Calves	1-9 weeks	96	100	Disk diffusion/MIC	CLSI or Eucast	10
Du, 2005	China	NI	NI	No design	Calves	NI	NI	100	MIC	CLSI or Eucast	13
Du, 2004	China	1982-1988	NI	No design	Calves	NI	NI	100	MIC	CLSI or Eucast	9
Elashmawy, 2016	Egypt	NI	Feces	Sectional	Buffalo calves	1 day - 2 month	120	66.66	Disk diffusion	CLSI or Eucast	18
Gamez, 2006	Brazil	2001 – 2002	Feces	Sectional	Calves	< 3 months	200	100	Disk diffusion	Bauer et al. 1966	53
Gharieb, 2019	Egypt	NI	Feces	No design	Calves	1-3 weeks	80	100	MIC	CLSI or Eucast	8
Giammanco, 2002	Italy	NI	Feces	No design	Calves	NI	37	NI	Disk diffusion	Other	37
Gonzalez, 1989	Spain	NI	Feces	No design	Calves	< 30 days	289	100	Disk diffusion	Other	84
Gonzalez, 2019	Argentina	2014-2015	Feces	No design	Calves	2-10 days	NI	100	Disk diffusion	Bauer et al. 1966	5
Gueler, 2008	Turkia	2001-2006	Feces	No design	Calves	< 2 months	NI	62.5	Disk diffusion	Other	66
Hakim, 2017	Egypt	NI	Feces	No design	Buffalo calves	NI	58	100	Disk diffusion	CLSI or Eucast	14
Holland, 1999	USA	NI	Feces	Sectional	Calves	< 3 months	215	53.02	Disk diffusion	Bauer et al. 1966	63
Islam, 2015	Bangladesh	2014	Feces	Sectional	Calves	6 days-2 months	100	100	Disk diffusion	Bauer et al. 1966	2
Iweriebor, 2015	South Africa	NI	Feces	Sectional	Calves	NI	400	0.00	Disk diffusion	CLSI or Eucast	95
Khalifa, 2019	NI	2016	Feces	Sectional	Calves	< 3 months	100	100	Disk diffusion	CLSI or Eucast	9
Kohansa, 2018	Iran	2015-2016	Rectal swabs	Sectional	Calves	< 30 days	540	100	Disk diffusion	CLSI or Eucast	71
Liao, 2019	China	NI	Feces	Sectional	Calves	NI	30	100	Disk diffusion	CLSI or Eucast	18

Lupindu, 2014	Tanzania	2010-2012	Feces	Sectional	Calves	NI	446	NI	Disk diffusion	CLSI or Eucast	10
Maciel, 2019	Brazil	2014-2015	Feces	Case control	Calves	21-60 days	60	50.00	Disk diffusion	CLSI or Eucast	9
Mahanti, 2014	India	NI	Feces	Sectional	Buffalo calves	NI	363	0.00	Disk diffusion	CLSI or Eucast	25
Manna, 2006	India	2003	Feces	Sectional	Calves	1-3 months	79	100	Disk diffusion	Bauer et al. 1966	11
Medina, 2011	Spain	1993-2005	Feces	No design	Calves	NI	NI	100	Disk diffusion	CLSI or Eucast	24
Mercado, 2004	Argentina	1995-200	Feces	No design	Calves	< 3 months	NI	100	Disk diffusion	Bauer et al. 1966	12
Mohammed, 2019	Egypt	2015-2016	Feces	Sectional	Calves	≤ 3 months	56	46.42	Disk diffusion	CLSI or Eucast	6
Montso, 2019	South Africa	2017	Feces	Sectional	Calves	NI	780	NI	Disk diffusion	Bauer et al. 1966	NI
Niraj, 2011	India	NI	NI	No design	Calves	NI	NI	100	Disk diffusion	Bauer et al. 1966	NI
Nizza, 2010	Italy	2006-2008	Feces	Sectional	Buffalo calves	≤ 30 days	169	100	Disk diffusion	CLSI or Eucast	94
Orden, 1999	Spain	1993-1995	Feces	No design	Calves	≤ 3 months	NI	100	MIC	CLSI or Eucast	137
Orden, 2000	Spain	1993-1995	Feces	No design	Calves	≤ 3 months	NI	100	MIC	CLSI or Eucast	137
Pereira, 2011	USA	2009	Feces	Sectional	Calves	2 days	117	47.86	Disk diffusion	CLSI or Eucast	117
Rigobelo, 2006	Brazil	2001-2002	Feces	Sectional	Calves	< 3 months	200	100	Disk diffusion	CLSI or Eucast	NI
Rusheeba, 2015	India	NI	Feces	Sectional	Calves	< 4 months	NI	100	Disk diffusion	NI	6
Shahrani, 2014	Iran	2010-2011	Feces	Sectional	Calves	2-30 days	8241	100	Disk diffusion	Bauer et al. 1966	419
dSharma, 2017	India	2013-2015	Feces	Sectional	Calves	≤ 3 months	350	100	Disk diffusion	NI	65
Smith, 1988	Chile	NI	Rectal swabs	Sectional	Calves	≤ 10 days	77	100	Disk diffusion	Bauer et al. 1966	32
Srivani, 2019	India	2014-2015	Feces	Sectional	Buffalo calves	< 3 months	375	100	Disk diffusion	Bauer et al. 1966	34
Srivani, 2017	India	2014-2015	Feces	Sectional	Buffalo calves	< 3 months	375	100	Disk diffusion	CLSI or Eucast	106
Umpierrez, 2017	Uruguay	2012-2014	Feces	No design	Calves	≤ 6 months	303	79.87	Disk diffusion	Bauer et al. 1966	26
Valat, 2012	France	2006-2010	Feces	No design	Calves	NI	204	NI	Disk diffusion	NI	NI
Valat, 2014	France	2001-2012	Feces	No design	Calves	NI	259	NI	Disk diffusion	Bauer et al. 1966	NI
Vargas, 2017	Brazil	NI	Feces	Sectional	Calves	≤ 6 months	40	37.50	Disk diffusion	Bauer et al. 1966	12
Verdier, 2012	Sweden	2004-2005	Rectal swabs	Case control	Calves	≤ 1 months	95	58.90	MIC	CLSI or Eucast	NI

NI: Not Informed **AMR:** Antimicrobial resistance

Appendix S5: Evaluation of possible limitations and bias in the methodology of the 56 articles selected for this systematic review on *Escherichia coli* from the intestinal tract of calves and buffalo calves, published between 1982 and 2020.

First author, Year	Test MIC	Test Disk Difusion	Reference protocol	Antibiotic []	Breakp/Halo diameter
Abdulgayeid, 2015	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Aly, 1996	0	Disk diffusion	CLSI or Eucast	Yes	Other
Aneela, 2018	0	Disk diffusion	Other	Yes	Other
Ary, 2008	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Awosile, 2020	Yes	MIC	CLSI or Eucast	Yes	CLSI or Eucast
Barigye, 2012	Yes	MIC	Other	No	Other
Borriello, 2012	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Bradford, 1999	Yes	Disk diffusion /MIC	CLSI or Eucast	No	CLSI or Eucast
Bumunang, 2019	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Cabal, 2013	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Çabalar, 2001	0	Disk diffusion	Bauer et al. 1966	No	Bauer et al. 1966
Das, 2005	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
De Rauw, 2019	0	Disk diffusion	NI	No	NI
De Rycke, 1982	0	Disk diffusion	Other	No	Other
Donaldson, 2006	Yes	Disk diffusion/MIC	CLSI or Eucast	Yes	CLSI or Eucast
Du, 2005	Yes	MIC	CLSI or Eucast	No	CLSI or Eucast
Du, 2004	Yes	MIC	CLSI or Eucast	No	CLSI or Eucast
Elashmawy, 2016	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Gamez, 2006	0	Disk diffusion	Bauer et al. 1966	No	Bauer et al. 1966
Gharieb, 2019	Yes	MIC	CLSI or Eucast	Yes	CLSI or Eucast
Giammanco, 2002	0	Disk diffusion	Other	No	Other
Gonzalez, 1989	0	Disk diffusion	Other	Yes	Other
Gonzalez, 2019	0	Disk diffusion	Bauer et al. 1966	No	Bauer et al. 1966
Gueler, 2008	0	Disk diffusion	Other	Yes	CLSI or Eucast
Hakim, 2017	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Holland, 1999	0	Disk diffusion	Bauer et al. 1966	Yes	Bauer et al. 1966
Islam, 2015	0	Disk diffusion	Bauer et al. 1966	Yes	Bauer et al. 1966
Iweriebor, 2015	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Khalifa, 2019	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Kohansal, 2018	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Liao, 2019	0	Disk diffusion	CLSI or Eucast	Yes	NI
Lupindu, 2014	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Maciel, 2019	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Mahanti, 2014	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast

