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CARACTERIZAÇÃO DE *Escherichia coli* PRODUTORAS DE BETA-LACTAMASES DE ESPECTRO ESTENDIDO ISOLADAS DE BEZERROS

YASMIN GARCIA MARANGONI

Campo Grande – MS
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Characterization of** Escherichia coli **producers of extended spectrum beta-lactamases isolated from calves.

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Universidade Federal de Mato Grosso do Sul, como requisito parcial para a obtenção do título de Mestre em Ciências Veterinárias.

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CERTIFICADO

Certificamos que a proposta intitulada "Caracterização epidemiológica, patológica e molecular de diarreias em bezerros nos biomas Cerrado e Pantanal sul-matogrossense", registrada com o nº 1.134/2020, sob a responsabilidade de **CARLOS ALBERTO DO NASCIMENTO RAMOS** - que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA DA UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL/UFMS, na 5ª reunião ordinária do dia 30/07/2020.

FINALIDADE	( ) Ensino (x) Pesquisa Científica
Vigência da autorização	01/09/2020 a 31/07/2023
Espécie/Linhagem/Raça	Bos taurus/ Bos indicus/ Nelore e/ou cruzamentos
Nº de animais	400
Peso/idade	Não se aplica/ Até 90 dias
Sexo	Fêmeas e machos
Origem	Pantanal e Cerrado

Fábio José Carvalho Faria
Coordenador da CEUA/UFMS
Campo Grande, 11 de agosto de 2020.

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RESUMO

A resistência antimicrobiana não é um fenômeno recente, mas é um problema crítico de saúde pública mundial. A produção de beta-lactamases de espectro estendido (ESBL), pelo grupo das Enterobacteriales, é o mecanismo de resistência bacteriana adquirida cada vez mais citado nas últimas décadas, presentes na interface humano-animal-ambiente. Este estudo teve como objetivo verificar em amostras fecais de bezerros, a presença de cepas de *Escherichia coli* diarreiogênicas produtoras de ESBL, identificando os genes de resistência *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV}}$ e *bla*$_{\text{TEM}}$ pelo método de Reação em Cadeia da Polimerase (PCR) e fenotipicamente pelo método de triagem de disco de aproximação. Também se objetivou a padronização de um método de identificação rápido, para isolados de *E. coli* multirresistentes, usando o método de Espectroscopia de Infravermelho com Transformada de Fourier (FT-IR). Duzentos e oito isolados de *E. coli* diarreiogênicas foram submetidos à PCR, sendo a presença de pelo menos um dos genes citados verificada em 35,6% do total (n=74). A distribuição dos genes nas amostras positivas ocorreu da seguinte forma: 3,84% positivas para *bla*$_{\text{CTX-M-2}}$, 5,28% para *bla*$_{\text{SHV}}$ e 29,3% para o gene *bla*$_{\text{TEM}}$. Os genes bla$_{\text{CTX-M-15}}$ e *bla*$_{\text{CTX-M-8}}$ não foram observados no estudo. Quanto à suscetibilidade aos antimicrobianos, as amostras apresentaram altas taxas de resistência ao grupo dos beta-lactâmicos, bem como para tetraciclina (80%), no qual 50,9% (106/208) foram fenotipicamente multirresistentes, mostrando que animais portadores de cepas bacterianas com a enzima ESBL, podem ser um reservatório constante de disseminação. Adicionalmente, 40 isolados de *E. coli* diarreiogênicas sabidamente multirresistentes foram selecionados para serem mensurados no FT-IR, com espectros colhidos na faixa de 4000 a 700 cm^{-1}, 3000 a 2800 cm^{-1} e 1800 a 800 cm^{-1}. O método de FT-IR para o aprendizado de máquina foi capaz de identificar os isolados de *E. coli* diarreiogênicas multirresistentes com 70% de precisão no teste de validação, demonstrando que o FT-IR poderia ser uma ferramenta de triagem para estudos bacteriológicos e diagnóstico.

Palavras-chave: beta-lactâmicos, ESBL, FT-IR, pecuária e resistência à antibióticos.

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ABSTRACT

Antimicrobial resistance is not a recent phenomenon, but it is a critical problem of world public health. The production of extended spectrum beta-lactamase (ESBL) by the Enterobacteriales group is the increasingly cited bacterial resistance mechanism in recent decades, present in the human-amimal-environment interface. This study aimed to verify in fecal calf samples, the presence of ESBL-producing *Escherichia coli* strains, identifying *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ by the Polymerase Chain Reaction method (PCR) and phenotypically by the approach disc screening method. It also was an aim of a rapid identification method for multiresistant *E. coli* isolates, using the Fourier Transform Infrared Spectroscopy (FTIR) method. Diarrheagenic *E. coli* isolates, and eight isolates were subjected to PCR, the presence of at least one of the genes mentioned in 35.6% of the total (n = 74). A distributed genes from positive samples occurred as follows: 3.84% were positive for *bla*$_{\text{CTX-M-2}}$, 5.28% for *bla*$_{\text{SHV}}$ gene and 29.3% for the *bla*$_{\text{TEM}}$ gene. The *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-8}}$ genes were not observed in the study. Regarding susceptibility to antimicrobials, the samples showed high resistance rates to the beta-lactam group, as well as for tetracycline (80%), in which 50.9% (106/208) were phenotypically multi-resistant, showing that strains carriers with strains bacterial with ESBL enzyme can be a constant dissemination reservoir. Additionally, 40 isolates of *E. coli* diarrheagenic multiresistant were selected to be measured in the FTIR, with spectra harvested from 4000 to 700 cm^{-1}, 3000 to 2800 cm^{-1}, and 1800 to 800 cm^{-1}. The FTIR method for machine learning was able to identify the isolates of *E. coli* diarrheagenic multi-resistant with 70% accuracy in validation tests, demonstrating that the FTIR could be a screening tool for bacteriological and diagnostic studies.

Keywords: beta-lactams, ESBL, FTIR, livestock and antibiotic resistance.

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1. INTRODUÇÃO GERAL

A identificação de bactérias multirresistentes e seus genes de virulência na pecuária tem grande importância em saúde pública mundial, assim como a identificação de possíveis genes de resistência à antimicrobianos (AGRs) (HAN et al., 2017).

O rebanho comercial brasileiro cresce com taxas altas, chegando a 218,2 milhões de cabeças de gado. A região Centro-Oeste, respondeu por mais de 75 milhões dessas cabeças. Em termos municipais, Corumbá, Mato Grosso do Sul, segue em segunda posição com o maior rebanho nacional (IBGE, 2020).

A enterite em bezerros, provocada pela bactéria *Escherichia coli* tem sido relatada desde o início do século XX como um dos principais agentes envolvidos nas diarreias de origem infecciosa. Considerando que o estado de Mato Grosso do Sul é uma importante região de pecuária de corte, este agente etiológico e suas cepas diarreiogênicas passaram a ser estudadas e identificadas no estado (SMITH; ORCUTT, 1925; TUTIJA et al., 2022).

A Organização Mundial da Saúde, OMS (2017) lista agentes bacterianos e suas respectivas resistências antimicrobianas na medicina humana, como um alerta à população mundial a crescente resistência global aos medicamentos antimicrobianos, entretanto, dentro da medicina veterinária já podemos observar a cosmopolitização destes AGRs listados pela OMS, como a produção de beta-lactamase de espectro estendido (ESBL) pela bactéria *E. coli* na interface humano-animal-ambiente (MELO et al., 2018; PALMEIRA et al., 2020).

Atualmente, por não existirem programas de vigilância epidemiológica de abrangência nacional ativos referentes à resistência bacteriana e a seus mecanismos, torna-se difícil estimar a proporção de cepas multirresistentes e aquelas produtoras de ESBL na bovinocultura (SANTAJIT; INDRAWATTANA, 2016; CHONG et al., 2018).

Com o surgimento de ferramentas da biologia molecular, podemos realizar análises detalhadas das sequências de ácidos nucleicos para identificação de AGRs, porém sua abrangência, seu poder discriminatório e simplicidade são variáveis e, para muitas análises, o alto custo e o tempo dificultam sua implementação na rotina laboratorial (SABAT et al., 2013).

Hoje é altamente desejável a busca pela implementação de novos métodos para detectar microrganismos e AGRs, com resultados rápidos, reduzido custo com consumíveis e fácil portabilidade (BENGTSSON-PALME et al., 2017).

Os estudos de espectroscopia óptica, como a Espectroscopia no Infravermelho com Transformada de Fourier (FT-IR), representam uma ferramenta poderosa para atingir esses objetivos, bem como em estudo futuros, nos processos de vigilância epidemiológica nacional referentes à resistência bacteriana e a seus mecanismos, usando abordagens baseadas em análises vibracionais em conjunto com a bioinformática (NOVAIS et al., 2019).

Diante deste contexto relatado, este trabalho objetivou estudar a presença de cepas de *E. coli* diarreiogênicas produtoras de ESBL no rebanho do estado de Mato Grosso do Sul, Brasil, em conjunto com o aperfeiçoamento do diagnóstico de resistência antimicrobiana por técnicas de fotônica.

1.1 OBJETIVO GERAL

O presente estudo teve por objetivo investigar fenotipicamente e genotipicamente a presença de linhagens de *E. coli* produtoras de ESBL das famílias CTX-M (*bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8}}$ e *bla*$_{\text{CTX-M-15}}$), SHV (*bla*$_{\text{SHV}}$) e TEM (*bla*$_{\text{TEM}}$) na microbiota intestinal de bezerros, provenientes de fazendas das quatro macrorregiões do estado de Mato Grosso do Sul, Brasil, bem como realizar a padronização para o método de FT-IR em isolados de *E. coli* multirresistentes.

1.2 OBJETIVOS ESPECÍFICOS

- Avaliar *in vitro* o perfil de suscetibilidade aos antimicrobianos em isolados de *E. coli* diarreiogênicas pelo método de disco difusão;
- Identificar fenotipicamente a presença de estirpes produtoras de ESBL;
- Identificar genotipicamente por meio da técnica de Reação em Cadeia da Polimerase (PCR), os genes codificadores de ESBL: *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-15,}}$ *bla*$_{\text{SHV}}$ e *bla*$_{\text{TEM}}$;
- Avaliar o desempenho da técnica de Espetroscopia de Infravermelho por Transformada de Fourier (FT-IR) para identificar cepas multirresistentes de *E. coli.*

2 REVISÃO DE LITERATURA

2.1. Resistência antimicrobiana

A introdução de antibióticos no uso clínico foi indiscutivelmente o maior avanço médico do século 20 (KATZ; BALTZ, 2016).

Em 1904, Paul Ehrlich e Alexander Fleming iniciaram a moderna “era dos antibióticos”. Ehrlich iniciou um programa de rastreamento sistemático para encontrar um medicamento contra a sífilis, baseadas em arsênico sintético salvarsan, porém os primeiros relatos de resistência ao salvarsan foram em 1930 (EHRLICH, 1913).

Salvarsan foi substituído pela sulfonamida Prontosil (OTTEN, 1986), considerado o primeiro antimicrobiano de amplo espectro com uso clínico, entretanto foi amplamente substituído pela descoberta da penicilina por Alexander Fleming em 1928 (FLEMING, 1929). A partir de 1945, a penicilina foi purificada por um grupo de pesquisadores da Universidade de Oxford, e após esse período, houve relatos crescentes de resistência a este medicamento (ABRAHAM et al., 1941).

O início da “era de ouro” da descoberta de antibióticos, ocorreu entre as décadas de 1940 e 1960. A maioria desses compostos ainda está em uso clínico, mas sua eficácia foi prejudicada pelo aumento da resistência microbiana. Na verdade, a descoberta rápida e relativamente fácil de várias classes de antibióticos durante um período relativamente curto, levou ao uso excessivo desses medicamentos (KATZ; BALTZ, 2016).

Existem diferentes mecanismos de resistência antimicrobiana: a resistência intrínseca e a resistência adquirida. A resistência intrínseca ocorre de forma natural, como parte de um processo de evolução bacteriana. Alguns exemplos são membros da ordem Enterobacteriales, na qual a bactéria *E. coli* está inserida, resistentes as classes dos macrolídeos, glicopeptídeos, bem como ao ácido fusídico, linezolida e a penicilina G, considerados antimicrobianos de grande uso na rotina clínica (WHO, 2012; EUCAST, 2020; CLSI, 2021).

