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VIRULENCE FACTORS OF ENTEROBACTERIACEAE ISOLATED FROM DIFFICULT TO TREAT INFECTIONS

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In some selected cases, the infections might become persistent with a difficult treatment. The prolonged evolution may be due to the characteristics of the host such as comorbidities and other predisposing diseases, and also to the bacterial features, like antimicrobial resistance or production of virulence factors. The phenotypical evaluation of the soluble virulence factors was evaluated through streaking the strains on different media. The results were evaluated at 24, 48 and 72 hours. All Enterobacteriaceae strains intensly produced soluble virulence factors, the most frequent production being observed in *Klebsiella pneumoniae.* The soluble virulence factor production could be one of the reasons for which the infections had a prolonged evolution.

Key-words: Enterobacteriaceae*,* virulence factors, difficult to treat infections.

INTRODUCTION

The causes which lead to the occurrence of difficult to treat infections are not only host dependent, they also may be due to the characteristics of the bacteria, such as the ability to produce biofilm, increased adhesion to the substrate, the development of soluble virulence factors or resistance to various antimicrobial[1].

Bacterial virulence factors may influence the type an localisation of infection, for example, in some studies it has been observed that the most virulent bacterial strains have been isolated from blood cultures, performed from systemic infections, and from peritoneal fluid[1]. Also, strains that expressed fewer virulence factors were isolated from localized eye infections[1].

The production of virulence factors is a strategy for survival and avoidance of the body's immune response, promoting bacterial development and affecting tissue[2]. The production of virulence factors may be different between microorganisms isolated in acute infections from those isolated in chronic infections; the second producing more frequent virulence factors[3].

MATERIALS AND METHODS

Regarding the phenotypic expression, the bacterial strains were evaluated for the production of the following soluble enzymatic virulence factors: hemolysins, lecithinase, lipase, caseinase, gelatinase, esculinase, DNase and amylase. The method of phenotypic determination of virulence factors consisted in the preparation of a bacterial inoculum of 0.5 McFarland in 0.9% saline sterile water. The strains were "spot" streaked with a sterile disposable 10 μL loop on media containing appropriate enzymatic substrates[4,5].

To evaluate the production of hemolysins, the bacterial strains were streaked on 5% blood agar[4,5]. The strains were incubated at 36 °C for the first 24 hours and at 25 °C for the next 48 hours[4,5]. After 24, 48 and 72 hours, the appearance of a clear transparent halo around the streaked strains was evaluated as beta hemolysis, and a greenish, opaque halo, as incomplete alpha hemolysis[4,5].

For the production of lecithinase, the medium was prepared from nutrient agar in which 2.5% egg yolk was incorporated, the strains were streaked in "spot", and the plates were incubated at 36 °C for the first 24 hours. and at 25 °C for the next

48 hours (4,5). The reading was performed at 24, 48 and 72 hours, and the result was considered positive in case of an area with precipitate around the streaked spot, thanks to the degradation of lecithin by lecithinase and accumulation of insoluble diglyceride, and / or a clear area due to release of lecithin from the lipid complex[4,5].

For the the production of gelatinase, the strains were streaked in “spot” on agar medium with the addition of 3% gelatin[4,5]. The plates were incubated at 36 °C for the first 24 hours and at 25 °C for the next 48 hours, and the reading was performed at 24, 48 and 72 hours (4,5). We considered positive the appearance of a clear halo around the streaked spot, due to the liquefaction of the gelatin[4,5].

To evaluate lipase production the nutrient agar was supplemented with 1% Tween 80 (sorbitol monooleate), the strains were streaked in “spot”, the plates were incubated at 36 °C for the first 24 hours and at 25 °C for the next 48 hours, and the reading was done at 24, 48 and 72 hours[4,5]. It was considered positive result the appearance of a precipitate around the streaked spot[4,5]. This more opaque, precipitation area, is due to the release of fatty acids from sorbitol monooleate, which reacts with calcium ions in the environment and leads to the formation of insoluble crystals of calcium, calcium oleate, which will precipitate in the environment[4,5].