Manna, 2006	0	Disk diffusion	Bauer et al. 1966	Yes	Bauer et al. 1966
Medina, 2011	0	Disk diffusion	CLSI or Eucast	No	CLSI or Eucast
Mercado, 2004	0	Disk diffusion	Bauer et al. 1966	Yes	Bauer et al. 1966
Mohammed, 2019	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Montso, 2019	0	Disk diffusion	Bauer et al. 1966	Yes	Bauer et al. 1966
Niraj, 2011	0	Disk diffusion	Bauer et al. 1966	No	Bauer et al. 1966
Nizza, 2010	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Orden, 1999	Yes	MIC	CLSI or Eucast	No	CLSI or Eucast
Orden, 2000	Yes	MIC	CLSI or Eucast	No	CLSI or Eucast
Pereira, 2011	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Rigobelo, 2006	0	Disk diffusion	CLSI or Eucast	Yes	CLSI or Eucast
Rusheeba, 2015	0	Disk diffusion	NI	Yes	NI
Shahrani, 2014	0	Disk diffusion	Bauer et al. 1966	Yes	CLSI or Eucast
Sharma, 2017	0	Disk diffusion	NI	Yes	Bauer et al. 1966
Smith, 1988	0	Disk diffusion	Bauer et al. 1966	Yes	NI
Srivani, 2019	0	Disk diffusion	Bauer et al. 1966	No	Bauer et al. 1966
Srivani, 2017	0	Disk diffusion	CLSI or Eucast	No	Bauer et al. 1966
Umpierrez, 2017	0	Disk diffusion	Bauer et al. 1966	No	CLSI or Eucast
Valat, 2012	0	Disk diffusion	NI	No	CLSI or Eucast
Valat, 2014	0	Disk diffusion	Bauer et al. 1966	No	Other
Vargas, 2017	0	Disk diffusion	Bauer et al. 1966	Yes	Bauer et al. 1966
Verdier, 2012	Yes	MIC	CLSI or Eucast	Yes	CLSI or Eucast

NI: Not Informed.

CHAPTER 2

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Antimicrobial resistance and public and animal health risks associated with pathogenic *Escherichia coli* isolated from calves

Dircéia Aparecida da Costa Custódio[a], Carine Rodrigues Pereira[a], Maysa Serpa Gonçalves[a], Anna Cecília Trolesi Reis Borges Costa[a], Pedro Felipe Rodrigues de Oliveira[a], Bruna Henrique Pinto da Silva[a], Giovanna Botelho Carneiro[a], Fernanda Morcatti Coura[b], Andrey Pereira Lage[c], Geraldo Márcio da Costa[a], Elaine Maria Seles Dorneles[a*]

[a]Departamento de Medicina Veterinária, Faculdade de Zootecnia e Medicina Veterinária, Universidade Federal de Lavras. Campus Universitário S/N, Caixa Postal 3037, 37200-900, Lavras, MG, Brazil.

[b]Departamento de Ciências Agrárias do Instituto Federal de Minas Gerais - Campus Bambuí. Rodovia Bambuí/Medeiros Km 05, Caixa Postal 05, 38900-000, Bambuí, MG, Brazil.

[c]Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais. Av. Pres. Antônio Carlos, 6627, São Luiz, 31270-901, Belo Horizonte, Minas Gerais, Brazil.

*Corresponding author: elaine.dorneles@ufla.br. postal address: Departamento de Medicina Veterinária, Faculdade de Zootecnia e Medicina Veterinária, Universidade Federal de Lavras, Campus Universitário S/N, Caixa Postal 3037, Lavras, MG, Brazil. Code: 37200-900.

Highlights

High resistance rates to several classes of antimicrobials among *E. coli* from calves

High levels of multidrug resistance among *E. coli* from calves, Minas Gerais, Brazil

ESBL-producing *E. coli* strains from calves, Minas Gerais, Brazil

EHEC/STEC pathotypes of *E. coli* involved in gastrointestinal infections in calves

Abstract

The aims of the present study were to determine the antimicrobial susceptibility profile of pathogenic *E. coli* strains isolated from fecal samples of calves and buffalo calves from 2008 to 2013 in Minas Gerais, Brazil, as well as to determine the frequency of O157 gene and strains carrying extended-spectrum beta-lactamases (ESBL) and mobile colistin resistance (*mcr*) genes. Five hundred and eighteen *E. coli* strains were tested for susceptibility against ten different antimicrobials, using the broth microdilution technique. Tetracycline was the antimicrobial with the highest percentage of resistance among isolates [73.74% (382/518)]; followed by ampicillin [61.9% (321/518)], sulfamethoxazole/trimethoprim [60.23% (312/518)]; chloramphenicol [37.06% (192/518)]; gentamicin [24.32% (126/518)], ciprofloxacin [28.57% (148/518)], cefazolin [17.18% (89/518)], colistin [10.42% (54/518)] and cefoxitin [6.56% (34/518)]. Multidrug resistance, considered as the resistance to three or more antimicrobial classes, it was observed in 66.79% (346/518) of the isolates while extensively resistant strains defined as not susceptible to one or more antimicrobial agents in all .it was observed in 11.58% (60/518) of the isolates. The presence of genes *mcr1*, *mcr2*, *mcr3* or *mcr5* was not observed in any of the isolates resistant to colistin *in vitro*. Among the isolates resistant to cephalosporins (cefazolin or cefoxitin) 19.11% were ESBL producing strains, of which 94.74% (18/19) were also multidrug resistant. All enterohemorrhagic *E. coli* (EHEC) and Shiga toxin-producing *E. coli* (STEC) isolates were tested by PCR for the presence of O157 gene and they were all negative. Overall, the tested pathogenic *E. coli* strains showed high rates of resistance to penicillin, tetracyclines and folate inhibitors, in addition to an alarming rate of multidrug resistance and strains able to produce ESBL.

Keywords: pathogenesis, virulence genes**,** diarrhea.

1. Introduction

Antimicrobial resistance (AMR) is one of the most significant threats to human and animal health, being considered an emerging global issue [1]. In fact, the implementation of control and prevention strategies on AMR and the rational use of antimicrobials, is one of the priorities of the One Health initiative, which recognize that both human and animal health are interconnected [2, 3]. In the United States, more than 2.8 million antimicrobial-resistant infections occur each year and more than 35,000 people die by infections caused by resistant bacteria [4]. Likewise, in the European Union, AMR is estimated to cost € 1.5 billion annually in healthcare costs and are responsible for around 33,000 deaths per year [5, 6]. For other regions/countries, there are no reliable statistics/data in this regard, which prevents the drawing of a real picture of the situation worldwide [7].

Animal production systems aligned with the inappropriate use of antimicrobials have been identified as one of the main factors responsible for the emergence of AMR bacteria [8, 9]. In animal health, antimicrobials are used for therapy, metaphylaxis, prophylaxis, and growth promotion, being enteric diseases one of the main animal infections that demands the use of these drugs [8]. Among enteric diseases, diarrhea is one of the most frequent, responsible for up to 75% of deaths in calves younger than three weeks, additionally to economic losses, such as reduced animal weight gain, treatment costs and lower development of affected individuals [10].

Diarrhea is considered a multifactorial disease with the association of several etiological agents that can act alone or in combination [11], among them, *Escherichia coli*, a facultative anaerobe bacterium present in the intestinal microbiota of humans and animals [11]. Depending on the virulence factors harbored by *E. coli* strains, they are

classified in pathotypes, which have different pathogenic/zoonotic potentials and are responsible for distinct clinical manifestations and fatality rates [12, 13].

