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# O brilho verde metálico no ágar emb possui acurácia na identificação de *Escherichia coli*?

## Is green metallic sheen in emb agar accurate to assist the *Escherichia coli* identification?

## ¿El brillo verde metálico en el agar emb tiene precisión en la identificación de *Escherichia coli*?

***Hosana Dau Ferreira de Souza[1], Paula Fernanda Alves Ferreira[2], Thereza Cristina da Costa Vianna[3], Gabrielli Stefaninni Santiago[4], Irene da Silva Coelho[5], Miliane Moreira Soares de Souza[6], Kayo Bianco [7], Shana de Mattos de Oliveira Coelho[8]***

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## Resumo

*Escherichia coli* é uma bactéria comensal em seres humanos e animais, embora esta espécie também esteja disseminada no meio ambiente. Os laboratórios clínicos utilizam ágar EMB (Eosin Metilene Blue) para identificar *E. coli* a partir da observação do fenômeno brilho verde metálico. No entanto, outras espécies bacterianas podem apresentar brilho quando semeadas no ágar EMB, ou em alguns casos, a acidez influencia a presença deste brilho por *E. coli*. Neste estudo, o MALDI-TOF foi utilizado como padrão-ouro para detectar *E. coli* de amostras humanas, animais e ambientais analisando a presença de brilho verde no ágar EMB. Os valores preditivos positivos (VPP) foram maiores (acima de 85%) em amostras humanas, animais e ambientais estudadas, e o valor preditivo negativo (VNV) foi variável entre as amostras. Especificidade e sensibilidade também foram variáveis sugerindo a influência do pH das amostras na presença de brilho verde metálico. Este trabalho demonstrou que é necessário implementar testes bioquímicos ou quando possível, ensaios moleculares para confirmar *E. coli*, uma vez que apenas a observação da produção do brilho verde metálico em EMB pode gerar resultados falso negativos na identificação de *E. coli*.

***Palavras-chave:*** Testes bioquímicos; *Escherichia coli*; Análise Laboratorial; MALDI-TOF.

## Abstract

*Escherichia coli* is an essential bacteria as a human and animal pathogen, although this species can also be disseminated in the environment. The clinical laboratories use EMB agar (Eosin Methylene Blue) to identify E. coli from the observation of metallic green sheen. However, other bacterial species may show sheen when sown on EMB agar, or in some cases, acidity influences the presence of this sheen by *E. coli*. In this study, MALDI-TOF was used as the gold standard to detect *E. coli* from human, animal, and environmental samples by analyzing the presence of green glow on EMB agar. The positive predictive values (PPV) were higher (above 85%) in human, animal, and environmental samples studied, and the negative predictive value (NPV) was variable between the samples. Specificity and sensitivity were also variables suggesting the influence of the pH of the samples in the presence of metallic green brightness. This work demonstrated that it is necessary to implement biochemical tests or, when possible, molecular assays to confirm E. coli since only the observation of the production of metallic green glow in EMB can generate false negative results in the identification of *E. coli*.

***Key words:*** Biochemical tests; *Escherichia coli*; Laboratory Analysis; MALDI-TOF.

## Resumen

*Escherichia coli* es una bacteria comensal en humanos y animales, aunque esta especie también está muy extendida en el medio ambiente. Los laboratorios clínicos utilizan agar EMB (eosina metileno) para identificar *E. coli* a partir de la observación del fenómeno del resplandor verde metálico. Sin embargo, otras especies bacterianas pueden exhibir brillo cuando se siembran en agar EMB o, en algunos casos, la muestra de pH influye en la presencia de este brillo por *E. coli*. En este estudio, MALDI-TOF se utilizó como el estándar de oro para detectar *E. coli* a partir de muestras humanas, animales y ambientales mediante el análisis de la presencia de brillo verde en el agar EMB. Los valores predictivos positivos (VPP) fueron mayores (por encima del 85%) en las muestras humanas, animales y ambientales estudiadas, y el valor predictivo negativo (VNV) fue variable entre las muestras. La especificidad y la sensibilidad también fueron variables, lo que sugiere la influencia del pH de las muestras en la presencia de brillo verde metálico. Este trabajo demostró que es necesario implementar pruebas bioquímicas o, cuando sea posible, ensayos moleculares para confirmar *E. coli*, ya que solo la observación de la producción de brillo verde metálico en EMB puede generar resultados falsos negativos en la identificación de *E. coli*.