Com frequência as bactérias utilizam mais de uma estratégia para evitar a ação dos antimicrobianos. Na resistência adquirida, a bactéria altera seu material genético de duas formas: pela mutação no seu material genético; ou introdução de um material genético estranho, como genes de resistência, que podem ser

transferidos entre gêneros ou espécies diferentes de bactérias, principalmente devido à pressão seletiva exercida pelo uso indiscriminado de antimicrobianos (LIVERMORE et al., 2001; WHO, 2012; EUCAST, 2020).

O relatório O'Neill (2016) previu que, sem ação urgente, dez milhões de pessoas, por ano morrerão de infecções causadas por bactérias multirresistentes à antimicrobianos até 2050.

Como um alerta à população mundial a crescente resistência aos medicamentos antimicrobianos, a Organização Mundial da Saúde (OMS), lista os agentes bacterianos e suas respectivas resistências antimicrobianas (Tabela 1), utilizando critérios para a seleção das bactérias, como: a) quão letal as infecções que esses microrganismos causam são; b) se o seu tratamento requer longas estadias hospitalares; d) como eles se espalham facilmente entre os animais, dos animais aos seres humanos, e de pessoa para pessoa; e) se os microrganismos podem ser prevenidos; f) quantas opções de tratamento ainda existem para esses microrganismos (WHO, 2017).

Tabela 1: Agentes bacterianos e suas respectivas resistências antimicrobianas na medicina humana, segundo a Organização Mundial da Saúde (2017).

Prioridade crítica	**Prioridade alta**	**Prioridade média**
Acinetobacter baumannii resistente à carbapenêmicos	*Enterococcus faecium* resistente à vancomicina	*Streptococcus pneumoniae* não penicilina suscetível
Pseudomonas aeruginosa resistente à carbapenêmicos	*Helicobacter pylori* resistentes à claritromicina	*Haemophilus influenzae* resistentes à ampicilina
Enterobacteriaceae resistentes à 3ª geração de cefalosporinas e carbapenêmicos.	*Salmonella* sp resistentes à fluoroquinolonas	*Shigella* sp resistentes à fluoroquinolona
	Staphylococcus aureus resistente à vancomicina e meticilina	
	Campylobacter sp resistentes à fluoroquinolona	
	Neisseria gonorrhoeae resistentes à 3ª geração de cefalosporinas e fluoroquinolona	

Fonte: WHO (2017).

Dentro da medicina veterinária já podemos observar a cosmopolização de *A. baumannii* (POMBA et al., 2015; EWERS et al., 2017), *P. aeruginosa* (FERNANDES et al., 2018; ELSHAFIEE et al., 2019)*, Staphylococcus aureus*

(OLIVEIRA et al., 2016); *Salmonellas* species (COLOBATIU et al., 2015; PRIBUL et al., 2017), *Campylobacter* species (FRASAO et al., 2015), bem como algumas bactérias membros do grupo Enterobacteriales, como a *E. coli* (MELO et al., 2018; PALMEIRA et al., 2020) na interface humano-animal-ambiente.

A bactéria *E. coli* é considerada comensal uma vez que, apenas uma pequena parte das cepas apresentam patogenicidade responsável por enfermidades. A diferenciação de cepas patogênicas (diarreiogênicas) de cepas não patogênicas, é baseada na produção de fatores de virulência, sendo cinco os principais patótipos de *E. coli* diarreiogênica para bezerros: *E. coli* enterotoxigênica (ETEC), *E. coli* enteropatogênica (EPEC), *E. coli* enterohemorrágica (EHEC), *E. coli* produtora de toxina Shiga (STEC), *E. coli* necrotoxigênica (NTEC). Cada categoria possui capacidade de causar síndrome clínica, com características epidemiológicas e patológicas específicas (BLANCHARD, 2012; COURA et al., 2015).

Quando há disseminação de cepas de *E. coli* diarreiogênicas no rebanho bovino, podem surgir cepas bacterianas capazes de expressarem enzimas que conferem resistência a múltiplos antimicrobianos, em resposta ao amplo uso destas moléculas para tratar os casos de diarreia (BLANCHARD, 2012; COURA et al., 2015; POIREL et al., 2018).

A classe de antimicrobianos beta-lactâmicos constitui a primeira via de escolha para o tratamento de diarreia, devido a sua boa eficácia terapêutica e baixa toxicidade. Representantes dessa classe são o grupo das penicilinas (amoxicilina, associada ou não à clavulanato de potássio e ampicilina) e cefalosporinas (1ª e 3ª geração), ambos com a presença do anel beta-lactâmico em sua estrutura molecular (CONSTABLE, 2004; SPINOSA et al., 2014).

O mecanismo principal de ação dos beta-lactâmicos é a inibição da última etapa de síntese de peptídeoglicano para a formação da parede celular bacteriana, logo estes antimicrobianos somente são capazes de atuar em bactérias durante a fase de crescimento logarítmica, quando, então, há necessidade da síntese da parede celular (SPINOSA et al., 2014; ANDRADE; DARINI, 2016).

O mecanismo de resistência mais importante para os beta-lactâmicos é a produção de betalactamases pelas bactérias. As betalactamases produzidas por diferentes bactérias possuem propriedades físicas, químicas e funcionais

variadas, sendo algumas betalactamases específicas para penicilinas (penicilinases), outras para cefalosporinas (cefaloporinases) e outras que podem atuar em ambos os grupos de antimicrobianos, como as beta-lactamases de espectro estendido (ESBL) (SPINOSA et. al, 2014).

Diversos trabalhos têm apontado para o crescimento mundial das ESBL em bactérias Gram-negativas, como membros do grupo Enterobacteriales (ELLER et al., 2014; ROCHA et al., 2016; GUNDRAN et al., 2019; SUKMAWINATA et al., 2020; WIDODO et al., 2020). Estas enzimas quando produzidas pelas bactérias Gram-negativas são secretadas no espaço periplasmático (SPINOSA et. al, 2014) podendo hidrolisar o anel beta-lactâmico irreversivelmente da ligação amina do anel beta-lactâmico (Figura 1), impedindo a ação dos beta-lactâmicos na síntese da parede celular bacteriana (BUSH; JACOBY, 2010), hidrolisando todas as penicilinas, cefalosporinas, incluindo as de terceira e quarta geração, e o aztreonam (COQUE et al., 2008).

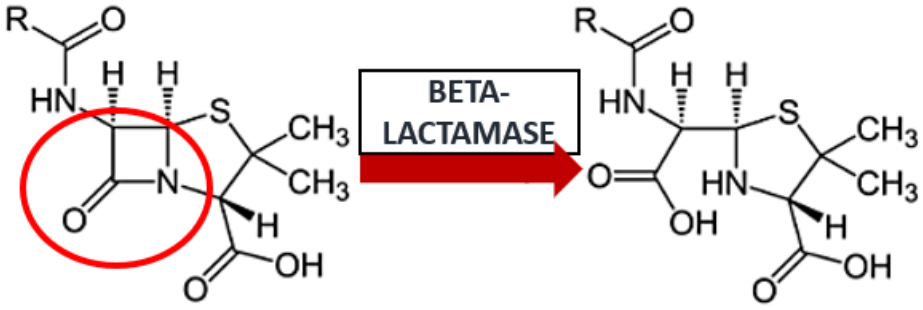

Figura 1: Esquema bioquímico demonstrando a ação da enzima beta-lactamase na ligação amina do anel beta-lactâmico. Arquivo pessoal.

A presença de ESBL tem um significado clínico importante, pois são codificadas por plasmídeo, e estes frequentemente carregam genes com co-resistência a outras classes de antimicrobianos, como, por exemplo, tetraciclinas, aminoglicosídeos e fluoroquinolonas. Portanto, as opções de antimicrobianos no tratamento de organismos produtores de ESBL são extremamente limitadas (PATERSON; BONOMO, 2005; ADEYANKINNU et al., 2014).

Dentre as variantes enzimáticas descritas, as enzimas da família SHV e TEM foram inicialmente reconhecidas em diferentes espécies bacterianas Gram-negativas, descritas em humanos a partir da década de 1960 (CANTÓN et al.,

2012), porém as enzimas da família CTX-M têm despertado atenção por sua alta incidência e grande capacidade de propagação (PITOUT et al., 2005; WIDODO et al., 2020).

As fezes de animais de produção, que possuem cepas com ARGs, como a enzima ESBL, podem ser reservatórios constantes de disseminação aos humanos (CARATTOLI, 2008; SCHMIDT et al., 2013), ao meio ambiente e a outros animais (BOONYASIRI et al., 2014; TEKINER; OZPINAR, 2016), levando à atrasos na terapia apropriada, aumento da morbidade, mortalidade, e consequentemente, em perdas econômicas aos produtores (SANTAJIT; INDRAWATTANA, 2016; CHONG et al., 2018).

Padrões de resistência entre bactérias têm sido estudados utilizando o cultivo a partir de métodos fenotípicos, como o teste de triagem de disco de aproximação (JARLIER et al., 1988) (Figura 2), bem como métodos moleculares, como a Reação em Cadeia da Polimerase (PCR), localizando os genes *bla*$_{\text{CTX-M}}$*, bla*$_{\text{SHV}}$ *e bla*$_{\text{TEM}}$ (PATERSON; BONOMO, 2005).

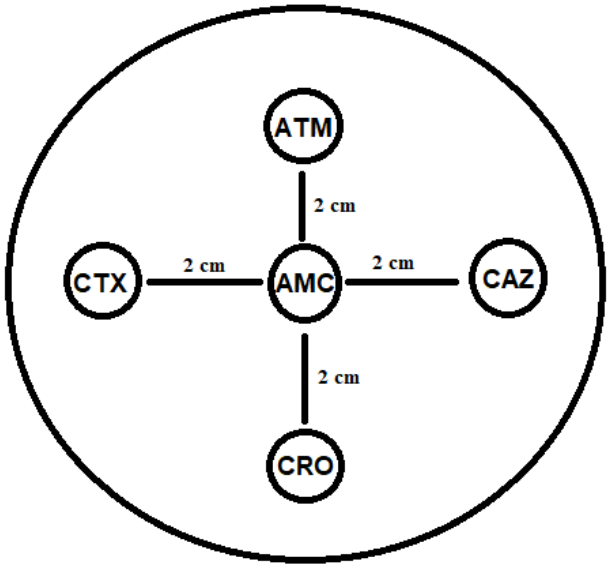

Figura 2: Ilustração da posição ideal das cefalosporinas, como cefotaxima (CTX), ceftazidima (CAZ) e ceftriaxona (CRO), o monobactâmico aztronam (ATM), e o inibidor de beta-lactamase amoxicilina + ácido clavulônico (AMC) no teste de triagem de disco de aproximação, padronizado pelo CLSI (2021). Arquivo pessoal.

Entretanto, hoje é altamente desejável a busca pela implementação de novos métodos de identificação bacteriana, dos quais não utilizem uma série de testes

para a identificação definitiva do organismo. Uma abordagem diferente para a identificação rápida de microrganismos é baseada em técnicas espectroscópicas (MAQUELIN et al., 2002).

2.2 Espectroscopia óptica

Do ponto de vista histórico, entre 1665 e 1666, Isaac Newton, um físico inglês, passou a introduzir a espectroscopia ao observar a luz do sol. A luz ao passar de meio de propagação para outro, como um prisma de vidro, poderia ser decomposta em diversas cores (NEWTON, 1972).

No ano de 1802, o interesse pela espectroscopia aumentou consideravelmente, quando um grupo passou a observar que substâncias emitiam cores diferentes quando colocadas sob a chama, com o objetivo de identificar os elementos químicos presentes. A partir de 1859, os químicos Robert Wilhelm Bunsen e Gustav Kirchhoff tiveram um papel fundamental na construção do primeiro espectroscópio, descobrindo que cada elemento químico produz um padrão único de linhas espectrais, distribuída ao longo dos comprimentos de onda (BUNSEN; KIRCHHOFF, 1862).

Esses e muitos estudos posteriores abriram caminhos para a utilização dessa metodologia para a análise de substâncias, como a descoberta do cloreto de césio e o cloreto de rubídio, bem como para a identificação de bactérias em diferentes níveis taxonômicos, desde gêneros, espécies a sorotipo/sorogrupo (BUNSEN; KIRCHHOFF, 1862; HELM et al. 1991; MAQUELIN et al., 2002).

Durante a década de 1990, houve novos desenvolvimentos de equipamentos, procedimentos experimentais e análise de dados em software, o que impulsionaram a novas publicações sobre a técnica de espectroscopia no infravermelho com transformada de Fourier (FT-IR) para identificação bacteriana (HELM et al. 1991).