According to the World Health Organization [14] (WHO), about 11 million children under 5 years of age die from gastroenteritis caused by *E. coli*, mainly in developing countries [9]. In calves, *E. coli* infections cause lesions in the gastrointestinal tract of animals, impairing the absorption of nutrients, additionally to a systemic condition due to dehydration, prostration and anorexia [15]. *E. coli* strains and other Gram-negative bacteria that enter the intestinal tract via exposures to contaminated food, water, and other external sources; therefore, risk factors for fecal carriage of drug-resistant commensal *E. coli* and antimicrobial resistance genes (ARGs) could include exposures to environmental sources of drug-resistant bacteria in addition to traditional risks such as prior use of antimicrobials [13]. Associated with the diversity of virulence factors, *E. coli* is also an important agent considering the dissemination of AMR, since the bacteria can easily transfer drug resistance-associated genes on mobile genetic elements, such as bacteriophages, plasmids, and pathogenicity islands, to different species and habitats [16, 17].

In this context, monitoring the virulence and antimicrobial resistance of diarrheal *E. coli* from animal origin provides useful information about the epidemiology of the disease, which is especially important considering that cattle and other ruminants can be reservoirs of pathotypes, mainly O 157 of public health importance. Therefore, the aims of the present study were to determine the antimicrobial susceptibility profile of pathogenic *E. coli* strains isolated from calves and buffalo calves from 1990 to 2013 in Minas Gerais, Brazil, as well as the frequency of O157 strains and strains carrying extended-spectrum beta-lactamases (ESBL) and mobile colistin resistance (*mcr*) genes.

2. Material and methods

2.1. Bacterial strains and culture conditions

In the present study, 518 pathogenic *E. coli* strains isolated from feces of calves (n = 483) and buffalo calves (n = 35), from 1990 to 2013, were tested, from different regions of the State of Minas Gerais, Brazil. The strains are the entire collection of the Laboratório de Bacteriologia Aplicada (LBA), Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG) and were identified using standard methods by previous studies [18-23]. Detailed information about the strains, regarding geographical location, host, year of isolation and occurrence of diarrhea are shown in the Table 1.

The strains were maintained frozen at - 80 ºC in Brain Heart Infusion (BHI) broth plus 20% glycerol (Synth, Brazil). The isolates were cultured onto BHI agar (Merck, Germany) and MacConkey agar (Oxoid, England) plates, incubated at 37°C for 24 h for molecular and phenotypic tests.

2.2.Antimicrobial susceptibility test

The antimicrobial susceptibility test was performed using the broth microdilution technique to assess the minimal inhibition concentration (MIC), according to Clinical and Laboratory Standards Institute (CLSI) [24]. Ten different antimicrobials from eight different classes were tested as described in Table 2. All tests were performed in duplicate, and *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212 were used as quality controls strains in all assays. The results were interpreted according to CLSI [25]. The MIC_{50} and MIC_{90} values were defined as the lowest concentration of the antibiotic at which 50% and 90% of the strains were inhibited, respectively. Strains were defined as multidrug-resistant (MDR) when they were not susceptible to at least one agent in three or more antimicrobial classes, while extensively resistant (XDR) strains were defined as not susceptible to one or more

antimicrobial agents in all but in two or less of the following antimicrobial classes: fluoroquinolones, folate pathway inhibitors, penicillins, phenicols and polymyxins [26].

2.3.Production of extended-spectrum beta-lactamase (ESBL)

To assess the production of ESBL, isolates resistant to cefoxitin or cefazolin (cephalosporins) were tested according to the methodology proposed by CLSI [24], using the following antimicrobials disks: cefotaxime (30 μg) or ceftazidime (30 μg) with or without acid clavulanate (10 μg) (Liofilchem®, Italy). A difference of ≥ 5 mm between the zone diameters of any of the cephalosporin disks and their respective cephalosporin/clavulanate disks was considered a phenotypic confirmation of ESBL production.

2.4.DNA extraction

Strains were submitted to genomic DNA extraction according to the protocol previously described by Pitcher et al [27]. The quantity and quality of DNA extracted were assessed by spectrophotometry using the NanoVue™ spectrophotometer (GE Healthcare, USA). DNA samples were kept at − 20 °C until the analysis.

2.5.Pathotypes and phylogroups

Virulence genes (*Stx1, Stx2, east, cnf2, saa, int, Sta, F41, F5* and *ehl*) from all strains were previously identified in previous studies [20-23]. According to these data, the following criteria were used to classify the pathotypes [18]: enterotoxigenic *E. coli* (ETEC) was classified mainly for the presence of one of the fimbrial adhesins F5 or F41 or production of heat-stable toxin (*STa*); enteropathogenic *E. coli* (EPEC) was defined by the presence of intimin gene (*eae*); enterohemorrhagic *E. coli* (EHEC) should harbored the genes coding for stx1 and/or stx2 toxins and for intimin (*eae*); Shiga toxin-producing *E. coli* isolates (STEC) were classified based on the presence of the toxins genes *stx1* and/or

stx2; necrotoxigenic *E. coli* (NTEC) was characterized for the presence of the CNF2 toxin; and enteroaggregative *E. coli* (EAEC) by the presence of *east1* enterotoxin gene. Strains carrying virulence factors that characterize more than one pathotype were classified as hybrid. Complete information on pathotype classification is shown in Supplementary Table S1.

Regarding the classification of phylogenetic groups, part of the isolates [20, 22] was previously classified following the methodology described by Clermont et al [28]; whereas the others [21, 23] were classified in the present study using the same method (Table 3).

2.6. Detection of E. coli O157 gene and mobile colistin resistance (mcr) genes

All strains classified as EHEC and STEC, according to the virulence genes identified, were tested by PCR to evaluate the presence O157 antigen, according to Paddock et al [29]. The detection of genes *mcr-1, mcr-2, mcr-3,* and *mcr-5* was performed using a multiplex PCR described by Rebelo et al [30]. Primers, fragment sizes and positive controls used in all assays are listed in Table 3.

2.7. Statistical analysis

The frequency distributions for the categorical variables were calculated. Logistic multivariable models using ten dependent variables (multidrug resistance, aminoglycoside, penicillin, cephem, fluoroquinolones, phenicol, polymyxin, folate inhibitor, tetracycline and ESBL) and 20 independent variables (age, sex, sampling year, diarrhea, pathotypes, aminoglycosides, penicillin, cephem, quinolone, phenicol, polymyxin, folate inhibitor, tetracycline, EHEC, EPEC, ETEC, hybrid, NTEC, STEC, EAEC) were built in the STATA 14.0 (http://www.stata.com) to assess the factors

associated with the occurrence of multidrug resistance and resistance in pathogenic *E. coli* strains. Independent variable selection was performed analyzing frequency distribution of its categories and the association between them by χ^2 and Fisher exact test (Supplementary Table S2 and S3). Variables were considered for inclusion in a multivariable model if significance was $p < 0.24$ [31]. The multivariate models were evaluated by testing the predictive ability of the model (sensibility and specificity) and the goodness-of-fit were accessed with Pearson χ^2 and Hosmer-Lemeshow tests [31].

Data were also imported into R statistical software version 4.2.1 (cran.r-project.org) and figures were constructed using the ggplot2[31] and cyclize [32].

3. Results

3.1. Antimicrobial susceptibility tests

Tetracycline was the antimicrobial with the highest percentage of resistant isolates [73.74% (382/518)], followed by ampicillin [61.9% (321/518)], sulfamethoxazole/trimethoprim [60.23% (312/518)], chloramphenicol [37.06% (192/518)], gentamicin [24.32% (126/518)] and ciprofloxacin [28.57% (148/518)]. Isolates also showed resistance to cefazolin [17.18% (89/518)], colistin [10.42% (54/518)] and cefoxitin [6.56% (34/518)]. In contrast, amikacin was the antimicrobial with the highest percentage of susceptible strains [97.87% (507/518)]. Detailed information on antimicrobial resistance, as well MIC_{50} and MIC_{90} are described in Table 2 and Fig.1.