To evaluate the production of caseinase, the medium consisted of nutrient agar and 15% casein, the strains were streaked in “spot”, and the plates were incubated at 36 °C for the first 24 hours and at 25 °C for the next 48 hours. The reading was done at 24, 48 and 72 hours[4,5]. The result was considered positive when a clear halo and a precipitate appeared around the streaked spot, due to calcium paracaseinate[4,5].

The ability to produce amylase was assessed by inoculating the strains in a medium with a 1% starch content, the inoculated strains were incubated at 36 °C for the first 24 hours and at 25 °C for the next 48 hours[4,5]. The test was considered positive in case of a clear yellow halo, intensified after the addition at 72 hours of lugol solution in the Petri dish[4,5].

To evaluate the production of DNase, a medium was made from a commercially available product with the addition of DNA and methyl green, the strains were streaked in “spot”, then incubated at 36 °C for the first 24 hours and at 25 °C for the next 48 hours. The reading was performed at 24, 48 and 72 hours[4,5]. The test was considered positive when a pink halo appeared (4,5).

Esculinase production was assessed by spot streaking of bacterial strains in medium containing 1% esculin and ammoniacal Fe^{3+} citrate, then incubation at 36 °C for the first 24 hours and at 25 °C for the next 48 hours[4,5]. The reading was performed at 24, 48 and 72 hours, being considered positive the appearance of a black precipitate due to the release of esculetol under the action of esculinase, which in the presence of iron citrate in the environment can form a black precipitate of ferric esculetin[4,5].

The results were quantified by assigning grades from 0 to 4, 0 meaning the absence of the described phenomenon, 1 low intensity, 2 medium intensity, 3 high intensity, and 4 maximum intensity[4,5]. For each bacterial strain, we calculated the virulence index by summing the values assigned to each virulence factor[4,5].

RESULTS AND DISCUSSION

The ability to produce soluble virulence factors was evaluated by spot streaking for 79 strains of *Enterobacteriaceae*. The strains were streaked on a plate and analyzed at 24, 48 and 72 hours for their ability to produce: lipase, lecithinase, DNase, esculinase, gelatinase, caseinase, hemolysins and amylase (Fig. 1).

The strains of *Escherichia coli* produced most frequently lipase and gelatinase (more than 90% of the strains). A reduced number of strains produced amilase (less than a third) and esculinase (almost half of the strains) (Figure 2).

Of the evaluated Enterobacteriaceae, *K. pneumoniae* strains had the most intense production of virulence factors. Most of the strains produced gelatinase, caseinase, esculinase and lipase. (Figure 3)

Compared to other studies which reported the production of soluble virulence factors and found the production of hemolysins in 17% of the strains of *E. coli,* 7% of the strains of *K. pneumoniae* and 18% of the strains of *Enterobacter* spp. (6), the present study presented a higher percent of strains producing virulence factors. This finding might be due to the origin of the strains, since they were isolated from persistent and difficult to treat infections.