2.2.1 Espectroscopia no Infravermelho com Transformada de Fourier (FT-IR).

Técnicas espectroscópicas destrutivas, como a Espectrometria de Massa com Ionização por Dessorção a Laser Assistida por Matriz (MALDI-TOF MS) tem mostrado resultados promissores para a identificação de grupos bacterianos Gram-negativos e Gram-positivos, porém inconsistências entre os protocolos

experimentais ou de análise de dados adotados podem dificultar o alcance de reprodutibilidade, padronização e, principalmente, a portabilidade para laboratórios de rotina de microbiologia clínica (SAUGET et al., 2017).

Considerando o bom desempenho em diferenciação de cepas, alto rendimento, custo-benefício, mínimo tempo de trabalho, e fácil portabilidade, métodos não destrutivos - àqueles que deixam a amostra intacta durante a análise - podem ser desenvolvidos com base em espectroscopias vibracionais, como o FT-IR (MAQUELIN et al., 2002; NOVAIS et al., 2019).

Na região do infravermelho (100 a 10000 cm^{-1}), ocorre a absorção da radiação infravermelha por um determinado tipo de amostra como, por exemplo, células bacterianas, causando excitação e vibração dos seus diferentes compostos químicos, com o objetivo de determinar a composição molecular (ácidos nucleicos, proteínas, lipídios e carboidratos) destas células bacterianas como um todo, semelhantes a impressões digitais ou espectro que podem ser utilizados para identificar a identidade do microrganismo (HELM et al. 1991; SETTLE, 1997; STUART, 2004).

O método de FT-IR, utiliza um equipamento chamado de espectrômetro (Figura 3). No espectrômetro, a fonte de luz emite um feixe de luz com energia e comprimento de onda bem definido. Este feixe de luz ao passar pela amostra analisada pode interagir com o meio e ser transmitido para um fotodetector. Logo, um espectro característico da absorção de radiação eletromagnética da amostra é obtido, e por meio de cálculos matemáticos, da transformada de Fourier, a distância do comprimento óptico pode ser convertida para o valor da frequência de radiação e vice-versa, garantindo a otimização das funções da espectroscopia no infravermelho, permitindo maior sensibilidade e velocidade de análise (STUART, 2004).

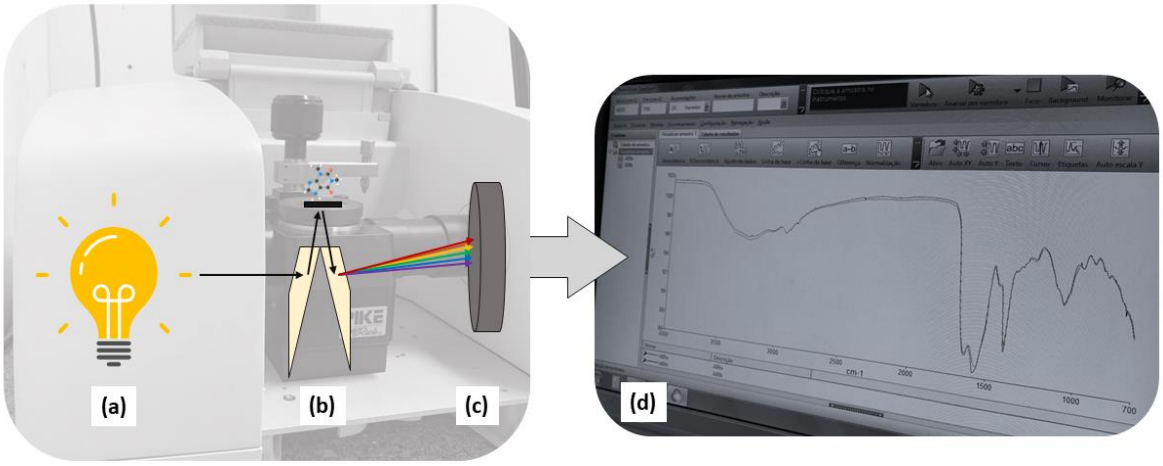

Figura 3: Ilustração básica da funcionalidade do espectrômetro, demonstrando a (a) fonte de luz, (b) interação da luz com a amostra analisada, (c) transmissão para um fotodetector, (d) registro do espectro da absorção de radiação eletromagnética, ou seja, “impressão digital. Arquivo pessoal.

O potencial do método de FT-IR para diferenciar e identificar grupos de cepas dentro de espécies Gram-positivas, como *Staphylococcus aureus* e Gram-negativas, principalmente em *E. coli*, sendo explorado inicialmente por Helm et al. (1991).

No estudo de Beutin et al. (2007), foi possível demonstrar que o FT-IR pode revelar diferenças estruturais de superfície entre dois grupos de cepas de *E. coli* O123. Para Abu-Aqil et al. (2022) e Abu-Aqil et al. (2023), foi utilizado o FT-IR e o aprendizado de máquina para identificar *E. coli* diretamente da urina de pacientes hospitalizados, bem como determinar o perfil de suscetibilidade aos antibióticos e a presença de isolados produtores de beta-lactamases de espectro estendido.

O estudo de Helm et al. (1991) determinou cinco janelas espectrais correspondentes à absorção expressa em números de onda (cm−1): janela 1, 3000-2800cm^{-1} dominada por vibrações presentes em ácidos graxos (lipídios); janela 2, 1800–1500^{cm-1}, dominada por vibrações das bandas amida I e amida II (proteínas e peptídeos); janela 3, 1500–1200cm^{-1}, com informações de proteínas, ácidos graxos e compostos de fosfato (região mista); janela 4, 1200–900cm^{-1}, bandas de carboidratos presentes na parede celular (polissacarídeos); janela 5, 900–700cm^{-1}, mostrando alguns padrões espectrais específicos, ainda não atribuídos a componentes celulares ou grupos funcionais (região de impressão digital).

Esses padrões específicos de impressão digital tornam este método uma alternativa para identificação rápida de bactérias, tipagem e a triagem no nível de subespécies, sendo as janelas 3 e 4, o ponto chave para a identificação bacteriana (MEYERS, 2006; SHI et al., 2020).

Apesar da grande aplicabilidade de FT-IR em microbiologia nos últimos anos, esta técnica pode ser considerada pouco convencional e seu potencial nas rotinas de microbiologia pode ser subestimado, pela aceitação e adaptação desta metodologia pela comunidade microbiológica. Entretanto, o método de FT-IR poderia agregar valor à caixa de ferramentas da identificação bacteriana e, em estudos futuros, nos processos de vigilância epidemiológica nacional referentes à resistência bacteriana e a seus mecanismos (NOVAIS et al., 2019).

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CAPÍTULO 2

4. (ARTIGO I)

[Artigo formatado de acordo com as normas da Revista Arquivo Brasileiro de Medicina Veterinária e Zootecnia]

IDENTIFICAÇÃO FENOTÍPICA E GENOTÍPICA DE *Escherichia coli* PRODUTORAS DE BETA-LACTAMASES DE ESPECTRO ESTENDIDO (ESBL) ISOLADAS DE FEZES DE BEZERROS

Phenotypic and genotypic characterization of Escherichia coli producing extended spectrum beta-lactamases (ESBL) isolated from calf feces.

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RESUMO

A produção de beta-lactamases por Enterobacteriales é o mecanismo de resistência mais comum aos antibióticos beta-lactâmicos. As beta-lactamases de espectro estendido (ESBL), são consideradas uma urgência clínica e epidemiológica mundial. Este estudo teve como objetivo verificar a presença de cepas de *Escherichia coli* diarreiogênicas, isoladas de bezerros, produtoras de ESBL. Amostras de fezes de 208 bezerros, de duas faixas etárias distintas, foram obtidas e cultivadas em meio de enriquecimento para isolamento de *E. coli*. A análise fenotípica foi realizada pelo método de triagem de disco de aproximação empregando discos de amoxicilina + ácido clavulônico (AMC), ceftriaxona (CRO), cefotaxime (CTX), ceftazidima (CAZ) e aztreonam (ATM). Foi realizada a Reação em Cadeia da Polimerase (PCR), para identificação dos genes de resistência $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CTX-M-15}}$, bla_{SHV} *e* bla_{TEM}. A presença de pelo menos um dos genes citados foi verificada em 35,6% do total (n=74). A distribuição dos genes nas amostras positivas ocorreu da seguinte forma: 3,84% (n=8) para $bla_{\text{CTX-M-2}}$, 5,28% (n=11) para bla_{SHV} e 29,3% (n=61) para o gene bla_{TEM}. Os genes $bla_{\text{CTX-M-15}}$ e $bla_{\text{CTX-M-8}}$

não foram observados no estudo. Quanto à suscetibilidade aos antibióticos, as amostras apresentaram altas taxas de resistência ao grupo dos beta-lactâmicos, bem como para tetraciclina (80%). Foi possível verificar que 50,9% (106/208) apresentaram perfil de multirresistência. Este estudo demonstra que bezerros podem ser reservatórios de cepas de *E. coli* produtoras de ESBL, constituindo um risco à saúde humana e animal, pelo risco de disseminação desses isolados no ambiente.

Palavras-chave: beta-lactâmicos, ESBL, pecuária e resistência à antibióticos.

ABSTRACT

Beta-lactamase production by Enterobacteriales is the most common resistance mechanism for beta-lactam antibiotics. Extended spectrum beta-lactamase (ESBL) are considered a world clinical and epidemiological urgency. This study aimed to verify the presence of diarrheagenic *Escherichia coli* strains, isolated from calves, ESBL producers. Fecal samples from 208 calves, from two distinct age groups, were obtained and cultivated in enrichment for *E. coli* isolation. Phenotypic analysis was performed by the approximation disc screening method employing amoxicillin discs + clavulonic acid (AMC), ceftriaxone (CRO), cefotaxime (CTX), ceptazidima (CAZ) and aztreonam (ATM). Polymerase Chain Reaction (PCR) was performed to identify the $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CTX-M-15,}}$ bla_{SHV} and bla_{TEM} resistance genes. The presence of at least one of the genes cited was verified in 35.6% of the total (n = 74). The distribution of genes in the positive samples occurred as follows: 3.84% (n = 8) to $bla_{\text{CTX-M-2}}$, 5.28% (n = 11) for bla_{SHV} and 29.3% (n = 61) for bla_{TEM} gene. The $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-8}}$ genes were not observed in the study. Regarding susceptibility to antibiotics, the samples showed high resistance rates to the beta-lactam group, as well as for tetracycline (80%). It was possible to verify that 50.9% (106/208) had multipurpose profile. This study demonstrates that calves can be *E. coli* strains reservoirs of ESBL producing, constituting a risk to human and animal health, due to the risk of dissemination of these isolates in the environment.

Keywords: beta-lactams, ESBL, livestock and antibiotic resistance.

INTRODUCTION

The identification of multiresistant bacteria and their virulence genes in livestock has great importance in public health worldwide, as well as the identification of possible

antibiotic resistance genes, resulting from exacerbated therapeutic conduct and selective pressure (Han *et al.*, 2017).

Diarrhea in calves is one of the main causes of economic losses in Brazilian and worldwide livestock. Since the beginning of the 20th century, the worldwide scientific community has pointed to the bacterium *Escherichia coli* as one of the main agents involved in diarrhea of infectious origin, therefore, this bacterial genus and its diarrheagenic strains have been studied and identified (Smith; Orcutt, 1925; Coura *et al.*, 2015; Tutija *et al.*, 2020).

The differentiation between *E. coli* pathogenic strains from non-pathogenic strains is based on the production of virulence factors, as well as on the identification of the mechanisms related to disease. In response to the widespread use of antimicrobials in the treatment of enteritis cases in calves, spread of virulence factors between herds may occur. This event may be relevant for the emergence and dissemination of bacteria capable of expressing enzymes that confer resistance to multiple antibiotics (Coura *et al.*, 2015; Poirel *et al.*, 2018).

Several studies have pointed to the worldwide growth of this resistance by members of Enterobacteriales, such as the emergence of ESBL, which can irreversibly hydrolyze the beta-lactam ring of the amine bond, preventing its action on transpeptidases (Bush and Jacoby, 2010). They can hydrolyze all penicillins, cephalosporins including third and fourth generation and aztreonam (Coque *et al.*, 2008).