One hundred and twenty-two different antimicrobial resistance profiles were observed among the tested isolates. Only 8.69% (45/518) of the isolates were sensitive to all classes tested, whereas MDR was observed in 66.79% (346/518) of the isolates, with 2.34% (12/518) resistant to seven different antimicrobial classes. XDR was observed in

11.58% (60/518) of the isolates, of which 60% (36/60) were resistant to one class and 40% (24/60) to two classes of antimicrobials. Among XDR isolates, most were resistant to folate inhibitor class [41.66% (15/36)], followed by polymyxin [25% (9/36)], phenicol [13.88% (5 /36)], penicillin [11.11% (4/36)] and fluoroquinolone [8.33% (3/36)]. The antimicrobial class most frequent among the strains classified as XDR was folate inhibitor [62.5% (15/24)], and the most frequent combinations of resistance were folate inhibitor + phenicol [25% (6/24)], folate inhibitor + fluoroquinolone 16.66% (4/24)], folate inhibitor + penicillin [12.5% (3/24)], and folate inhibitor + polymyxin [8.33% (2/24)].

Different levels of AMR were observed between the pathotypes, with the EHEC pathotype being the most associated 28.32(98/346) followed by STEC with 25.0% (87/346), EAEC 24.57% (84/346), ETEC 8.67% (30 /346), EPEC 6.06% (21/346), NTEC 5.49% (19/346) and Hybrid *E. coli* with 2.02% (7/346). Temporal distribution of the resistance profiles observed are shown in Figure 2A. Most MDR strains were isolated in 2008 [225/346 (65.03%)], followed by 2012 [95/346 (27.46%)] and 2013 [15/346 (4.34%)]. For the XDR isolates, 80% (48/60) belonged to the year 2008, followed by 10% (6/60) in 2012, 6.66% (4/60) in 2013 and 3.33% (2/100) isolated without information regarding the year of isolation.

3.2. Detection of ESBL production, O157 gene and mcr genes

Among the cephalosporin resistant strains 19.19% (19/99) of isolates were ESBL producing, from which 94.74% (18/19) were also MDR. These results are described in the Table 4.

All EHEC isolates and STEC isolates were negative in the PCR for detection of O157 antigen. Likewise, although 10.42% (54/518) of the strains showed a colistin resistance phenotype, they were all negative in the PCR for *mcr* genes (*mcr1, mcr2, mcr3*

and *mcr5*).

3.3. E. coli pathotypes and phylogroups

Distribution of phylogroups and pathotypes according to year of isolation is shown in Fig. 2 (B and C). The most prevalent pathotype was STEC [26.64% (138/518)], followed by EHEC [24.90% (129/518)], EAEC [24.32% (126/518)], ETEC [7.91% (41/518)], NTEC [7.52% (39/518)] and EPEC [5.59% (29/518)]. Hybrid *E. coli* pathotypes were observed in 3.08% (16/518) of the isolates. Most *E. coli* strains belong to the phylogroup B1 [58.49% (303/518)], followed by E [13.12% (68/518)], A [5.40% (28/518)], C [4.44% (23/518)], F [3.47% (18/518)], D [1.35% (7/518)], B2 [0.77% (4/518)], and Clade I and II with 0.38% (2/518) each. Relation between pathotypes and phylogroups is shown in Fig.3. Forty-seven isolates [9.07% (47/518)] did not belong to any of the tested phylogroups and 3.09% (16/518) were not typeable.

The presence of diarrhea among the animals was more associated with the pathotype EAEC [27.02% (60/222)], followed by EHEC [26.57% (59/222)], STEC [19.36% (43/222)], ETEC [10.36% (23/222)], NTEC [5.85% (13/222)], EPEC [5.85% (13/222)] and 4.05% [(9/ 222)] *E. coli* hybrid pathotype. On the other hand, [57.14% (296/518)] strains isolated from animals without clinical signs of diarrhea were more associated with STEC [32.09% (95/296)], followed by EHEC [23.64% (70/296)], EAEC [22.29% 66/296], NTEC [8.10% (24/296)], ETEC [6.08% (18/296)], EPEC [5.40% (16/296)] and hybrid *E. coli* [2.36% (7/296)].

Among the strains isolated from animals with diarrhea, 58.10% (129/222) belonged to phylogroup B1, followed by E [14.41% (32/222)], A [5.40% (12/222)], C [3.60% (8/222)], F [3.15% (7/222)], D [1.35% 3/222(D)] and Clade I and II [0.45% 1/ 222)]. Four isolates [1.80% (4/22)] were non-typeable and 9.90% (22/222) were negative according the Clermont et al.[28] technique. Regarding to animals without clinical signs,

the most common phylogroup was also B1 (58.78% 174/296), followed by phylogroup E [12.16% (36/296)], A [5.40% (16/296)], C [5.06% (15/296)], F [3.71% (11/296)], D [1.35% (4/296)] and Clade I and II [0.33% (1/296)], whereas 6.12% (12/296) were non-typable and 8.44% (25/296) were negative.

3.4. Logistic multivariable models

The logistic multivariable model using AMR as outcome was the only model that showed a good fit [Pearson χ^2 = 273.85 (P-value = 0.9504) and the Hosmer-Lemeshown χ^2 = 11.24 (P-value = 0.1884)], with age (months), some antimicrobial classes (aminoglycoside, penicillin, cephem, quinolone, phenicol and tetracycline) and the EHEC pathotype been found as significantly associated with AMR. The final multivariate logistic model for AMR is shown in Table 5. All significant variables in the final model showed positive association with AMR, being the higher odds ratios (OR) exhibited by fluoroquinolones and penicillin. The sensitivity of the model was 100% and the specificity was 51.16%, correctly classifying 83.78% the AMR. The area under the curve for the ROC curve was 0.9628.

4. Discussion

In the present study, we investigated the antimicrobial susceptibility profile of pathogenic strains of *E. coli* isolated from calves and buffalo calves from 1990 to 2013 in Minas Gerais. The results showed high rates of resistance to several classes of antimicrobials, mainly tetracyclines, penicillin and folate inhibitor, as well as high levels of MDR, which is a concern from two point of views, animal health and public health, since most of the tested *E. coli* strains exhibited great zoonotic potential (EHEC/STEC), despite O157 antigen has not been observed in any of the isolates. Additionally, it is important to note that the antimicrobials to which resistance was observed are generally also the first choice for treatment of *E. coli* infections and have a low economic cost and

belong to the same class as those administered in for enteric infections caused by *E. coli* in humans [10, 32, 33]. On the other hand, from the animal health point of view, the vast resistance and MDR rates observed among the tested strains also show a huge problem in treating enteric infections caused by *E. coli* in cattle [34]. The intensive use of antimicrobial in food-producing animals, for different purposes, such as therapy, metaphylaxis, prophylaxis, and growth promotion [35, 36], are probably the explanation these findings. Indeed, data from the United States and European Union all countries have shown that tetracycline followed by penicillin are the two best-selling antimicrobial classes, considered to be easily accessible, abundant and inexpensive antimicrobials, contributing to their overemployment [7, 37].

In this sense, it is important to note that all the antimicrobial classes that remained significant in the final logistic model are also among the most sold antimicrobials [37] to produce food from animals, strongly suggesting the relationship of their overuse with the increasing AMR in *E. coli* from animal origin, demonstrated herein and elsewhere [38]. This association is reinforced considering that, except for tetracyclines, all the antimicrobials' classes significantly associated with MDR in the final model, showed the same profile: low rates of resistance only to the class but intensely related to MDR. As an example, the fluroquinolones were the antimicrobial class most significantly associated with MDR (Table 5), although most of the tested *E. coli* were susceptible to ciprofloxacin (69.30%), the assessed fluroquinolone. However, among the fluroquinolone's resistant strains 91.89% (136/148) were also MDR, which suggest that the resistance to this class is probably given by unspecific mechanisms in the evaluated population, such as efflux pumps, able to confer resistance simultaneously to several different antimicrobial classes [39]. Although the genetic bases of resistance were not assessed in the present study, these findings all together, point to the wide dissemination

of generic MDR mechanisms in the studied population, which make the results observed even worrier. In addition, age was also associated with MDR in the final model, probably because older animals have longer exposure time to both the use of antimicrobials and infections.