***Palabras clave:*** Pruebas bioquímicas; *Escherichia coli*; Análisis de laboratorio; MALDI-TOF.

Afiliação dos autores: [1]Discente de Doutorado pelo Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brasil. E-mail: dfs.hosana@gmail.com; ORCID: https://orcid.org/0000-0002-0221-1333. [2]Discente de Doutorado pelo Programa de Pós-Graduação em Agronomia - Ciências do Solo, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brasil. E-mail: paula.faf@hotmail.com; ORCID: https://orcid.org/0000-0001-7533-1216. [3]Mestre pelo Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brasil. E-mail: therezacristina8466@gmail.com; ORCID: https://orcid.org/0000-0001-6166-6736. [4]Docente da Faculdade Anhanguera, São José dos Campos, São Paulo, Brasil. E-mail: gabriellissantiago@outlook.com.br; ORCID: https://orcid.org/0000-0001-8761-6008. [5]Docente Associada, Departamento de Microbiologia e Imunologia Veterinária - Universidade federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brasil. E-mail: irenecoelho@ufrrj.br; ORCID: https://orcid.org/0000-0003-1357-2529. [6]Docente Titular, Departamento de Microbiologia e Imunologia Veterinária - Universidade federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brasil. Email: milianemss@gmail.com; ORCID: https://orcid.org/0000-0001-8325-9322 [7]National Institute of Quality Control in Health - INCQS, Oswaldo Cruz Foundation - FIOCRUZ, Rio de Janeiro, RJ 4365, Brazil. E-mail:kayo.bianco@fiocruz.br ORCID: https://orcid.org/0000-0002-7520-4097. [8]Docente Associada, Departamento de Microbiologia e Imunologia Veterinária - Universidade federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brasil. E-mail: shana@ufrrj.com.br; ORCID: https://orcid.org/0000-0003-4165-5735.
* E-mail de correspondência: dfs.hosana@gmail.com

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## Introduction

The order *Enterobacterales* is a large group of Gram-negative, facultative anaerobes, non-sporulating, rod-shaped bacteria of the class Gammaproteobacteria. Members of this group inhabit many different ecological niches and are found in soil, water, and associated with organisms including plants, insects, animals, and humans[1]. Many members of the order *Enterobacterales* are considered human and animal pathogens, such as *Escherichia coli*, *Salmonella enterica*, and *Yersinia pestis*, and economically harmful plant pathogens, such as *Dickeya*, *Pectobacterium*, *Brenneria*, *Erwinia*, and *Pantoea* [2,3,4,5,6,7,8].

*Escherichia coli* is a bacterium belonging to the *Enterobacteriaceae* family, morphologically characterized by bacilli, gram-negative, lactose fermenters, sucrose, and glucose, gas production, oxidase-negative, catalase-positive, and due to the presence of periflagels, does not use sodium citrate as the only carbon source due to the lack of citrate permease[9]. It can grow from a variety of carbon sources, with a temperature range of 5ºC to 45ºC, with an ideal growth temperature of 37ºC. They are heat tolerant microorganisms that survive 15 minutes at 60ºC or 1 hour at 55ºC and tolerate pH changes in the range of 4.4 to 7.0[10]. Strains of this species are part of the intestinal microbiota of mammals, but some strains are associated with intestinal and extraintestinal pathologies in humans and animals[5,11]. Among several microorganisms of clinical importance, *Escherichia coli* is one of the most important as it presents high values of morbidity and mortality both in humans and in animals all over the world. Besides, the lack of adequate sanitary conditions also allows soil, water, and food to be contaminated because of contact with animal waste that harbors pathogenic and non-pathogenic strains. This bacterium's high dissemination and infectious capacity in different hosts is a great alert for public health since it connects beings from different environments considered in unique health[12].