Feces of production animals, which have strains with antimicrobial resistance genes, such as the ESBL enzyme, can be constant reservoirs of dissemination to humans (Carattoli, 2008; Schmidt *et al.*, 2013), the environment and other animals (Boonyasiri *et al.*, 2014; Tekiner; Ozpinar, 2016; Melo *et al.*, 2018; Brisola *et al.*, 2019), leading to delays in appropriate therapy, increased morbidity, mortality, and consequently, economic losses to producers and industry (Santajit; Indrawattana, 2016; Chong *et al.*, 2018) .

Thus, the aim of this study was to evaluate the presence of diarrheagenic *E. coli* strains producing ESBL, from an important beef cattle producing region in Brazil.

MATERIAL AND METHODS

Two hundred and eight isolates (n = 208) of diarrheagenic *E. coli* were used in this study. These isolates were previously characterized as diarrheagenic *E. coli* by Tutija *et al.* (2020).

These isolates came from stool and rectal swabs, from beef calves, with ages varying from one to 60 days. The study was conducted at the Veterinary Bacteriology Laboratory at the Universidade Federal de Mato Grosso do Sul (UFMS), from March 2021 to December 2022.

In vitro susceptibility to antimicrobials was verified, according to the antibiogram method standardized by the CSLI (Clinical Laboratory Standard Institute) (2021), which is based on the method originally described by Bauer *et al.* (1966). The most used antibiotics in the field reported by the veterinarians themselves were used, such as: amoxicillin + clavulanic acid (AMC), amoxicillin (AMO), cephalexin (CFE), enrofloxacin (ENO), florfenicol (FLF), gentamicin (GEN), norfloxacin (NOR), penicillin G (PEN), sulfamethaxazol+trimethoprim (SUT) and tetracycline (TET).

As for the phenotypic analysis of ESBL-encoding genes, the approximation disk screening technique according to Jarlier *et al.* (1988), was performed using amoxicillin + clavulanic acid (AMC), ceftriaxone (CRO), cefotaxime (CTX), ceftazidine (CAZ) and aztreonam (ATM) discs. A sample of *E. coli* ATCC® 25922 was used as a control, verifying the interaction of the AMC antibiotic with the others.

Subsequently, molecular analysis of the diarrheagenic *E. coli* isolates was performed to identify the genes encoding the ESBL enzyme, such as $bla_{\text{CTX-M}}$ (including $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-15}}$), bla_{SHV} and bla_{TEM}, using Reaction Polymerase in Chain (PCR).

The isolates were suspended in BHI broth (brain and heart infusion), incubated in a culture oven for 24 hours. After this time, the centrifugation was performed and the extraction of the sediment DNA started, according to the protocol described by Adwan (2014). For the analysis of DNA purity and its quantification, the NanoDrop ™ OneC (Thermo Fischer Scientific) was used.

For the amplification of target genes (Tab. 1), the reactions were performed with a final volume of 25µL, containing 2.5µL of 10X buffer (20 Mm Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U of *Taq* DNA polymerase (5 U/uL), 10 pmol of each primer (100 ng/uL) and 2µL of DNA (approximately 50 ng).

Amplification conditions for the CTX-M-like enzyme included: 1) initial denaturation at 94°C for 5 minutes; 2) denaturation at 94°C for 1 minute; 3) annealing at

706 a specific temperature for each primer, for 1 minute; 4) extension at 72°C for 1 minute;
707 5) repetitions of steps 2, 3 and 4, 29 times; ending with step 6, the final extension at 72ºC,
708 for 5 minutes.

709 As for the SHV-type enzyme, they included: 1) initial denaturation at 94°C for 5
710 minutes; 2) denaturation at 94°C for 30 seconds; 3) annealing at a specific temperature
711 for each initiator, for 30 seconds; 4) extension at 72°C for 1 minute; 5) repetitions of steps
712 2, 3 and 4, 32 times; with final extension at 72°C for 10 minutes.

713 For the TEM-type enzyme, they included: 1) initial denaturation at 94°C for 5
714 minutes; 2) denaturation at 95°C for 30 seconds; 3) annealing at a specific temperature
715 for each primer, for 1 minute; 4) extension at 72°C for 2 minutes; 5) repetitions of steps
716 2, 3 and 4, 32 times; with final extension at 72°C for 5 minutes.

717

718 Table 1. Initiators to identify the genes encoding ESBLs.

Genes	Sequence 5'-3'	T (°C) *	bp**	References
bla$_{\text{CTX-M-2}}$	F5'-GCGACCTGGTTAACTACAATC-3' R5'-CGGTAGTATTGCCCTTAAGCC-3'	55	351	Tollentino *et al.*, 2011
bla$_{\text{CTX-M-8}}$	F5'-CTGGAGAAAAGCAGCGGGGG-3' R5'-ACCCAGGATGTGGGTAGCCC-3'	55	320	Bonnet *et al.*, 2000
bla$_{\text{CTX-M-15}}$	F5'-CACACGTGGAATTTAGGGACT-3' R5'-GCCGTCTAAGGCGATAAACA-3'	56	550	Muzaheed *et al.*, 2008
bla$_{\text{SHV}}$	F5'-AGGATTGACTGCCTTTTTG-3' R5'-ATTTGCTGATTTCGCTCG3'	54	392	Colom *et al.*, 2003
bla$_{\text{TEM}}$	F5'-TTGGGTGCACGAGTGGGTTA-3' R5'-TAATTGTTGCCGGGAAGCTA-3'	54	465	Gangoue-Pieboji *et al.*, 2005

719 *T (°C): annealing temperature; ** bp: base pairs.

720

721 Subsequently, the amplified products were analyzed by electrophoresis in agarose
722 gel 1%, together with *E. coli* ATCC® 25922. Molecular weight marker (Ladder 100bp,
723 Ludwing Biotec, Brazil), and Gel Red nucleic acid stain (Biotium, USA) were used. The
724 amplified produts were visualized under ultraviolet light, with the aid of a transilluminator
725 (Transiluminator UV for gel - LTB HE, Loccus, Brazil).

726

727 **RESULTS**

728

In the phenotypic analysis, an increase in the diameter of the inhibition zone towards the AMC disk or the appearance of the phantom zone was observed in 31.25% (65/208) samples, as shown in the figure 1.

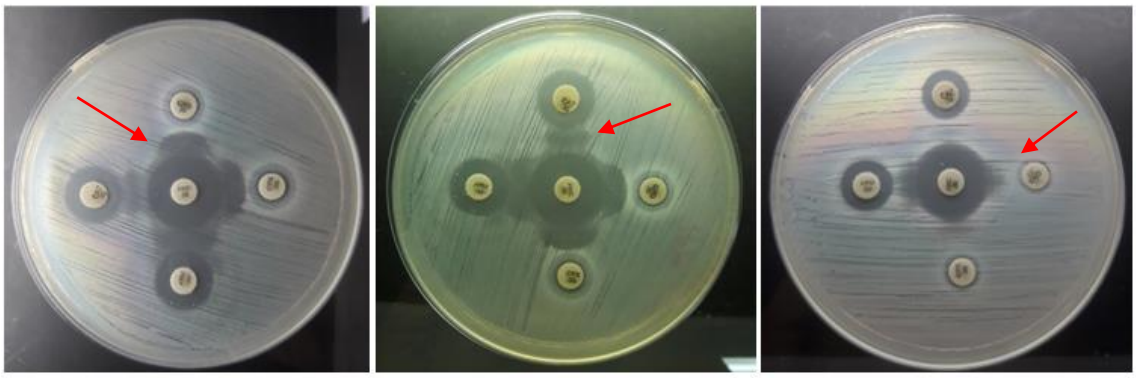

Figure 1: Positive approximation disk screening technique demonstrating the phantom zone or absence of inhibition halo (red arrow) to verify samples possibly producing the ESBL enzyme.

Genotypic analysis by the PCR method for the search for ESBL coding enzymes showed positivity in 3.84% (8/208) of the samples for the *bla*$_{\text{CTX-M-2}}$ enzymes, 5.28% (11/208) for the gene *bla*$_{\text{SHV}}$ and 29.3% (61/208) for the *bla*$_{\text{TEM}}$ gene (Figure 2). The *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M8}}$ genes were not observed in the study.

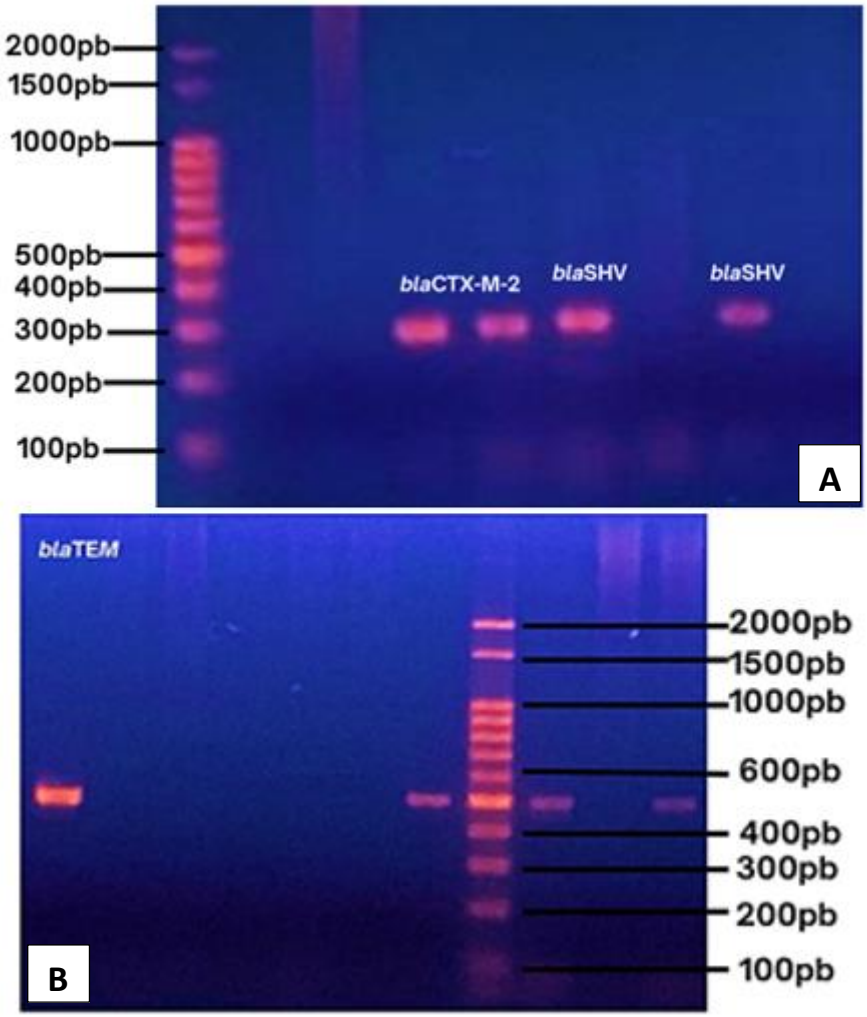

Figure 2: Electrophoresis of positive samples on a 1% agarose gel, using a molecular marker of 100 base pairs, visualizing the amplification of the *bla*$_{\text{CTX-M-2}}$ and *bla*$_{\text{SHV}}$ (A), as well as the *bla*$_{\text{TEM}}$ gene (B).

Through genetic identification, it was possible to observe that in the four macro regions of the state of Mato Grosso do Sul, at least one ESBL coding gene was located (Figure 3).