The production of ESBL by *Enterobacteriaceae* is also a finding of great clinical and epidemiological relevance, as these isolates have the ability to hydrolyze the structures of beta-lactam rings, that act hampering the synthesis in the cell wall, thus promoting resistance against the antimicrobial [40]. Furthermore, the presence of this type of resistance may be an indication of resistance to other classes of antimicrobials [41]. Indeed, among the cephalosporin-resistant isolates that were ESBL-producing, 94.7% were also MDR, which is of great concern, since humans can acquire these strains through consumption of contaminated food or water, or through soil contamination and occupational activity [42]. The indiscriminate use of antimicrobials, aligned with several other factors, such as lack of adopted management measures in animal farms, lack of preventive measures, insufficient training of personnel, all together can lead to a high prevalence of ESBL production [4]. Moreover, it is also important to mention that most 52.63% (10/19) of the ESBL-producing strains were EHEC/STEC [43, 44], which have cattle as its main reservoir and are of significant clinical importance in public health, especially considering the O157 strains [45]. This role of cattle as carriers of these pathotypes may also explain the high prevalence and the absence of clinical signs for EHEC/STEC [46, 47] pathotype observed, considering the whole population. In the present study, the search for serogroup O157 among STEC/EHEC *E. coli* isolates resulted negative for the molecular test performed, indicating the absence of the marker among the tested strains. Nonetheless, it is worth to emphasize the importance of surveillance and control of this marker, mainly among bovine strains, as well as of MDR and ESBL

producers, as central components in the strategy to fighting the spreading of highly pathogenic *E. coli* and AMR.

Another important marker related to AMR is the plasmid-mediated colistin resistance carrying *mcr* genes, which threat animal health and public health, due to the possibility of quickly spread [48]. However, in the present study, albeit phenotypic resistance to colistin was found in 10.42% of the isolates, none exhibited the tested *mcr* genes (*mcr-1, mcr-2, mcr-3* and *mcr-5*). It is possible that other chromosomal mechanisms or different *mcr* genes not tested may be involved in the colistin resistance observed in vitro, such as membrane alteration, efflux pump and even cross-resistance with other antimicrobials [49]. Also, the phenotypic resistance to colistin observed, even possible considered low (10.42%) and having the genetic basics unknown, it can be pondered important from the public health perspective, since colistin is an antimicrobial commonly used as a last resort in infections caused by multidrug-resistant Gram-negative bacteria [50]. In addition, it is important to mention that resistance to colistin among *E. coli* (6.3%) and *Salmonella* enterica (21%) strains isolated from pigs was previously reported in Brazil [51].

Regarding to *E. coli* phylogroups, which is a combinations of genes associated with different sources of infections [28, 52], our stud showed phylogroup B1 as the most frequent, followed by phylogroup E and phylogroup A, which are in accordance to literature data, being most frequently associated with intestinal infections of cattle [53, 54].

Despite having a considerably expressive collection with 518 isolates, this sampling comes from a non-probabilistic collection, which brings a non-systematic error to the analysis that cannot be corrected. Another limitation of the study is that the collection came from samples that were isolated almost ten years ago, which limits the inferences today.

5. Conclusion

In conclusion, the pathogenic *E. coli* strains isolated from calves and buffalo calves from 1990 to 2013 in Minas Gerais assessed in the present study showed high rates of resistance to penicillin, tetracyclines and folate inhibitors, in addition to an alarming rate of multidrug resistance and strains able to produce ESBL, which altogether point to a non-negligible risk to public and animal health and for the need to build better strategies for monitoring bacterial infections caused by *E. coli* in animals.

Conflict of Interest Statement

The authors declare no conflict of interest

Acknowledgements

The authors are grateful to PPGCV-UFLA. DACC, ACRP and CRP are thankful the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) for the fellowships. The EMSD laboratory was supported by Fundação de Amparo à Pesquisa de Minas Gerais (Fapemig), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Capes. The control strains used were kindly provided by Dr. Roberta Hilsdorf Piccoli and Dr. Nilton Lincopan (Table 3).

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Figures

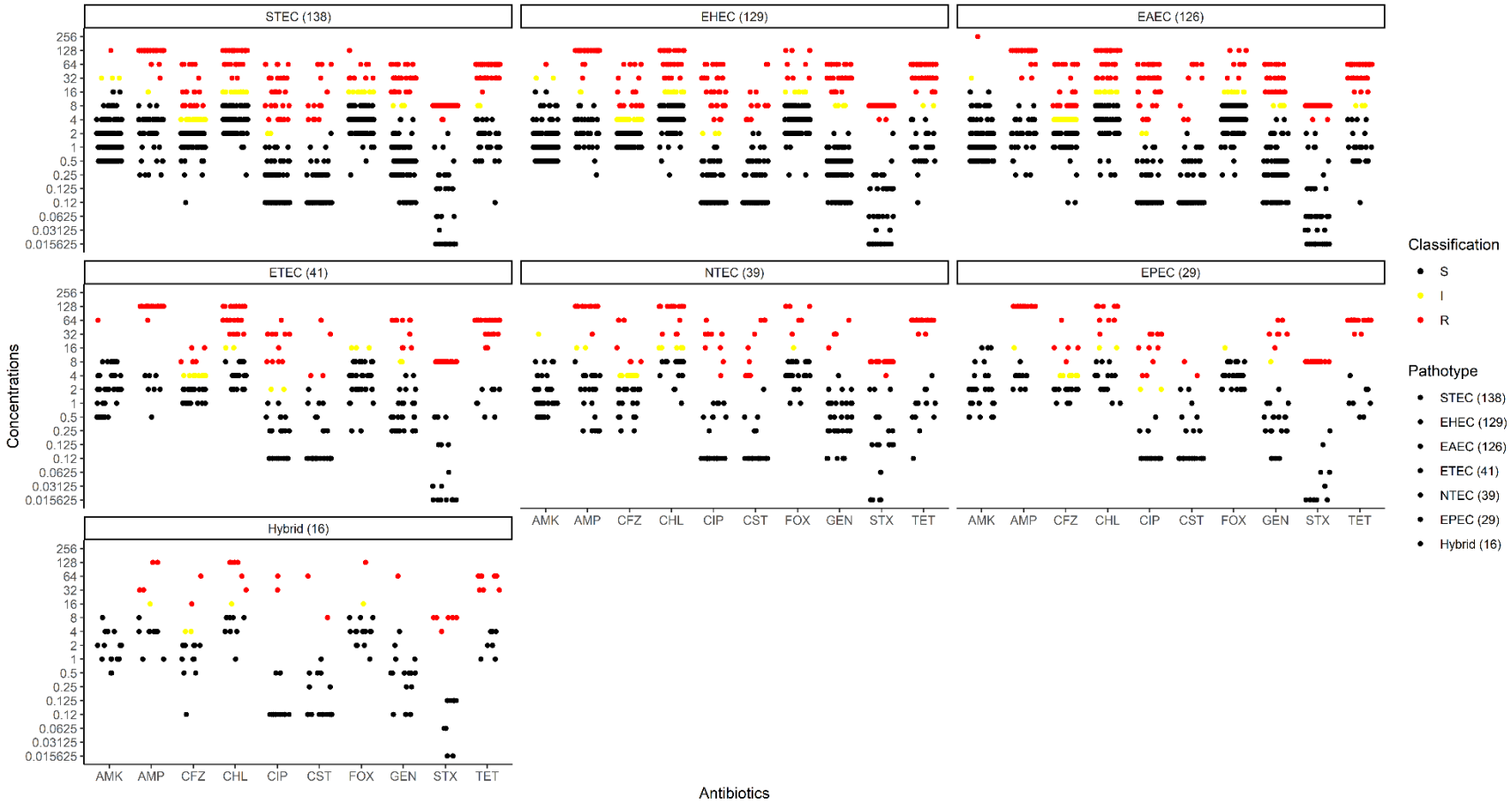

Fig 1. Susceptibility profile and minimal inhibitory concentrations (MIC) of several antimicrobials for *Escherichia coli* strains isolated from calves and buffalo calves from 1990 to 2013, in Minas Gerais, Brazil.