*E. coli* is transmitted by fecal-oral transmission through contaminated water and food. Usually isolated from feces, it hardly causes disease in the host. But in debilitated, immunocompromised, or altered gastrointestinal animals, nonpathogenic *E. coli* strains present in the intestine can cause infection[13,11]. The pathogenic strains of *Escherichia coli* can cause intestinal/diarrhea or extra-intestinal infection, testicular infections, mainly urinary tract infections also sepsis/meningitis[12]. These bacteria can be classified by serotyping according to the presence of different somatic, capsular, and fimbrial antigens. However, bacterial serotyping is limited, as only a few strains have antisera available for classification[14]. The analysis of the virulence of this species allows the current division into main categories or pathotypes: Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), *E. coli* enteroinvasive (EIEC), diffusively adherent *E. coli* (DAEC), uropathogenic *E. coli* (UPEC) and sepsis-associated *E. coli* (SEPEC), neonatal *E. coli* meningitis (NMEC) and pathogenic *E. coli* (APEC). Also, the bacterial group can be classified according to somatic (O), capsular (K) and flagellar (H) antigens, resulting in more than 700 *E. coli* serotypes, the most well-known and studied O157: H7. This serotype is found in foods of animal origin and has a significant impact on human health[15,16,17,18].

The identification of *Escherichia coli* goes through some internationally standardized methodologies for enterobacteria. Being, described by the National Health Surveillance Agency (ANVISA) - Manual of Detection and Identification of Bacteria of Medical Importance, methodologies that allow discriminate with 80% of correct genera and species, such as glucose fermentation, lactose fermentation, motility, use of citrate, decarboxylation of lysine, production of the hydrogen sulfide ($H_2S$), gas ($CO_2$), oxidase, indole, urease, phenylalanine deaminase or tryptophanase option, and gelatinase[19]. Complementary identification tests such as fermentation of other carbohydrates (sucrose, maltose, arabinose, salicin, dulcitol, mannitol), use of amino acids (arginine and ornithine), schooner hydrolysis, ONPG, acetate use can be used.

EMB agar (Eosin Methylene Blue) is a culture medium favorable to the growth and differential of this bacterial species. This differential and selective medium presented yellow eosin and methylene blue in its formulation, which can inhibit Gram-positive bacteria's growth and differentiate lactose and sucrose fermentation[20]. The traditional colonial characteristic of *E.coli* in this agar is large, bluish-black colonies with a metallic green luster, sometimes presenting colonies with a

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purple center[21]. According to the National Health Surveillance Agency (ANVISA - Manual of Clinical Microbiology for the Control of Infection Related to Health Care), EMB media and AMC agar (MacConkey) are selective and differential for the growth of *Escherichia coli*[22]. The samples should be incubated for 18 to 24 hours at 35ºC, observing that in the first medium, the colonies have a metallic green luster and, in the second, a dark red color, being necessary for the confirmation of the bacterial species later through biochemical tests. Another essential organ for microbiological analysis is the Food and Agriculture Organization of the United Nations (FAO - Manuals of food quality control), which describes the use of bright green broth to isolate this bacterium that appears as colonies with a bright green appearance after incubation 48 hours at 35°C[23]. Thus, clinical laboratories follow these protocols to identify the strains, often using the colonial characteristics in these media to suggest *E. coli* in the sample analyzed, being followed to the antibiogram stage without confirmation by biochemical tests.

The use of metallic green sheen in EMB as a screening method to identify *Escherichia coli* isolates is commonly found in the literature[24,15,25,18]. However, the use of the presence of metallic green sheen presented by some colonies to detect *E. coli* is problematic since other bacterial species are also capable of presenting this aspect, leading to false-positive results[26]. Thus, the present study aimed to evaluate the ability of the EMB agar to indicate the characteristic growth of *Escherichia coli*, helping in its laboratory identification.