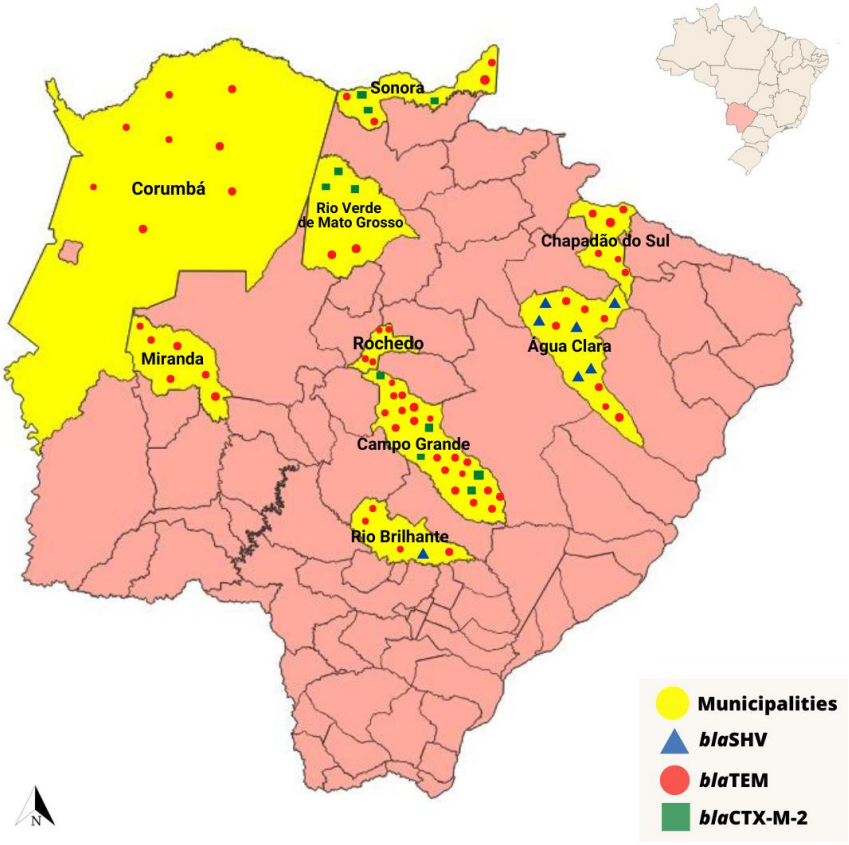

Figure 3: Map of the state of Mato Grosso do Sul, Brazil, showing the positive municipalities (yellow) for the genotypic identification of the ESBL-encoding genes: $bla_{\text{CTX-M-2}}$ (triangle), bla_{SHV} (square) and bla_{TEM} (circle).

In this study, it was possible to observe that 65.7% of the positive samples for ESBL coding genes are from calves between 1 and 30 days of age and 34.2% between 31 and 60 days.

In 35.6% (74/208) of the samples positive for the enzymatic variants CTX-M, SHV and TEM, the frequency of in vitro antimicrobial resistance of the class of beta-lactams tested can be observed (Tab. 2A) and, additionally, the correlation of the frequency of in vitro antimicrobial resistance of antimicrobials of the aminoglycosides, fluoroquinolones, tetracyclines, amphenicols and sulfonamides classes, with the localization of CTX-M, SHV and TEM family genes (Tab. 2B).

Table 2. Correlation of the frequency of antimicrobial resistance to beta-lactams (A) by the disk diffusion method and antimicrobials from the classes of aminoglycosides, fluoroquinolones, tetracyclines, amphenicols and sulfonamides (B), with the location of the $bla_{\text{CTX-M-2}}$ genes, bla_{SHV} and bla_{TEM} from diarrheagenic *E. coli* isolates by the PCR method.

A

Antibiotics	Number of resistant samples (%)		
	$bla_{\text{CTX-M-2}}$	bla_{SHV}	bla_{TEM}
Penicilin G	8 (100%)	11 (100%)	61 (100%)
Amoxicillin	7 (87.5%)	4 (36.36%)	43 (70.49%)

Cephalexin	7 (87.5%)	6 (54.54%)	23 (37.70%)
Amoxicillin + Clavulanic Acid	3 (37.5%)	0	16 (26.22%)
B			
Tetracycline	7 (87.5%)	7 (63.63%)	55 (90.16%)
Sulfamethoxazole+Trimethoprim	4 (50%)	4 (36.36%)	35 (57.37%)
Enrofloxacin	4 (50%)	6 (54.54%)	22 (36.06%)
Norfloxacin	3 (37.5%)	5 (45.45%)	17 (27.86%)
Gentamicin	3 (37.5%)	5 (45.45%)	14 (22.95%)
Florfenicol	2 (25%)	0	7 (11.47%)

After performing the antibiogram, the frequency of in vitro antimicrobial resistance of the 208 samples was observed (Tab. 3), in which the highest resistance rate was for penicillin G (100%), followed by tetracycline (80%), amoxicilin (61%), sulfametaxazole+trimethoprim (45.7%) and cephalexin (45%) and with less frequency of antimicrobial resistance, florfenicol (12%).

Table 3. Frequency of in vitro antimicrobial resistance in 208 diarrheagenic *Escherichia coli* isolates from stool and rectal swab samples.

Antibiotics	Number of resistant samples (%)
Penicilin	208 (100%)
Tetracycline	166 (80%)
Amoxicillin	126 (61%)
Cephalexin	94 (45%)
Sulfamethoxazole+Trimethoprim	95 (45.7%)
Enrofloxacin	66 (32%)
Norfloxacin	50 (24%)
Amoxicillin + Clavulanic Acid	43 (20.6%)
Gentamicin	39 (19%)
Florfenicol	25 (12%)

One hundred and six (50.9%) samples showed multidrug-resistance to three or more classes of antibiotics, in which no antibiotic was 100% effective, of these, 48.11% were positive for the phenotypic disk approximation test, and 42.45% molecularly positive for ESBL enzymes in relation to the presence of multidrug-resistance.

Twenty-eight (13.5%) samples of diarrheagenic *E. coli* were positive for the disk-proximation screening test and for the presence of one of the genes encoding ESBL. Forty-five samples (21.6%) did not express the phantom zone or lack of inhibition zone and were molecularly positive. However, 32 (15.4%) samples tested positive for the screening test and were molecularly negative.

DISCUSSION

Production animals, such as cattle, are considered one of the main reservoirs of *E. coli* producing ESBL (Chong *et al.*, 2018).

According to the age of the animals, Brunton *et al.* (2014), demonstrated in their study that resistance genes may be less prevalent in relation to the growth of the animals, due to the increase in the physical area in which they remain, hindering the transmission of resistance genes between them. In this case, the animals in the study were not weaned and were in the same physical area, according to the selected farm. The limitation of the age range in the present study, up to 60-days-old, may have influenced the non-alteration of the indices. When extending this age range to more than 60 days, it is possible that the variation mentioned by Brunton *et al.* (2014) could be noticed.

The situation of ESBL incidence in the world has undergone several changes, and in the new epidemiological scenario, the prevalence of enzymes of the CTX-M family stands out, both in hospital isolates, the community, and animals. This worldwide spread was described as "the CTX-M pandemic" (Cantón *et al.*, 2012).

In the present study, enzymes of the CTX-M family were highlighted in 8 (3.84%) of the 208 fecal samples from calves. The study by Sukmawinata *et al.* (2020) confirmed the presence of this family in the feces of thoroughbred horses in Japan, as well as the study by Gundran *et al.* (2019) which obtained 89.86% gene positivity in isolates from broiler chickens in the Philippines, confirming the cosmopolitanization of the production of ESBL type CTX-M.

In the molecular analysis, the prevalence of genes encoding ESBL was higher for the bla_{TEM} gene (29.3%), followed by bla_{SHV} (5.28%) and, lastly, by the $bla_{\text{CTX-M-2}}$ gene (3.84%). The $bla_{\text{CTX -M-15}}$ and $bla_{\text{CTX-M-8}}$ genes were not observed in this study.

In Brazilian cattle, the $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-2}}$ genes were found in the Southeast region, mainly in the states of São Paulo and Rio de Janeiro (Rocha *et al.*, 2016), as well as in the Northeast region (Palmeira *et al.*, 2020). Sukmawinata *et al.* (2020) reported a high prevalence of the $bla_{\text{CTX-M-2}}$ gene, however, among the few studies that investigated the prevalence of ESBL in calves, Eller *et al.* (2014) reported a high prevalence of $bla_{\text{CTX-M8}}$, unlike our study.

The studies reported in human and veterinary medicine by Cergole-Novella *et al.* (2010), Rocha *et al.* (2016), Widodo *et al.* (2020), and Ejaz *et al.* (2021) demonstrated that the prevalence of the $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} gene is increasing among ESBL-producing bacteria, that corroborates our study. They are common in humans (Aworh *et al.*, 2022), especially in nosocomial infections; in animals - dogs, cats, horses, chickens,

pigs, cattle, wild animals (Ewers *et al.*, 2010; Melo *et al.*, 2018; Brisola *et al.*, 2019) - and in the environment (Cantón *et al.*, 2012).

For Aworh *et al.* (2022), healthcare professionals should be aware that people who have close contact with calves and their feces are a high-risk group for spreading *E. coli* with ESBL, when compared to the general population. In their study, in addition to isolating *E. coli* with ESBL in 45.4% of calves' feces from an abattoir, they observed the presence of this enzyme in workers (41.2%) and in the work environment (13.4%).

The WHO (2017) lists bacterial agents and their respective antimicrobial resistance in human medicine, but within veterinary medicine we can already observe the cosmopolitanization of *Acinetobacter baumannii* (Pomba *et al*., 2015; Ewers *et al.*, 2017), *Pseudomonas aeruginosa* (Fernandes *et al.*, 2018; Elshafiee *et al.*, 2019), as well as ESBL-producing *E. coli* (Melo *et al.*, 2018; Brisola *et al.*, 2019; Palmeira *et al.*, 2020) in the critical priority group, and in the high priority group, *Staphylococcus aureus* (Oliveira *et al*., 2016), *Salmonella* species (Colobatiu *et al.*, 2015; Pribul *et al.*, 2017) and *Campylobacter* species (Frasao *et al.*, 2015), at the human-animal-environment interface.

The production of ESBL by *E. coli* bacteria is a major public health problem, as infections by these bacteria can result in failure of beta-lactam therapy (Chong *et al.*, 2018). Another reason is the phenomenon of co-resistance to other antibiotics, as they are encoded by plasmids. Profiles of resistance genes for aminoglycoside, macrolides, tetracyclines and fluoroquinolones antibiotics were observed by Carey *et al.* (2022) for the $bla_{\text{CTX-M}}$ gene, which may facilitate co-selection processes, limiting current therapeutic options (Paterson; Bonomo, 2005; Cantón *et al.*, 2012; Adeyankinnu *et al*., 2014).

According to a report by the OIE (2021), tetracyclines were the most used antibiotics in food-producing animals, followed by macrolides and penicillins.

Regarding antimicrobial resistance to the beta-lactam class and the location of the genes that encode ESBL, we could observe that the highest index of resistance was for penicillin G (100%), similar results to those of Shahrani *et al.* (2014), who evaluated the antimicrobial resistance profile correlated with the identification of the STEC virulence gene in samples of diarrhea from calves. However, it is known that members of the Enterobacterales group, such as *E. coli*, are intrinsically resistant to penicillin G, that is, this drug was being administered incorrectly, possibly due to the lack of knowledge of the bacteria's intrinsic resistance.

Brower *et al.* (2017), Rahmahani *et al.* (2020), and Jarrige *et al.* (2020), observed a profile of significant resistance to tetracycline in poultry farms (47%), domestic chickens (48%), from cloacal swab, and in milk dairy calves (68%), results that corroborate the findings of this study, presenting antimicrobial resistance in 80% (166/208) for tetracycline. In addition, records from the farms tested showed that, at some point during management, tetracycline was administered to the calves in the study.

Bacteria showed the lowest frequency of resistance to florfenicol, with only 12% (25/208) of inhibition failure in the tested samples, as well as the study by Nespolo *et al.* (2014), who analyzed 52 bovine isolates destined for slaughter, obtained a resistance index of 1.92% to florfenicol.

E. coli is also prone to developing broad drug resistance, as it has a great capacity to accumulate resistance genes, mainly by horizontal gene transfer, which leads to infections that are difficult to treat (Poirel *et al.*, 2018). In the present study it was observed three samples with two tested genes, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM,}}$ and three samples with *bla*$_{\text{CTX-M-2}}$ and *bla*$_{\text{TEM}}$, as well as their multidrug resistance to six classes of antibiotics, such as beta-lactams, fluoroquinolones, tetracycline, aminoglycoside, sulfonamides and amphenocol.

The multidrug-resistance profile, characterized by its resistance to three or more classes of antimicrobials (Magiorakos *et al.*, 2011), was found in 50.9% (106/208) of the isolates, results like Cergole-Novella *et al.* (2010) and Brisola *et al.* (2019), in which multidrug resistance was observed in eight of the 12 samples (66.7%) and 50 of the 135 samples (37.4%), respectively.

Al-Tamimi *et al.* (2019) proposes to use the phenotypic synergy test to detect the synergistic ability of clavulanic acid with cephalosporins and monobactam in ESBL-positive isolates. However, in our study there were divergences between positive samples in the phenotypic test (15.4%) and negative in the genotypic analysis, which could explain this phenomenon is the use of specific primers for the CTX-M, SHV and TEM families in this study, and the literature mentions other genes that can encode ESBL enzymes such as *bla*$_{\text{CMY}}$ (Brisola *et al.*, 2019) and *bla*$_{\text{OXA}}$ (Palmeira *et al.*, 2020).