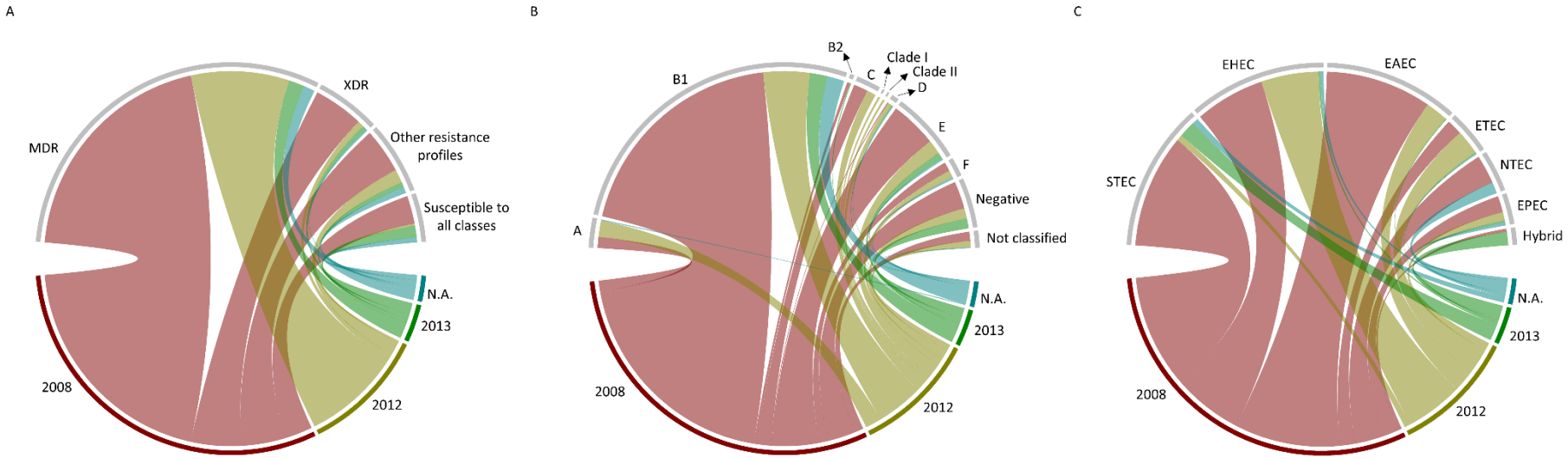

Fig.2 (A, B and C). Distribution of multi-resistance phenotype, phylogroups and pathotypes, according to year of isolation of *Escherichia coli* strains isolated from calves and buffalo calves from 1990 to 2013, in Minas Gerais, Brazil.

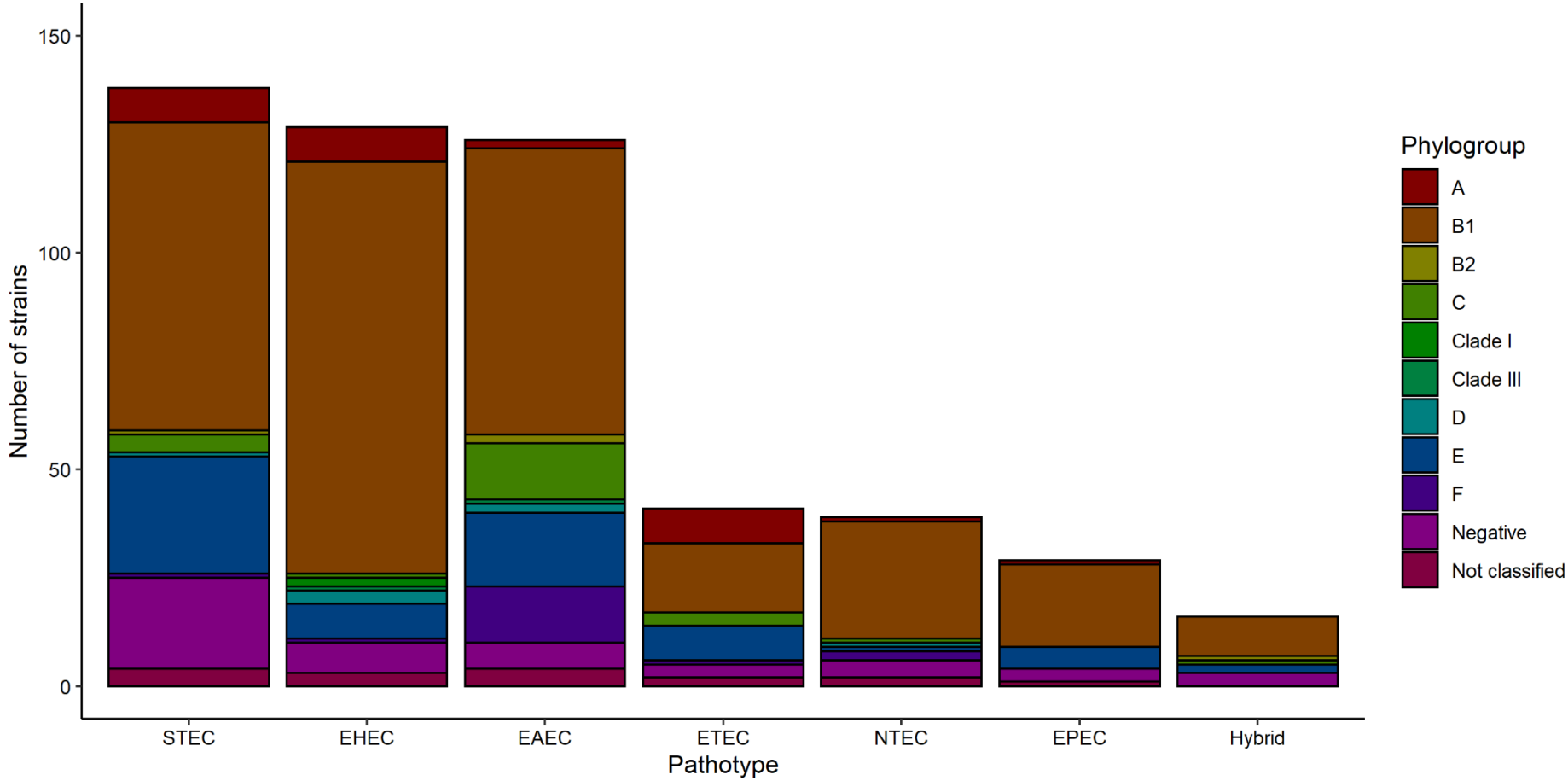

Fig.3 Relation between pathotypes and phylogroups of *Escherichia coli* strains isolated from calves and buffalo calves from 1990 to 2013, in Minas Gerais, Brazil.

Tables

Table 1. Detailed epidemiological information of *Escherichia coli* strains isolated from calves and buffalo calves in Minas Gerais, Brazil, 1990 to 2013.

Reference	N of isolates	Geographic location	Year of isolation	Host	Age of animals	Diarrhea* (%)
[18,19]	343	Martinho Campos	2008	Calves	1 to 9 weeks	123/343 (35.86 %)
[18,19]	80	Belo Horizonte	2012	Calves	1 to 6 weeks	57/80 (71.25%)
[18,19]	36	Belo Horizonte	2012	Calves	1 to 9 weeks	12/36 (33.33%)
[20]	35	Oliveira	2013	buffalo calves	1 to 12weeks	17/35 (48.57%)
[21]	24	NI	NI	Calves	1 to 9 weeks	13/24 (54.16%)

*The pathogenic strains of *Escherichia coli* were isolated from diarrheic and no diarrheic calves.

Table 2. Antimicrobial susceptibility of pathogenic *Escherichia coli* strains isolated from calves and buffalo calves in Minas Gerais, Brazil, 1990 to 2013.