## Materials and methods

Enterobacteria obtained from three different sources were tested: environmental, animal, and human. The strains of environmental origin came from samples of horse litter from a farm located in Nova Friburgo, and avian litter from a farm in São José do Rio Preto and Nova Friburgo, Rio de Janeiro-Brazil. Such samples were inoculated in *Escherichia coli* (EC) broth for turbidity analysis and gas production, and an aliquot of the broth was seeded on EMB agar. The human strains came from urine samples, provided by the partnership with the LabGelson Clinical Analysis Laboratory (Mendes, RJ, Brazil), with permission from the Federal Rural University of Rio de Janeiro (UFRRJ) Research Ethics Committee (COMEP - 340/2021). Such urine samples were sown directly on EMB agar. About animal samples, bacterial strains were recovered from the urine of dogs and cats processed at the Laboratory of Residency in Veterinary Microbiological Diagnosis - UFRRJ, with permission from the Ethics Committee on the Use of Animals at UFRRJ (CEUA – 6967290616-2017), identified biochemically as *E. coli*, stored in BHI broth (Brain Heart Infusion) with glycerol (45%) at -20ºC, were reactivated in BHI agar and subsequently soed in selective EMB agar.

All EMB plates were incubated for 24h to 48h at 35ºC (+/- 2 ºC), and the colonial growth in the medium was evaluated concerning the expression of the production of the metallic green sheen suggestive of *Escherichia coli*. In addition to phenotypic analyzes of the colony.

After analyzing colonial growth, the bacterial strains isolated in EMB medium were subjected to the Matrix-Assisted Laser Ionization / Desorption Flight Time (MALDI-TOF) technique at the Integrated Microbiology Laboratory (LIM) Instituto de Microbiologia Paulo Góes of the Federal University of Rio de Janeiro (UFRJ).

Matrix-assisted laser desorption mass spectrometry and ionization (MALDI-TOF) technology is a general study of proteins and enzymes or a comprehensive study of the sum of all proteins produced by an organism or biota. Generate qualitative information on proteins related to identification, distribution, interactions, structure, and function, as well as quantitative information such as abundance, distribution in different locations, time changes in abundance due to synthesis and degradation, or both[27,28]. The technique is based on a part of the sample overlapped by a matrix capable of providing protons (or H+) for the process of ionization of the sample components. The energy emitted by a laser is absorbed by the matrix, thus transferring protons from the matrix to the sample components and at the same time triggering a desorption process (substance is released through a surface), in which the sample passes from the solid state to the gaseous state. In this way, the ionized are directed to the TOF analyzer, where they are accelerated through an electric field inside a vacuum tube where the sample components are separated according to their m/z, reaching a detector at different times[29].

MALDI-TOF MS has been widely applied

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in studies identifying different genera of Gram-negative bacilli, such as *Escherichia coli* and other members of the Enterobacteriaceae family from clinical samples of humans, but authors have investigated MALDI-TOF ability to discriminate and characterize environmental isolates of *E. coli* obtained from feces samples from mammals and birds[30,31].

Each bacterial culture was transferred to the microplate (96 MSP, Bruker - Billerica, USA) and, to the bacterial pellet, a lysis solution (70% formic acid, Sigma-Aldrich®) was added in sufficient quantity to cover it. Then, one µL of the matrix solution (alpha-cyano-4-hydroxy-cinnamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, Sigma-Aldrich®) was used to cover the bacterial extract, to be processed finally. The spectra of each sample were generated in a mass spectrometer (MALDI-TOF LT Microflex Bruker, Bruker®) equipped with a 337nm nitrogen laser in linear mode controlled by the FlexControl 3.3 program (Bruker®). The spectra were collected in the mass range between 2,000 and 20,000 m / s and, subsequently, analyzed by the MALDI Biotyper 2.0 program (Bruker®), with standardized settings for bacterial identification. The program confronts the spectra of the unknown sample with reference samples in a database. The results obtained vary on a scale ranging from zero to three, and the higher the value on the scale, the more reliable the identification is. Those with values ≥ 2,000 were considered satisfactory identification, indicating, according to FlexControl software 3.3, as a safe identification of genus and probable species

The MALDI Biotyper 2.0 program compares the spectra of the unknown sample with the reference samples in the database and categorizes the results on a scale that goes from zero to three, as shown in table 1, being the highest values, the most reliable for sample identification. The Green metallic sheen in EMB was evaluated by the Chi-Square test ($\chi 2$) and Fisher's test with a 95% confidence interval, considering the MALDI-TOF as the gold standard technique specie identification. Previously, in our laboratory, a total of 183 enterobacteria were tested, and the *gyr*B sequencing confirmed the proteomic technique results in 100% [32].