In the phenotypic synergism test, the ideal distance for high synergistic activity is 20 to 25 mm between cephalosporins/monobactam and amoxicillin and clavulanic acid (Jarlier *et al.*, 1988; Bengtsson-Palme *et al.*, 2017). In the present study, 45 (21.6%) samples did not express the phantom zone or any zone of inhibition and were molecularly

positive, possibly greater distances could decrease the sensitivity of the test, hiding most of the resistance factors during the laboratory routine (Al-Tamimi *et al.*, 2019).

Many production animals, carrying these strains of ESBL, can be constant reservoirs of dissemination for humans, such as, for example, the contamination of products of animal origin (Tekiner; Ozpinar, 2016).

Currently, there are no national programs for monitoring bacterial resistance and its mechanisms in livestock, which makes it difficult to estimate the proportion of ESBL-producing strains in the federations. In turn, human and animal infections by ESBL-producing bacteria is associated with increased mortality, morbidity, high cost of hospitalization, delay in adequate therapy and, consequently, economic losses for producers (Santajit; Indrawattana, 2016; Chong *et al.*, 2018).

CONCLUSION

This study presents the first report on the identification of the $bla_{\text{CTX-M-2}}$, bla_{SHV} and bla_{TEM} genes in stool samples from calves from Mato Grosso do Sul, Brazil, alerting veterinarians about the use and knowledge of antibiotics in livestock, indicating the need to establish strategies for surveillance and tracking of antibiotic resistance in the country.

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5. (ARTIGO II)

[Artigo formatado de acordo com as normas da revista *Journal of Biophotonics*]

IDENTIFICATION OF MULTI-RESISTANT DIARRHEAGENIC *Escherichia coli* BY USING FTIR SPECTROSCOPY AND MACHINE LEARNING: A FEASIBLE STRATEGY TO IMPROVE THE GROUP CLASSIFICATION

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ABSTRACT

The identification of multidrug-resistant strains from *E. coli* species responsible for diarrhea in calves still faces many laboratory limitations and is necessary for properly monitoring the microorganism spread and control. Then, there is a need for the development of a screening tool for bacterial strain identification in microbiology laboratories, which must show easy implementation, fast response, and accurate results. The use of FTIR spectroscopy to identify microorganisms has been successfully demonstrated in the literature, including many bacterial strains, here we explored the FTIR potential for multidrug-resistant *E. coli* identification. First, we applied principal component analysis to observe the group formation tendency, the first results showed no clustering tendency with a messy sample score distribution, then we improved these results by properly selecting the main principal components. Finally, by using machine learning algorithms, a predicting model showed 70% overall accuracy in the validation test, which can be considered a great result for a screening tool. The present results

demonstrate the viability of the method for microorganism identification, which can significantly contribute to its control.

Keywords: multi-resistant bacteria, diarrheagenic, *Escherichia coli*, FTIR, machine learning.

INTRODUCTION

The introduction of antibiotics into clinical use was the greatest medical advance of the 20th century, and the rapid discovery of several classes of antibiotics in a relatively short period has led to the overuse of these drugs, which help promotes increasing antimicrobial resistance and antibiotics effectiveness loss [1]. The O'Neill report [2] predicted that without urgent action, ten million people a year will die from drug-resistant infections by 2050. As a warning to the world's population about the growing global resistance to antimicrobials, the WHO, World Health Organization [3] lists bacterial agents and their respective resistance to antimicrobials, divided into critical, high, and medium priority.

Important strategies have been adopted in this scenario for world public health maintenance, between them the identification of multidrug-resistant bacteria in herds of animals [4], since we have observed the migration of this microorganism to a human-animal-environment interface, such as *Acinetobacter baumannii* [5, 6], *Pseudomonas aeruginosa* [7, 8], *Staphylococcus aureus* [9]; *Salmonellas species* [10, 11], *Campylobacter species* [12], and *Escherichia coli* [13, 14, 15].

Besides, diarrhea in calves is one of the main causes of economic losses in Brazilian and worldwide livestock. Since the beginning of the 20th century, the worldwide scientific community points to the bacteria *E. coli* as one of the main agents involved in diarrhea of infectious origin; therefore, this bacterial genus and its pathogenic strains began to be studied and identified [16, 17, 18, 19].

The *E. coli* bacteria is considered a commensal of the intestinal flora, only a small part of the strains has pathogenicity responsible for diseases and the differentiation of pathogenic (diarrheagenic) strains from non-pathogenic strains is based on the production of virulence factors [17]. Once *E. coli* bacteria with virulence factors spread in the cattle herd, serious concerns arise due to its capability of expressing enzymes that confer resistance to multiple antimicrobials [20, 17, 21]. Animal feces, which have multiresistant

strains, serve as constant reservoirs for bacteria dissemination to humans [22, 23], the environment, and other animals [24, 25] which hinders the adequate therapy, increased morbidity, mortality, and cause economic losses to producers and industry [26, 27].

Phenotypic and molecular methods have been used in bacterial culture to analyze the nucleic acid sequences and identify microorganisms with multidrug resistance characteristics, but their variable discriminatory capacity, high cost, and time-consuming experimental routine are difficult the implementation as a standard laboratory procedure [28, 29].

In an attempt to overcome such limitations, several studies have been developed by using Fourier Transform Infrared Spectroscopy (FTIR) as a screening test [30, 31], which can obtain information about the sample chemical compounds thought their vibrational molecular modes, enabling the identification of sample molecular composition (nucleic acids, proteins, lipids, and carbohydrates) of these bacterial cells [32, 33, 34].

The potential use of FTIR spectroscopy to discriminate, classify and identify microorganisms has been successfully demonstrated in the literature, including many bacterial strains from Gram-positive, and Gram-negative species [32, 35, 36, 37, 38, 39, 40, 41]. The specific spectral patterns observed for each molecular group can be revealed with multivariate analysis and/or machine learning algorithms aid, which enable FTIR spectroscopy as an alternative for rapid bacterial identification at the subspecies level [42, 43]. Despite its great applicability for microorganism identification in recent years, this technique's potential in microbiology routines is still underestimated, due to the difficult acceptance and comprehension of the methodology by the microbiological community [31].

Therefore, here we explore the use of FTIR spectroscopy associated with machine learning algorithms for data analysis to identify and classify multidrug-resistant strains from *E. coli* species responsible for diarrhea in calves, which may add value to the microbiology laboratory toolbox and society [26, 27, 31]. Our study is focused on the development of a simple laboratory routine to sample preparation and data acquisition, then our data analysis aims to improve the method's accuracy for future implementation as a screening tool in bacterial strain identification in microbiology laboratories.

METHODS

Sample preparation and FTIR spectra acquisition

A total of 80 *Escherichia coli* isolates were analyzed. The sample pool was divided into 40 multidrug-resistant (MR) *E. coli* isolates and 40 replicates of *E. coli* ATCC® 25922. First, they were screened for analysis and placed in Brain Heart Infusion (BHI) broth for activation, subsequently, seeded on MacConkey Agar to verify purity and isolate selected colonies. Then, the colonies of MR *E. coli* and ATCC *E. coli* were suspended in 1 mL of BHI broth, and a resulting turbid inoculum was adjusted to the 0.5 McFarland scale (10^8 CFU/mL) before the measurements.

A small aliquot (30 µL) of the bacterial isolate was carefully deposited onto a flat silicon substrate (SiO2) by casting, followed by drying at 50°C per 30 minutes – this procedure was repeated three times until the obtention of a thick film. Each sample was produced in duplicate, and the samples were analyzed in two different spots from the center to the border, to avoid inhomogeneity in the sample composition due to the drying process [44]. Finally, the mid-infrared spectra were obtained by using an attenuated total reflectance accessory (ATR) at Fourier Transform Infrared Spectrophotometer (Spectrum 100, Perkin Elmer). The spectra were collected from 4000 to 700 cm^{-1}, with 4 cm^{-1} resolution and 10 scans. This entire experimental roadmap is illustrated in Figure 1.

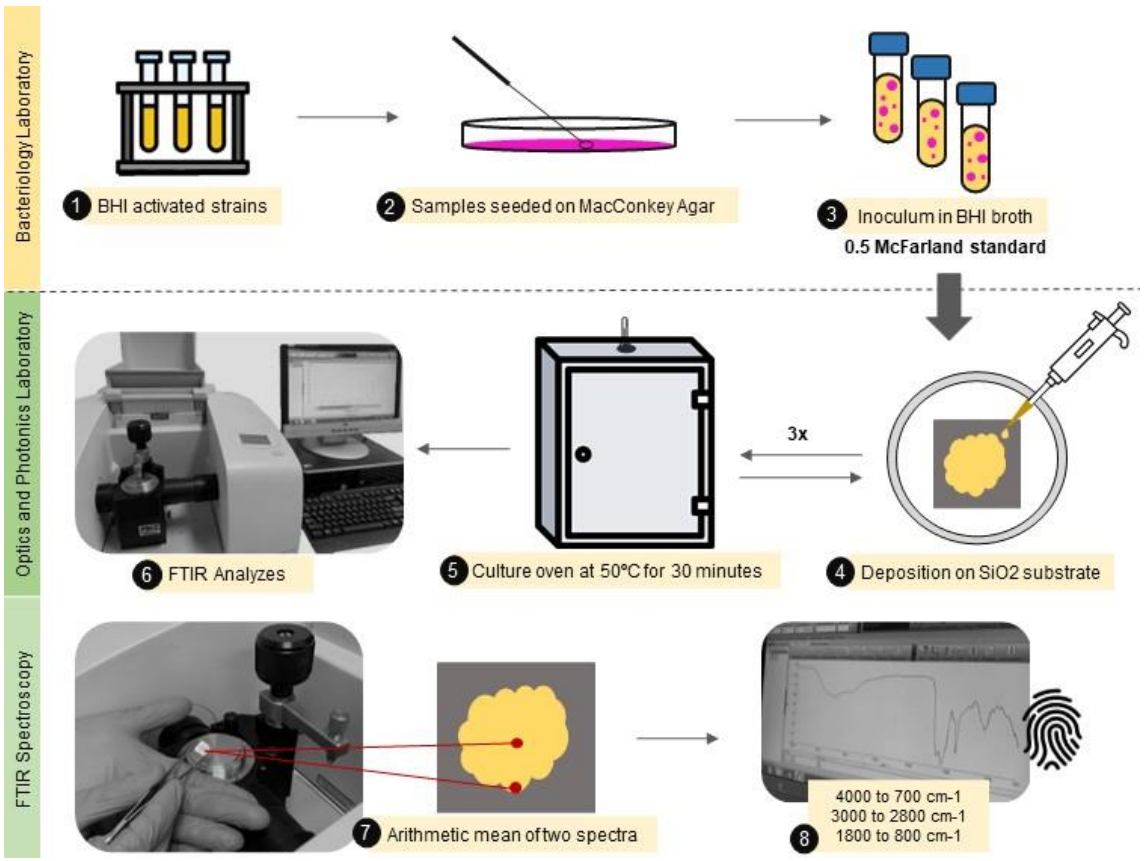

Figure 1: Experimental scheme describing the main steps from the sample (*E. coli*) preparation to FTIR spectra acquisition.

Data analysis and sample classification

The data analysis was performed in Python (version 3.9.12), using the Scikit-learn package (version 1.1.2) [45]. First, the FTIR spectra from two different spots of duplicate samples were averaged and then subjected to Standard Normal Variate (SNV) pre-processing method, which removes the variation from the baseline and rescales the spectral intensity, to prevent interference in the data analysis due to random experimental variations [46].

The average FTIR-SNV spectra for MR *E. coli* and ATCC *E. coli* group, in the 4000 to 800 cm^{-1} range, were submitted to principal component analysis (PCA) [47]. PCA is an unsupervised method that will project our pre-processed data set into a new dimension (PCs – principal components) which aims to maximize the data variance, this dimensionally reduced data set retains most of the information from the original variables and shows how each data sample is distributed in this new dimension (score plot), allowing cluster the similar samples and distinguish groups. Each PC represents a percentage of the data variance and, allows us to analyze the main spectral range that most contributes to the data variance percentage through the loading plot. PCA is an important step to visualize the group classification tendency. Here we analyzed three different ranges: (i) 4000 to 800 cm^{-1}; (ii) 3000 to 2800 cm^{-1}, and (iii) 1800 to 800 cm^{-1}, to use only those vibrational modes that improve the group clustering and classification and eliminate highly correlated data [48].