Antimicrobial	Class	Range MIC	Breakpoints			R (%)	I (%)	S (%)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
			S	I	R					
Amikacin	Aminoglycoside	0.5-256	≤ 16	32	≥ 64	0.77	1.35	97.87	1.15	5.80
Ampicillin	Penicillin	0.25-128	≤ 8	16	≥ 32	61.96	1.54	36.48	>128	>128
Cefazolin	Cephem	0.12-64	≤ 2	4	≥ 8	17.18	1.15	59.26	1.90	6.17
Cefoxitin	Cephem	0.25-128	≤ 8	16	≥ 32	6.56	6.17	87.25	2.02	11.83
Ciprofloxacin	Fluoroquinolone	0.12-64	≤1	2	≥ 4	28.57	2.12	69.30	0.17	23.90
Chloramphenicol	Phenicol	0.25-128	≤ 8	16	≥ 32	37.06	9.65	49.42	6.55	>128
Colistin	Polymyxin E	0.12-64	≤ 2	-	≥ 4	10.42	0.00	89.57	<0.12	2.94
Gentamicin	Aminoglycoside	0.12-64	≤ 4	8	≥ 16	24.32	3.28	72.39	0.42	24.63
Sulfamethoxazole/trimethoprim	Folate inhibitor	0.015/0.296-8/152	≤ 2/38	-	≥ 4/76	60.23	0.00	60.23	>8/152	>8/152
Tetracycline	Tetracycline	0.12-64	≤ 4	8	≥ 16	73.74	1.35	24.90	45.12	>64

S: susceptible; I: intermediate susceptible; R: resistant; MIC: minimal inhibitory concentration; MIC50: minimal inhibitory concentration that inhibited 50% of the tested strains; MIC90: minimal inhibitory concentration that inhibited 90% of the tested strains

Table 3. O157 serotype-specific and mobile colistin resistance (*mcr*) primers and positive controls tested in enterohemorrhagic, Shiga toxin-producing and cephalosporin resistant *Escherichia coli* strains isolated from calves and buffalo calves in Minas Gerais, Brazil, 1990 to 2013.

Target genes	Primers	Sequence 5'-3'	Product size (pb)	References	Positive controls
mcr-1	mcr1_fw	AGTCCGTTTGTTCTTGTGGC	320	[29]	*Klebsiella pneumonie* LEM2808
	mcr1_rev	AGATCCTTGGTCTCGGCTTG			
mcr-2	mcr2_fw	CAAGTGTGTTGGTCGCAGTT	715	[29]	*Escherichia coli* KP37
	mcr2_rev	TCTAGCCCGACAAGCATACC			
mcr-3	mcr3_fw	AAATAAAAATTGTTCCGCTTATG	929	[29]	*Escherichia coli* 2013-SQ352
	mcr3_rev	AATGGAGATCCCCGTTTTT			
mcr-5	mcr5_fw	ATGCGGTTGTCTGCATTTATC	1644	[29]	*Salmonella* Paratyphi 13-SAO1718
	mcr5_rev	TCATTGTGGTTGTCCTTTTCTG			
rfb-E (O157)	rfbE_fw	CAGGTGAAGGTGGAATGGTTGTC	296	[28]	*Escherichia coli* EHEC O157 LEM2807
	rfbE_rev	TTAGAATTGAGACCATCCAATAAG			
chuA	chuA.1b	5’-ATGGTACCGGACGAACCAAC-3’	288	[27]	*Escherichia coli* STEC 424 LEM 2808
	chuA.2	5’-TGCCGCCAGTACCAAAGACA-3’			
yjaA	yjaA.1b	5’-CAAACGTGAAGTGTCAGGAG-3’	211	[27]	*Escherichia coli* STEC 424 LEM 2808
	yjaA.2b	5’- AATGCGTTCCTCAACCTGTG-3’			
TspE4.c2	TspE4C2.1b	5’-CACTATTCGTAAGGTCATCC-3’	152	[27]	*Escherichia coli* STEC 168 LEM 2809
	TspE4C2.2b	5’- AGTTTATCGCTGCGGGTCGC-3’			
arpA	Acek.f	5’-AAGCCTATTCGCCAGCTTGC-3’	400	[27]	*Escherichia coli* EHEC 028 LEM 2810
	ArpA1.r	5’-TCTCCCCATACCGTACGCTA-3’			
arpA	ArpAgpE.f	5’-GATTCCATCTTGTCAAAATATGCC-3’	301	[27]	*Escherichia coli* ETEC 068 LEM 2811
	ArpAgpE.r	5’-GAAAAGAAAAAGAATTCCCAAGAG-3’			
trpA	trpAgpC.1	5’-AGTTTTATGCCCAGTGCGAG-3’	219	[27]	*Escherichia coli* EHEC 107 LEM 2812
	trpAgpC.2	5’-TCTGCGCCGGTCACGCCC-3’			

Table 4 – Detailed information of ESBL-producing *Escherichia coli* isolated from calves and buffalo calves in Minas Gerais, Brazil, 1990 to 2013.

Strain	Host	Year of isolation	Animal age	Diarrhea	Geographical location	Antimicrobial resistance profile										MDR	Virulence profile										PG	PT	Ref.
						AMC	AMP	CFZ	CTX	CIP	CLO	COL	GEN	STX	TET		stx1	stx2	east	cnf2	saa	eae	sta	F41	F5	ehl				
62	calves	2008	5 weeks	+	Martinho Campos	S	R	R	R	R	S	S	S	R	R	+	-	+	-	-	-	-	-	-	-	-	-	STEC	[18,19]
119	calves	2008	2 weeks	-	Martinho Campos	S	R	R	S	R	R	S	S	R	R	+	-	-	+	-	-	-	-	-	-	-	B1	east1 +	[18,19]
253	calves	2008	9 weeks	-	Martinho Campos	S	S	R	R	S	S	R	S	S	R	+	-	-	-	+	-	-	-	-	-	-	-	NTEC	[18,19]
254	calves	2008	4 weeks	+	Martinho Campos	S	R	R	S	S	S	S	S	R	R	+	+	-	-	-	-	+	-	-	-	+	B1	EHEC	[18,19]
271	calves	2008	4 weeks	+	Martinho Campos	S	R	R	S	R	R	S	R	R	R	+	-	-	+	-	-	-	-	-	-	-	B1	east1	[18,19]
305	calves	2008	5 weeks	-	Martinho Campos	S	S	R	R	I	S	R	S	R	S	+	+	-	-	-	+	-	-	-	-	-	B1	STEC	[18,19]
310	calves	2008	5 weeks	-	Martinho Campos	S	S	S	R	S	I	R	S	S	S	-	+	-	-	-	-	+	-	-	-	-	B1	EHEC	[18,19]
316	calves	2008	5 weeks	-	Martinho Campos	S	S	R	R	R	S	R	S	S	S	+	-	+	-	-	+	-	-	-	-	-	B1	STEC	[18,19]
332	calves	2008	6 weeks	-	Martinho Campos	S	R	S	R	S	R	S	S	R	R	+	-	-	+	-	-	-	-	-	-	-	-	east1 +	[18,19]
340	calves	2008	2 weeks	+	Martinho Campos	S	R	R	S	S	R	S	S	R	R	+	-	-	+	-	-	-	-	-	-	-	-	east1 +	[18,19]
342	calves	2008	1 weeks	+	Martinho Campos	S	R	R	S	R	R	S	S	R	R	+	-	-	+	-	-	-	-	-	-	-	B1	east1 +	[18,19]
355	calves	2008	2 weeks	-	Martinho Campos	R	R	R	R	S	R	R	R	R	R	+	-	-	+	-	-	-	-	-	-	-	-	east1 +	[18,19]
390	calves	NI	2 weeks	+	NI	I	R	R	R	S	I	S	S	R	R	+	+	-	NT	-	NT	+	-	-	-	NT	B1	EHEC	[21]
392	calves	NI	2 weeks	-	NI	S	I	R	S	S	S	S	S	R	R	+	-	-	NT	-	NT	+	-	-	-	NT	B1	EPEC	[21]
416	buffallo	2013	NI	-	Oliveira	S	R	R	S	S	I	S	S	S	R	+	+	-	-	-	-	+	-	-	-	+	B1	EHEC	[20]
458	calves	2012	5 weeks	+	Belo Horizonte	S	R	R	S	S	R	S	R	R	R	+	-	-	+	NT	NT	+	-	-	-	NT	E	EPEC	[18,19]
479	calves	2012	2 weeks	-	Belo Horizonte	S	R	R	I	R	R	S	I	R	R	+	+	-	-	NT	NT	+	-	-	-	NT	D	EHEC	[18,19]
493	calves	2012	2weeks	+	Belo Horizonte	S	R	R	S	S	S	R	R	R	R	+	+	-	+	NT	NT	+	-	-	-	NT	-	EHEC	[18,19]
510	calves	2012	3 weeks	+	Belo Horizonte	S	R	R	S	S	R	S	R	R	R	+	-	-	+	NT	NT	-	-	-	-	NT	A	east1	[18,19]

Antimicrobial resistance (AMR), Amikacin (AMC), ampicillin (AMP), cefazolin (CFZ), ciprofloxacin (CIP), chloramphenicol (CLO), colistin (COL), gentamicin (GEN), sulfamethoxazole/trimethoprim (STX), Tetracycline (TET).