## Results

All samples were inoculated in EMB medium to assess metallic green sheen. After incubation, 417 enterobacteria were counted, 158 from environmental samples, 157 and 102 from human and animal urine, respectively. Were submitted to MALDI-TOF MS for proteomic characterization of the species.

Of the total of 417 strains grown on EMB agar, different bacterial species were identified by MALDI-TOF, as shown in chart 1.

There were 63.7% (266/417) *Escherichia coli*, 9.83% *Proteus mirabilis*, 8.15% *Klebsiella pneumoniae,* 3.83% *Enterobacter cloacae*, 1.92% *Citrobacter freundii,* and the other species showed less than 1 %.

Thus, the results were grouped in *Escherichia coli* green metallic sheen producer, *E. coli* green metallic sheen not-producer, non-*E. coli* green metallic sheen producer and non-*E. coli* green metallic sheen not-producer for later comparison by the results with the proteomic technique, as shown in Figures 1 and 2.

**Table 1.** Information extracted from the Bruker Daltonik MALDI program describes the meaning of the values in relation to the score obtained in the analyzed sample.

| Score | Identification | Symbol | Color |
|---|---|---|---|
| 2.300 – 3.000 | Highly probable identification of species | (+++) | Green |
| 2.000 – 2.299 | Safe identification of the genus and probable of the species | (++) | Green |
| 1.700 – 1.999 | Probable gender identification | (+) | Yellow |
| 0.000 – 1.699 | Untrusted identification | (-) | Red |

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**Chart 1.** Distribution of Gram-negative rod species isolated from human, animal, and environmental samples.

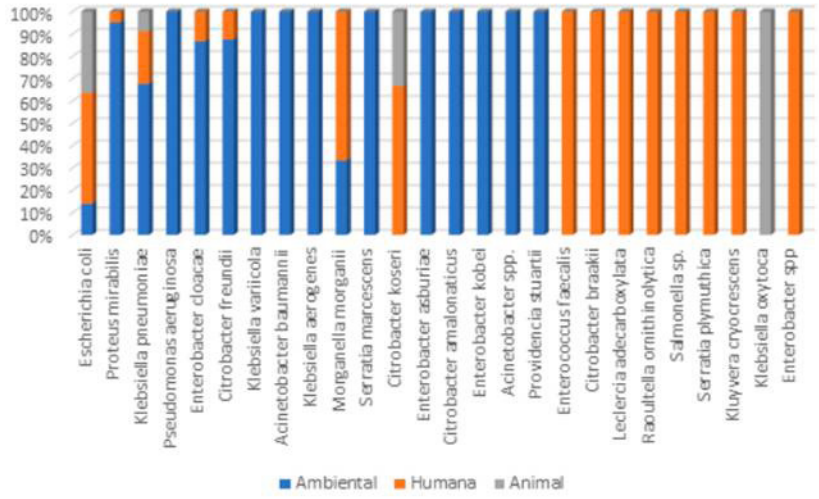


**Figure 1.** Evaluation of green sheen in *Escherichia coli* strains from animal samples on EMB agar. A - Growth of *Escherichia coli* with a metallic green sheen in EMB medium.

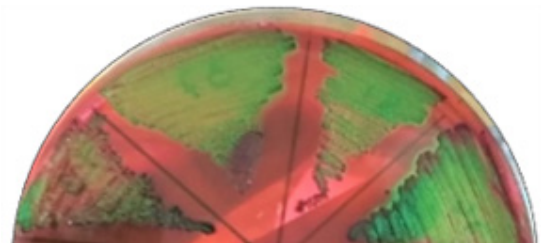

**Figure 2.** Evaluation of green sheen in *Escherichia coli* strains from animal samples on EMB agar. B - Growth of *Escherichia coli* without metallic green sheen in EMB medium.