The sample classification is performed by prediction models built by machine learning (ML) algorithms using PC output data from 70% sample set. Before sample classification tests, we must determine the ideal number of PCs used by ML algorithms to avoid overfitting and underfitting [49, 50]. Here we used the Feature Selection Recursive Feature Elimination (RFE), which selects the main PCs that most contribute to achieving high accuracy and remove other PCs with the weakest contribution to correct sample classification in ML tests [51]. The use or removal of a determined PC was made based on the accuracy achieved by using Linear Discriminant Analysis (LDA) to classify the samples in a Leave One Out Cross-validation (LOOCV) test.

In a brief description, Discriminant Analysis (DA) classifies the sample based on the distance between the sample data and the contour built by using a linear (L) or

quadratic (Q) function to separate the classes (group) [52]. In LOOCV, one sample is taken from the data set, and the others are used to build the prediction model (training). Then, the prediction model accuracy is tested by using the sample data withdrawal from the data set. The procedure is repeated until all sample data have been tested [53].

After determining the ideal number of PCs, and which PCs most contribute to sample classification in each spectral range analyzed, a LOOCV test was performed – by using the respective RFE-PCs data for each range – based on three different methods: (i) DA (described above); (ii) k-Nearest Neighbor (KNN), which uses the Euclidean distance between k closest neighbors to classify the sample [54]; and (iii) Support Vector Machine (SVM), which organizes each sample class through the optimization of a hyperplane – the hyperplane can be linear or nonlinear, being optimized to reach high performance – between the classes [55]. Finally, we were able to determine the best spectral range, ML algorithm, and PCs to build a predicting model, whose ability for generalization was tested in an external validation test by using 30% of the sample set.

RESULTS AND DISCUSSION

The average FTIR-SNV spectra for *E. coli* ATCC, and MR *E. coli* samples, Figure 2, exhibit great similarity between them, with a small standard deviation into the data set. It suggests that direct identification of the isolate is not possible, and the data set shows great coherence. There is a remarkable presence of the three most pronounced bands in the spectra, the first around 1600 cm^{-1} is the result of overlapped bands 1639 and 1583 cm^{-1}, assigned to Amides I and II from proteins, which is relatively wide, suggesting the presence of a third band, mainly due to the small shoulder observed around 1500 cm^{-1}. The vibrational bands assigned to Amides I (N-H and C=O) and II (N-H and C-N) have shown great contributions to biological sample classification in the literature [56, 57]. The second band around 1400 cm^{-1} can be assigned to C-H and C=O vibrational modes from lipids and proteins, while the third band around 1080 cm^{-1} is usually assigned to nucleic acid and phospholipids. Also, weak bands assigned to C-H, and C-H-O vibrational modes from carbohydrate below 1000 cm^{-1} was identified in the spectra [58, 59].

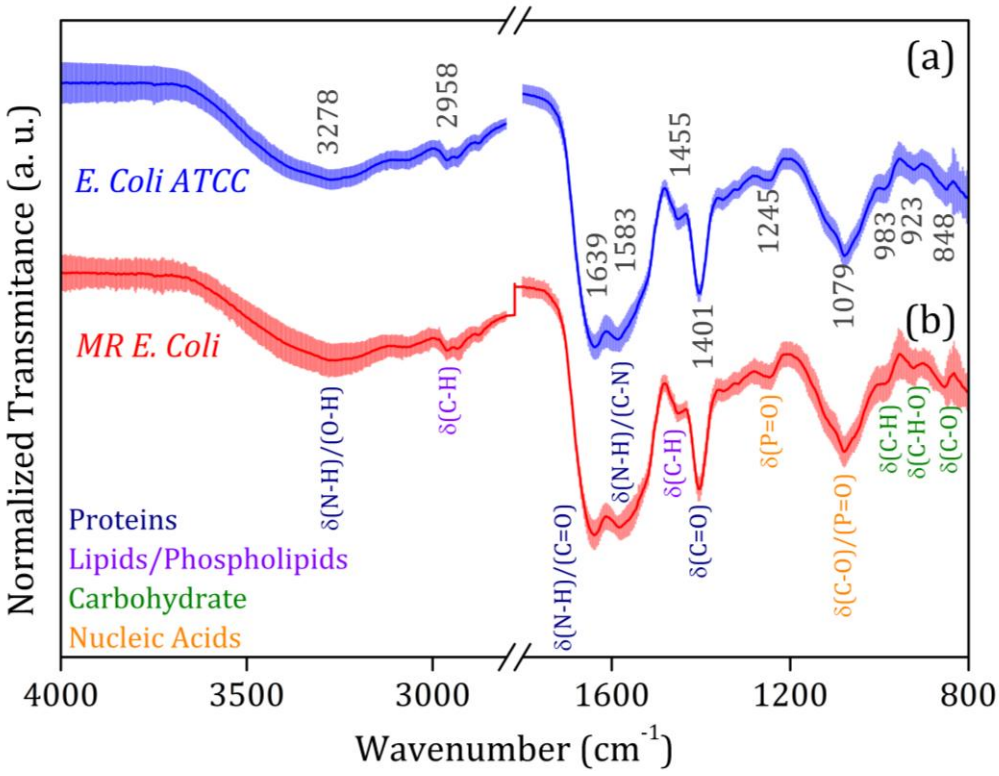

Figure 2: Average FTIR-SNV spectra for (a) *E. coli* ATCC (blue line), and (b) multidrug-resistant (MR) *E. coli* (red line) isolates. The main vibrational assignments are indicated in detail and their related molecular groups (proteins, lipids, carbohydrates, and fatty acids) are identified by colors. The symbol δ indicates the deformation vibrations.

Figure 3 shows the principal component analysis results for the FTIR-SNV spectral data from *E. coli* ATCC, and MR *E. coli* sample groups. On the left side, we can observe the score plot, and on the right side, the loading plot for (i) 4000 to 800 cm^{-1}; (ii) 3000 to 2800 cm^{-1}, and (iii) 1800 to 800 cm^{-1} range. For all cases analyzed two main characteristics stand out, the score plot exhibits a single cluster for both groups, which hinders the future group classification, and the loading shows few regions with a small contribution to the data variance, suggesting a small contribution of the bands for data variance in PC1 and PC2. We found PC1 and PC2 responsible for 79.2%, 96.8%, and 74.9% of data variance at 4000 to 800 cm^{-1}, 3000 to 2800 cm^{-1}, and 1800 to 800 cm^{-1} range, respectively, besides this great contribution for data variance previous studies have shown that the first PCs can be ignored, and high order PCs can be used to improve the group classification for ML algorithms [60].

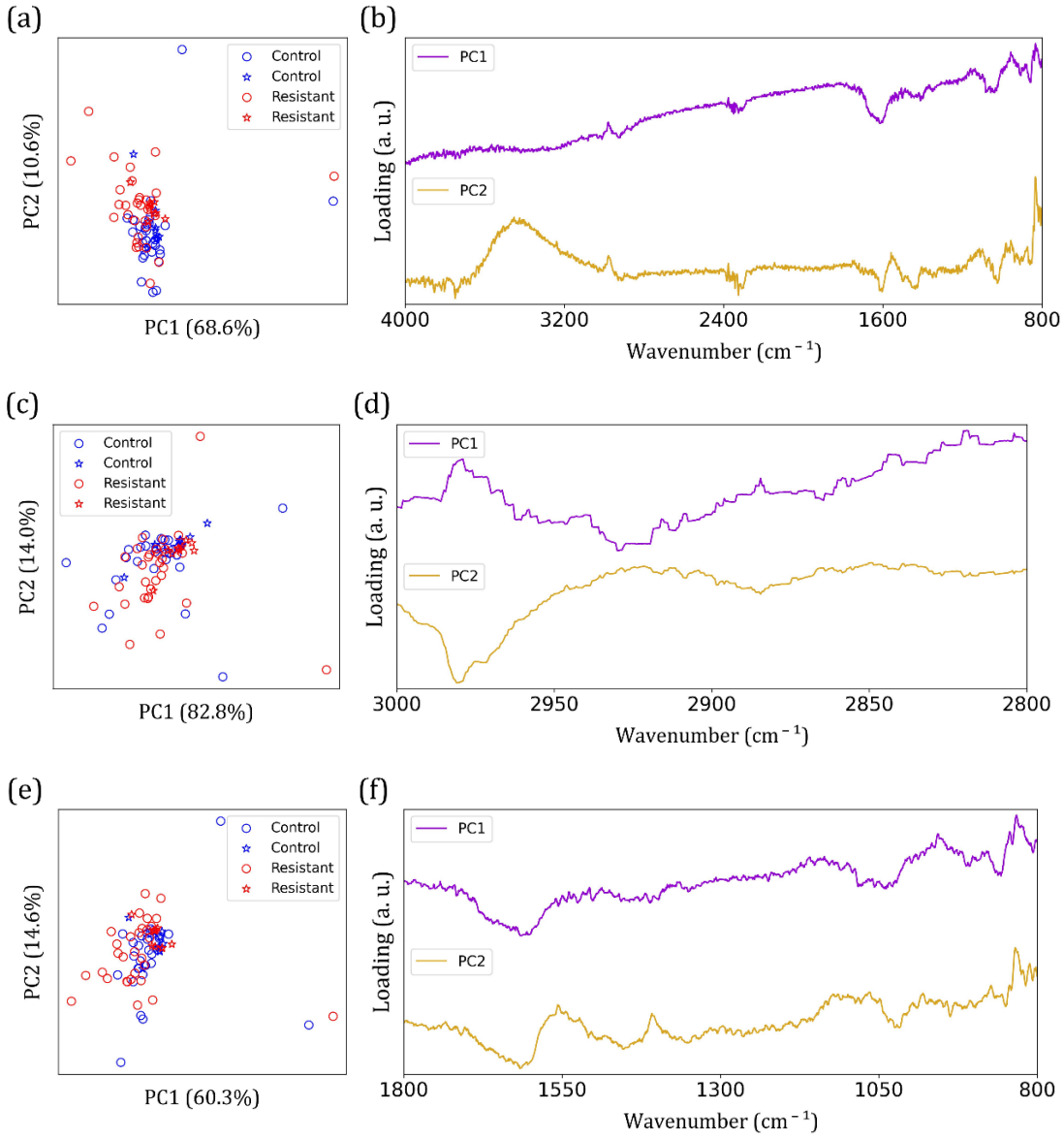

Figure 3: Principal Component Analysis for *E. coli* ATCC (blue circles/stars), and multidrug-resistant (MR) *E. coli* (red circles/stars) isolates average FTIR-SNV spectra. The score plot and respective loading for the three different ranges were analyzed: (a-b) 4000 to 800 cm^{-1}; (c-d) 3000 to 2800 cm^{-1}; and (e-f) 1800 to 800 cm^{-1}. The circles represent 70% of the sample data set used to build the predicting model, and the stars represent 30% of the sample data set used for external validation.

Usually, the proper choice of spectral range helps to improve group classification and clustering formation [61], since we use only spectral information that most contributes to clustering from those with highly correlated data, which hinders cluster formation. But here we couldn´t succeed with this strategy probably because of the high similarities between the groups involved, and the highly correlated data present. The presence of antibiotic resistance genes in the MR *E. coli* group was mainly identified as resulting of the ESBL enzyme – previous study [62] - which is responsible for amine

beta-lactam ring hydrolysis, resulting in antibiotics inactivation on the bacterial cell walls. Since it represents a break in the C-N bond (Amide group) and the appearance of N-H and O-H bonding, almost no difference in the IR spectra will be observed. Because, the C-N and N-H vibrational modes are superimposed around 1580 cm^{-1}, and O-H vibrational mode, around 3278 cm^{-1}, is too wide to provide enough information for group differentiation.

Then, an alternative to improve group clustering and separation is the use of Feature Selection Recursive Feature Elimination (RFE) to find the best PCs (data projection) that most contribute to data separation. The RFE can select PCs that most contribute to achieving high accuracy and remove those with a small contribution. Here, the main PCs were determined by the overall accuracy achieved in a Leave One Out Cross-validation (LOOCV) test by using Linear Discriminant Analysis (LDA) [60].The main PCs found for our data were: PC2, PC3, PC7, PC8, and PC19 for the 4000 to 800 cm^{-1} range, responsible for 20.26% of data variance; PC5, PC13, PC21, PC36, and PC46 for the 3000 to 2800 cm^{-1} range, responsible for 0.49% of data variance; and PC4, PC6, PC7, PC12, PC13, PC17, PC28, PC33, and PC48 for the 1800 to 800 cm^{-1} range responsible for 8.58% of data variance.