Table 5. Multivariable logistic regression model for the risk factors associated to AMR in Escherichia coli strains isolated from calves and buffalo calves in Minas Gerais, Brazil, 1990 to 2013.

AMR	*Odds Ratio*	p-value	95% CI	
Age (months)	1.5850	0.089	0.932	2.694
Aminoglycoside				
Susceptible	Base category			
Intermediary/Resistant	13.337	0.000	4.381	40.602
Penicillin				
Susceptible	Base category			
Intermediary/Resistant	28.481	0.000	12.263	66.147
Cephems				
Susceptible	Base category			
Intermediary/Resistant	4.993	0.000	2.354	10.593
Fluoroquinolones				
Susceptible	Base category			
Intermediary/Resistant	72.278	0.000	220.762	251.620
Phenicol				
Susceptible	Base category			
Intermediary/Resistant	3.895	0.000	1.851	8.195
Tetracycline				
Susceptible	Base category			
Intermediary/Resistant	17.482	0.000	6.623	46.142
EHEC				
Others	Base category			
Positive	2.431	0.028	1.099	5.373

Log likelihood = -110.833; Pseudo R2 = 0.6634. Sensitivity: 100.00%. Specificity: 51.16%. Correctly classified: 83.78%. *E. coli* enterohemorrhagic (EHEC).

Supplementary Material

Supplementary Table S1. Main criteria defined to classify pathotypes of *Escherichia coli* isolated from calves and buffalo calves in Minas Gerais, Brazil, 1990 to 2013.

Pathotype	Classification criteria	Reference
EPEC	(+) Intimin *eae*, and (-) *stx1/1tx2*	[16]
ETEC	+*F5(K99*) or F41 or Sta	[16]
STEC	(-) Intimin *eae* and (+) for (*stx1* or *stx2)*	[16]
EHEC	(+) Intimin *eae* and + for (*stx*1 or *stx2)*	[16]
NTEC	(+) *cnf2*	[16]
EAEC	(+) *east*	[16]
E. coli hybrid*	*combination of virulence factors from more than one of the pathotypes EPEC, ETEC, STEC, EHEC and NTEC	[16]

E. coli enteropathogenic (EPEC), *E. coli* enterotoxigenic (ETEC), *E. coli* Shiga toxin producer (STEC), *E. coli* enterohemorrhagic (EHEC), *E. coli* necrotoxigênic (NTEC) and *E. coli* enteroaggregative (EAEC)

Supplementary Table S2. Frequency distribution and percentage of categorical variables from the dataset of AMR in *Escherichia coli* strains isolated from calves and buffalos between 2008 and 2013 in Minas Gerais Brazil.

Variable	Frequency	Percentage (%)
Sampling Year		
2008	343	66.22
2012	116	22.39
2013	35	6.76
Not available	24	4.63
Phylogroup		
A	28	5.41
B1	303	58.49
B2	4	0.77
C	23	4.44
Clade I	2	0.39
Clade II	2	0.39
D	7	1.35
E	68	13.13
F	18	3.47
Negative	47	9.07
Not classified	16	3.09
Diarrhea		
Negative	296	57.14
Positive	222	42.86
Sex		
Female	472	95.55
Male	22	4.45
EHEC		
Others	389	75.10
EHEC	129	24.90
EPEC		
Others	489	94.40
EPEC	29	5.60
ETEC		
Others	477	92.08
ETEC	41	7.92
Hybrid		
Others	502	96.91
Hybrid	16	3.09
NTEC		
Others	479	92.47
NETC	39	7.53
STEC		
Others	380	75.68
STEC	138	24.32
EAEC		
Others	392	75.68

EAEC	126	24.32
AMR		
No	172	33.20
Yes	346	66.80
Aminoglycosides		
Susceptible	369	71.24
Intermediary/Resistant	149	28.76
Penicillin		
Susceptible	189	36.49
Intermediary/Resistant	329	63.51
Cephem		
Susceptible	291	56.18
Intermediary/Resistant	227	43.82
Fluoroquinolones		
Susceptible	359	69.31
Intermediary/Resistant	159	30.69
Phenicol		
Susceptible	276	53.28
Intermediary/Resistant	242	46.72
Polymyxin		
Susceptible	-	-
Resistant	518	100
Folate Inhibitor		
Susceptible	-	-
Resistant	518	100
Tetracycline		
Susceptible	129	24.90
Intermediary/Resistant	389	75.10

AMR: Antimicrobial resistance, *E. coli* enterohemorrhagic (EHEC), *E. coli* enteropathogenic (EPEC), *E. coli* enterotoxigenic (ETEC), *E. coli* hybrid, *E. coli* necrotoxigênic (NTEC). *E. coli* Shiga toxin producer (STEC) and *E. coli* enteroaggregative (EAEC)

Supplementary Table S3. P-values resulting from X^2 and/or Exact Fisher tests verifying the independence among the independent variables in the dataset of AMR in *Escherichia coli* strains isolated from calves and buffalos, from 2008 to 2013, in Minas Gerais, Brazil.

Variable	Year	Diarrhea	Sex	AMR	EHEC	EPEC	ETEC	Hybrid	NTEC	STEC	EAEC	Aminoglycosides	Penicillin	Cephems	Fluoroquinolones	Phenicol	Polymyxin	Folate Inhibitor	Tetracycline
Year	1																		
Diarrhea	0	1																	
Sex	0	0.235	1																
AMR	0	0.017	0	1															
EHEC	0	0.446	0.022	0.011	1														
EPEC	0.273	0.825	0.621	0.508	0	1													
ETEC	0	0.074	0.242	0.366	0	0.155	1												
Hybrid	0	0.271	0	0.047	0.016	1	0.628	1											
NTEC	0	0.564	0.627	0.013	0	0.154	0.061	0.623	1										
STEC	0	0.001	0.042	0.275	0	0	0	0.009	0	1									
East	0	0.214	0.002	0.972	0	0	0	0.016	0	0	1								
Aminoglycosides	0	0.047	0.002	0	0.186	1	0.664	0.049	0.055	0.244	0.463	1							
Penicillin	0	0.001	0.005	0	0.034	0.573	0.044	0.014	0.002	0.046	0.056	0	1						
Cephems	0.029	0.003	0.538	0	0.913	0.377	0.991	0.199	0.19	0.004	0.015	0.058	0	1					
Fluoroquinolones	0	0.721	0.005	0	0.567	0.199	0.884	0.167	0.283	0.171	0.012	0.01	0.003	0.018	1				
Phenicol	0.067	0.446	0.711	0	0.092	0.834	0.21	1	0.207	0.092	0.037	0.001	0	0	0	1			
Polymyxin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1		
Folate Inhibitor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	
Tetracycline	0	0	0.004	0	0.15	0.731	0.405	0.018	0.042	0.287	0.131	0	0	0	0.006	0	-	-	1

AMR: Multiresistence, *E. coli* enterohemorrhagic (EHEC), *E. coli* enteropathogenic (EPEC), *E. coli* enterotoxigenic (ETEC), *E. coli* hybrid, *E. coli* necrotoxigênic (NTEC). *E. coli* Shiga toxin producer (STEC) and *E. coli* enteroaggregative (EAEC)

General conclusion

In conclusion, high rates of antimicrobial resistance among virulent *E. coli* from intestinal tract of calves and buffalo calves were observed, in both chapters of the present thesis. These findings point to a non-negligible risk to public and animal health and to the need to build better strategies for monitoring bacterial infections caused by *E. coli* in animals.