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The colonial aspect of *Escherichia coli* strains on EMB agar shows that only 74% (197/266) produced the metallic green sheen. The values of sensitivity, specificity, positive and negative predictive value of the culture medium were 74%, 91%, 93%, and 66%, respectively. Only 8.6% (13/151) of non-*E.coli* produced metallic green, being *Citrobacter freundii* (n = 6), *Klebsiella pneumoniae* (n = 3), *Citrobacter braakii* (n = 1) , *Enterobacter cloacae* (n =1) and *Kluyvera cryocrescens* (n = 1), which contributed to a high specificity of the culture medium (91%).

Regarding the different sources of origin, the statistical values were also established separately, as explained in chart 2.

The samples from the environment showed the most reliable indicative rates, with 100% and 95% of sensitivity and specificity, respectively. Only the PPV was lower than the others sources once six strains of *Citrobacter freundii* (6/115) showed a metallic green colony. Despite this, all statistical indications were higher than 86%.

Strains of animal origin had the lowest specificity rate (40%) since 60% (3/5) of non-*Escherichia coli* strains revealed a metallic green colony, producing false negatives, as shown in Figure 3.

The lowest sensitivity rate was concerning samples of human origin (55%), which also had a low negative predictive value (25%) because many

**Chart 2.** Statistical rates of evaluation of the EMB agar in identifying *Escherichia coli* from different samples

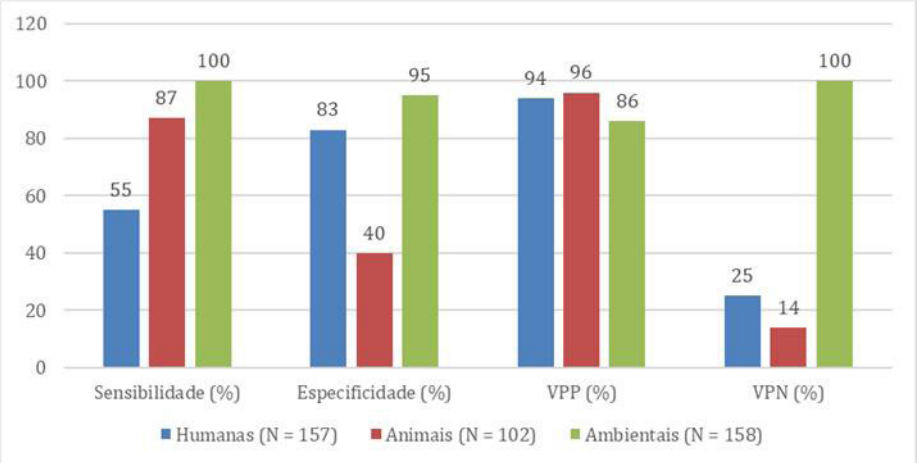
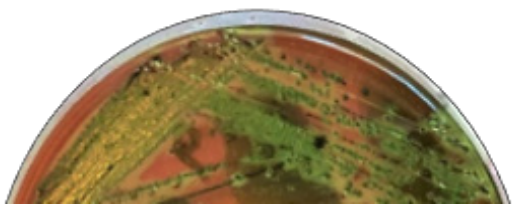



**Figure 3. Strains** of *Citrobacter* spp. showing metallic green coloring on EMBagar.



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strains of *Escherichia coli* of human origin did not have a metallic green luster (57/132).

## Discussion

As reported by Antony et al. (2016) *apud* Parisi & Marsik (1969), the authors warn that, in some cases, strains of *E. coli* may present with pink color in the EMB medium, which may impair the correct laboratory identification course[33,34]. A possible explanation for the lack of brightness production by these isolates could be because the urine samples were sown directly on the EMB agar, differently from the animal and environmental strains that were re-isolated after being stocked in microbiological broths. Microbiological broths have a neutral pH, and the pH of the urine with an indication of urinary infection is commonly measured above 7.5, which could influence the production of the shine[35].