Figure 4 shows the violin plot for the 9 most relevant PCs, in 1800 to 800 cm^{-1} range, with a clear monomodal distribution for the score data project over its respective PC for both sample groups, except for PC7, which exhibits a wide distribution for MR *E. coli* group, and a double peak maximum for *E. coli* ATCC, similar behavior observed for MR *E. coli* group in PC6. But the more important characteristic to be observed is the median value (dashed thick line around the peak center), which assumes a better distinct position projection over the axis for these PCs compared to the others and improves the clustering and group classification of our data in the LOOCV test with LDA.

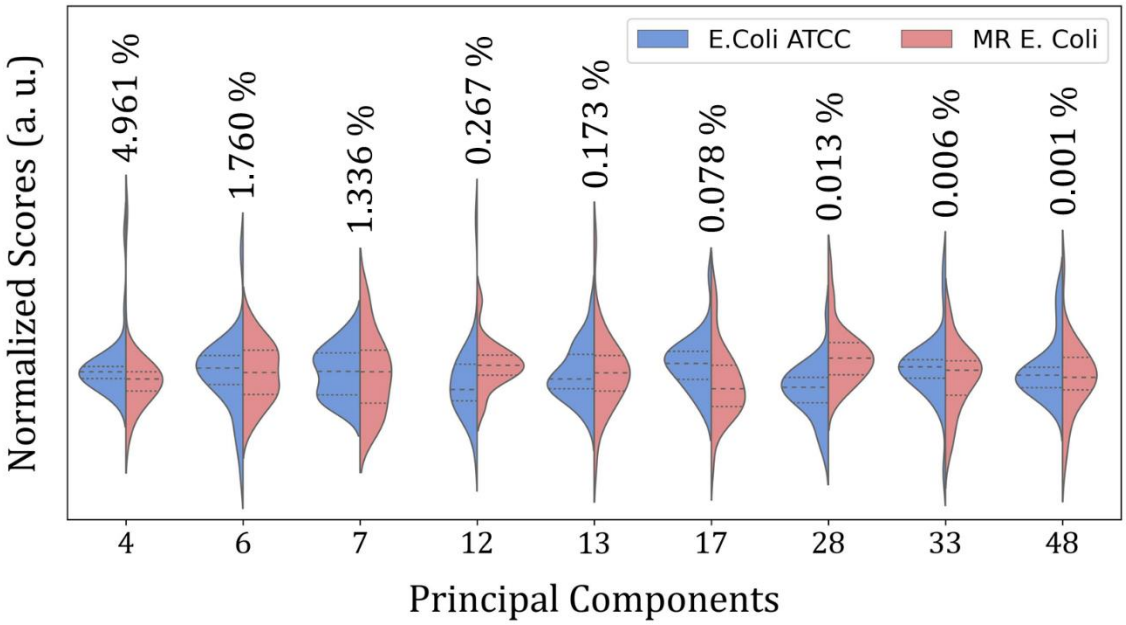

Figure 4: Violin plot for nine main PCs normalized scores selected by RFE in 1800 to 800 cm^{-1} range from *E. coli* ATCC® 25922 (blue), and multidrug-resistant (MR) *E. coli* (red) isolates. The thick dashed line represents the data distribution median. The data variance percentage of each PC is described above each plot.

Figure 5 exemplifies the importance of these RFE-PCs. The score plot and loading for PC4, PC17, and PC28 demonstrate the improvement of data clustering and group separation. They were responsible for only 5.05% of the data variance, similar results have been reported in the literature [60]. Besides that, the loading plot for each PC, Figure 5 (right column), shows a contribution for data variance in the 1600 to 1000 cm^{-1} range in accordance with the main bands identified in the FTIR-SNV spectra (Figure 2) assigned to protein and phospholipids molecules. In this case, with great similarity among the samples, then between the spectra – high data correlation – the first PCs can be responsible for a great percentage of data variance, with a small contribution to group separation. However, low data variance PCs can still be useful for group classification and better influence the algorithm performance, as demonstrated by Figure 5 results.

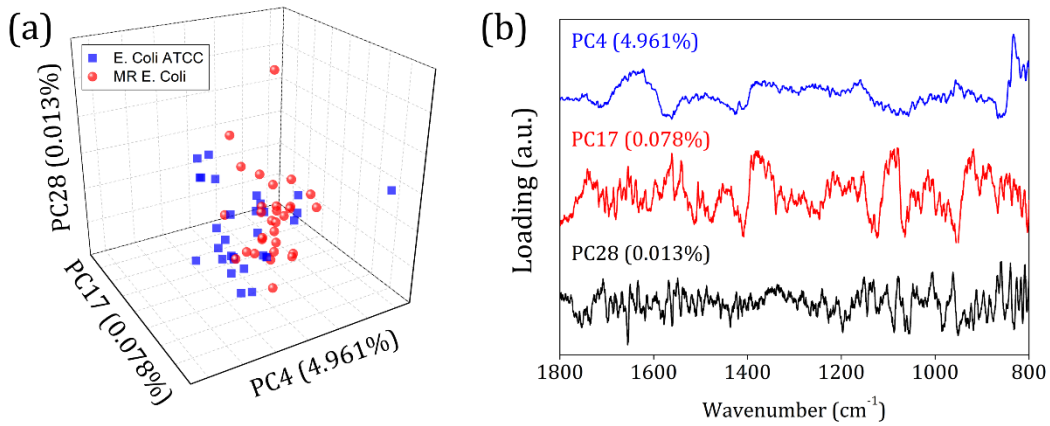

Figure 5: (a) score plot clustering improvement due to PCs selection by RFE., and **(b)** loading for PC4, PC17, and PC28 from *E. coli* ATCC® 25922 (blue square), and (b) multidrug-resistant (MR) *E. coli* (red circles) isolates.

The RFE-PCs were submitted to machine learning algorithms (DA, SVM, and KNN) to build a prediction model for sample classification, the overall accuracy achieved for each model by using different functions for classification in the LOOCV test is summarized in Figure 6 (a). The maximum overall accuracy achieved by the predicting models was 85% for Linear SVM by using 9 PCs – responsible for 8.59% of data variance – within the 1800 to 800 cm^{-1} range, and 5 PCs in the 4000 to 800 cm^{-1} range. The Weighted KNN also achieved an overall accuracy of 85% in the 4000 to 800 cm^{-1} range with 5 PCs, which was responsible for 20.26% of data variance.

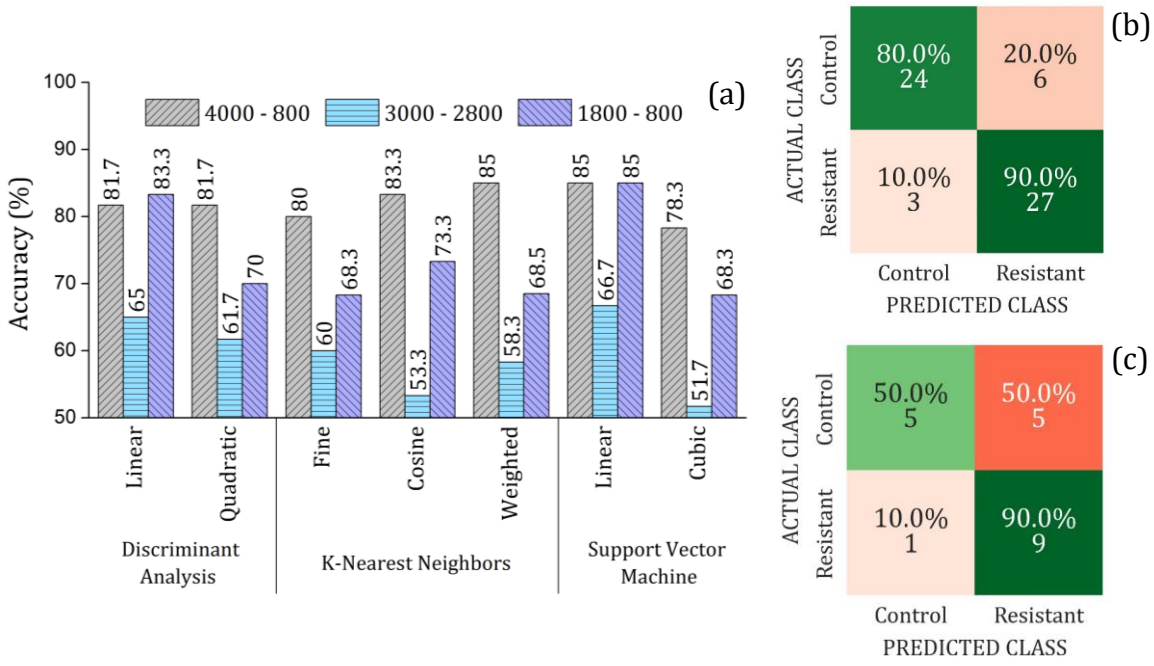

Figure 6: (a) Overall accuracy obtained in the LOOCV tests for Discriminant analysis (DA), K-nearest neighbor (KNN), and Support vector machine (SVM) algorithms. Different ranges were

analyzed with respective RFE-PCs: (i) 4000 to 800 cm^{-1} (gray bar color with right inclined line pattern) using 5 PCs; (ii) 3000 to 2800 cm^{-1} (light blue color with horizontal line pattern) by using 5 PCs; (iii) 1800 to 800 cm^{-1} (purple color with left inclined line pattern) by using 9 PCs. The highest overall accuracy was 85% for Linear SVM at (4000 to 800 cm^{-1}) and (1800 to 800 cm^{-1}) ranges, and Weighted KNN at (4000 to 800 cm^{-1}) range. Confusion matrix for the performance of Linear SVM algorithm in 1800 to 800 cm^{-1} range by using 9 PCs **(b**) LOOCV test with 70% data set, and **(c)** external validation test with 30% data set. The input PCA data from average FTIR-SNV spectra of *E. coli* ATCC® 25922, and multidrug-resistant (MR) *E. coli* isolates.

Besides both models achieving the same overall accuracy of 85% in the 4000 to 800 cm^{-1} range, the W-KNN and L-SVM showed a much lower generalizability of the model in the external validation test, when compared to L-SVM in the 1800 to 800 cm^{-1} range. The external validation overall accuracy for L-SVM was 55%, and only 50% for W-KNN (see Supp. Mat.). However, the L-SVM with 85% of overall accuracy in the 1800 to 800 cm^{-1} range, achieved 90% and 80% of sensitivity and specificity, respectively, Figure 6(b). Then, in the external validation test, Figure 6(c), the overall accuracy decreased to 70%, and achieved 90% and 50% of sensitivity and specificity, respectively. As can be observed in Figure 6(c), the prediction model failed to identify *E. coli* ATCC samples, which is less problematic for a trial method considering the need for large-scale tests to a fast decision in livestock management.

The current study presented an easy and suitable methodology for the identification of MR-*E. coli* bacteria by FTIR spectroscopy and machine learning algorithms but must keep in mind that the methodology and results can be still improved, so the possibility for new studies is open. Here, we discussed the FTIR limitations to evidenced spectral changes due role played by ESBL enzyme to create MR bacteria, so new photonic techniques must be explored for better data acquisition. In our study, we explore a promising route to build a prediction model with good performance in the external validation test, but we can still search for the best hyperparameters for the algorithm or even explore new pre-processing data methods and algorithms to build the prediction model and improve its generalization capacity in the external validation test.

CONCLUSIONS

The FTIR spectra, in the 1800 to 800 cm^{-1} range, of multi-resistant diarrheagenic *Escherichia coli*, isolates obtained from calves' feces showed great potential for microorganism identification. The usual principal component analysis was not able to provide a promising clustering formation for future sample classification. Then, we

applied the Feature Selection Recursive Feature Elimination (RFE) algorithm, from which the most important PCs were chosen before being used in machine learning algorithms. The score plot of PC4 x PC17 x PC28 clearly demonstrated the improvement of clustering tendency and group separation, here 9 PCs were selected by RFE and used to build a prediction model by using Linear SVM. The prediction model showed an 85% overall accuracy in the LOOCV test, and achieved 70% overall accuracy, with 90% sensitivity and 50% specificity in the external validation test.

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