According in his work using direct inoculation of milk on EMB agar, *E. coli* did not produce a green metallic luster as expected, indicating that this coloration could be sensitive to changes in pH, that is, the absence of luster could be due to the alkalinity of mastitic milk. On the other hand, when using apple cider, whose pH is acidic, the authors detected an increase in the production of brightness by the colonies present in the medium leading to false-positive results compared to the isolation of colonies inoculated directly on the EMB agar[36]. Therefore, the isolation methodology and the pH of the sample can be crucial factors for more accurate identification of *E. coli* in this culture medium, which is worrying since many routine laboratories sow urine directly in EMB medium to assess the colonial aspect.

Also, the PPV occurred in a high value since few non-*E. coli* isolates were error-inducing. So, once metallic green producer strain, the higher is the chance of belonging to the *E. coli* species. In a general aspect, the evaluated medium had a low sensitivity because many *E. coli* did not produce the metallic green luster, but a high specificity since few non-*E. coli* strains presented the phenomenon.

It is also worth mentioning that the use of EMB agar is indicated for prior identification that must be followed by other techniques, such as growth on AMC agar, morphological and staining evaluation after Gram method, and evaluation of bacterial metabolism in biochemical tests such as Indol production, Red Metila, Voges-Proskauer, and Citrato, for example. However, these techniques demand time to complete, taking around 24 to 48 hours to allow the tests to be examined. As an alternative to reduce errors in the identification of *Escherichia coli*, the use of chromogenic media for the detection of bacterial species would be indicated, such as Chromoagar Orientation. Chromogenic media are intended to correctly identify morefrequently occurring bacteria and yeasts or organism groups on primary culture with no further testing or a minimum number of confirmatory tests. Substrates present in chromogenic media target specific classes of enzymes produced by certain bacteria and yeasts[37.] Target enzymes hydrolyze chromogenic substrates generating colored products which allow for easy identification of specific organisms[37,38].

After incubation for 24h at 35 ° C (+/- 2 ºC), this medium identifies *E. coli* by the presence of colony pink or reddish[33] or the Hicrome ECC agar, whose detection of *E. coli* is made by the presence of reference bluish-green colonies.

In a study[33], observed the production of metallic green glow in EMB agar, of a large number of colonies, typical of *E. coli* characteristics, while in the Hicrome *E. coli* agar produced white and non-blue-green colonies. And when biochemically tested they proved not to be *E. coli*. It found false-positive results in EMB agar isolate, revealing the inefficiency of EMB agar in differentiating *E. coli* from other enterobacteriaceae members that exhibit similar phenotypic and biochemical characteristics.

Besides, there are molecular techniques, such as flow cytometry, polymerase chain reaction (PCR), DNA microarrays, enzyme-linked immunosorbent assay (ELISA), fluorescent in situ hybridization (FISH), which despite being technical more sensitive devices, require expensive equipment and reagents, in addition to a technoscientific team to perform and read the tests, and are not accessible to many routine clinical laboratories[12] .

## Conclusion

In the present study, of the total of 417 strains grown on EMB agar, different bacterial species were identified by MALDI-TOF there were 63.7% (266/417) *Escherichia coli*, 9.83% *Proteus mirabilis*, 8.15% *Klebsiella pneumoniae,* 3.83% *Enterobacter cloacae*, 1.92% *Citrobacter freundii,*

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and the other species showed less than 1 %. The EMB agar showed low sensitivity in the indication of *Escherichia coli* colonies, considering 26% of the strains did not produce the metallic green sheen-probably due to the pH change of the samples, as described by Leininger et al. (2001), in its work using direct inoculation of milk in EMB agar. However, it showed high specificity since few non-*E. coli* strains showed such a phenomenon.

Rapid and accurate identification of bacteria is extremely important in clinical microbial laboratory testing. The presence of a metallic green glow in EMB should not be used exclusively as a confirmatory test for the identification of *E. coli* strains, being necessary to implement other identification methods to confirm this bacterial species, such as growth in the evaluation of AMC agar, morphological and staining after Gram method, and evaluation of bacterial metabolism in biochemical tests or molecular techniques.

Therefore, it is up to the routine clinical laboratory to apply the most accessible test to its panorama.

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## Conflito de interesse

Os autores declaram não haver conflitos de interesse de nenhuma natureza.

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