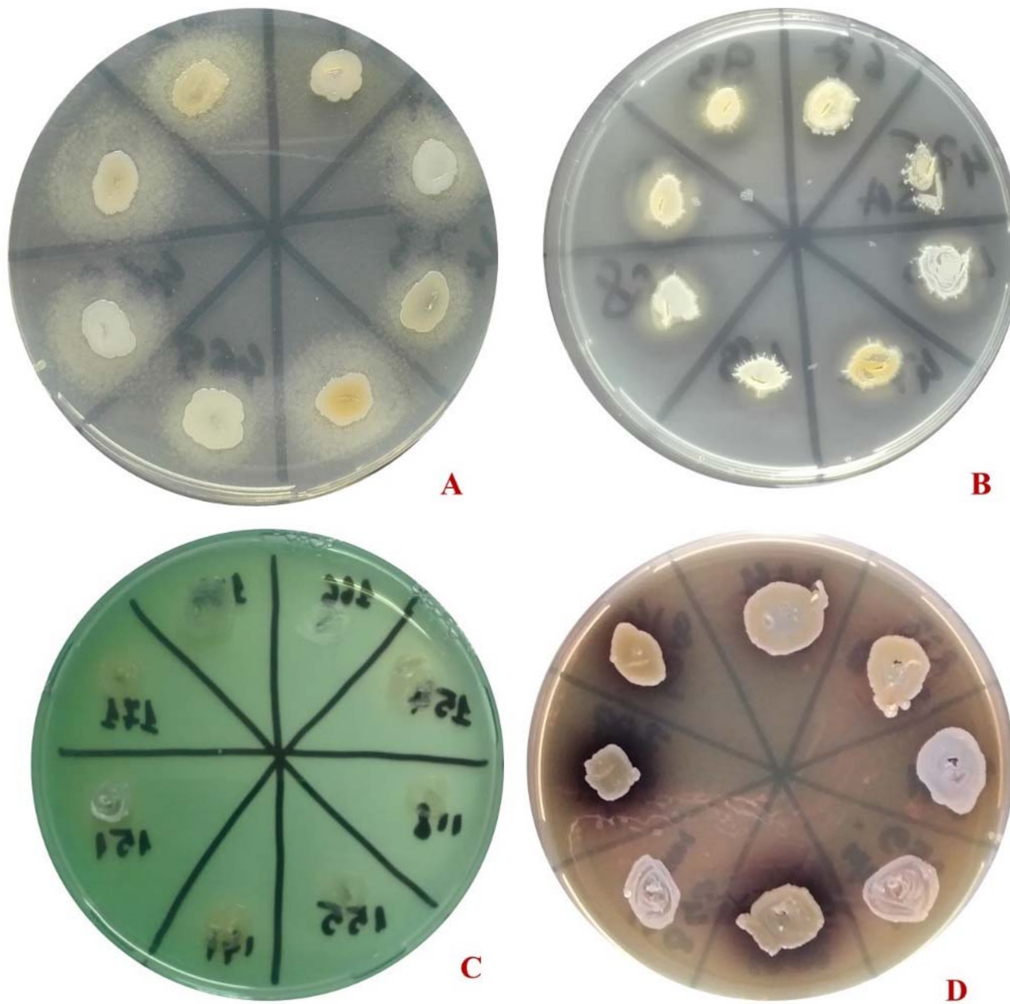

Figure 1. Evaluating the capacity to produce soluble virulence factors at 72 hours: A. lipase; B. lecitinase; C. DN-ase; D. Esculinase.

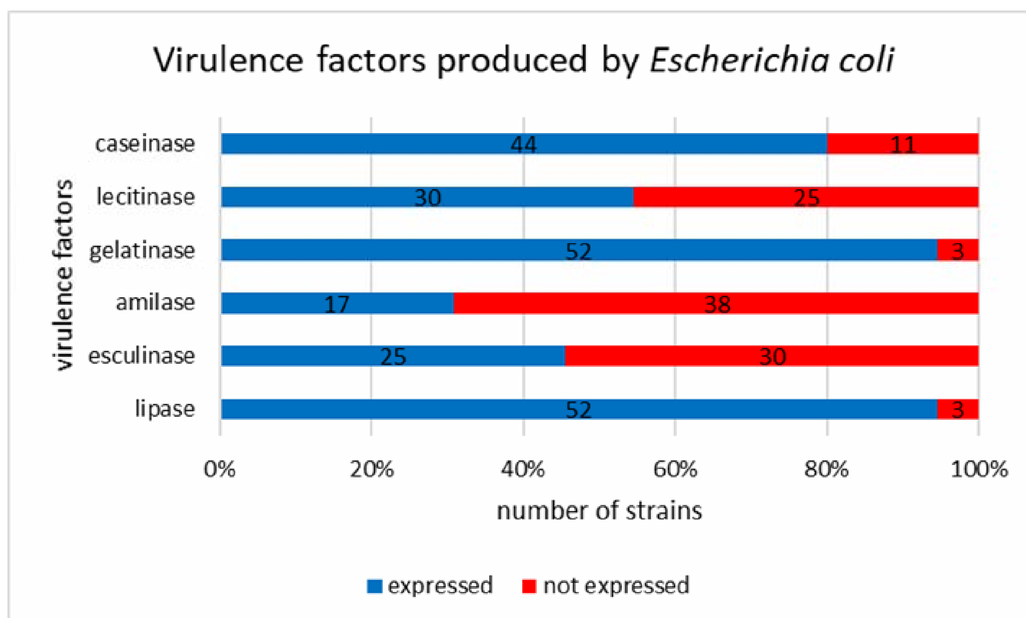

Figure 2. Soluble virulence factors produced by *E. coli*.

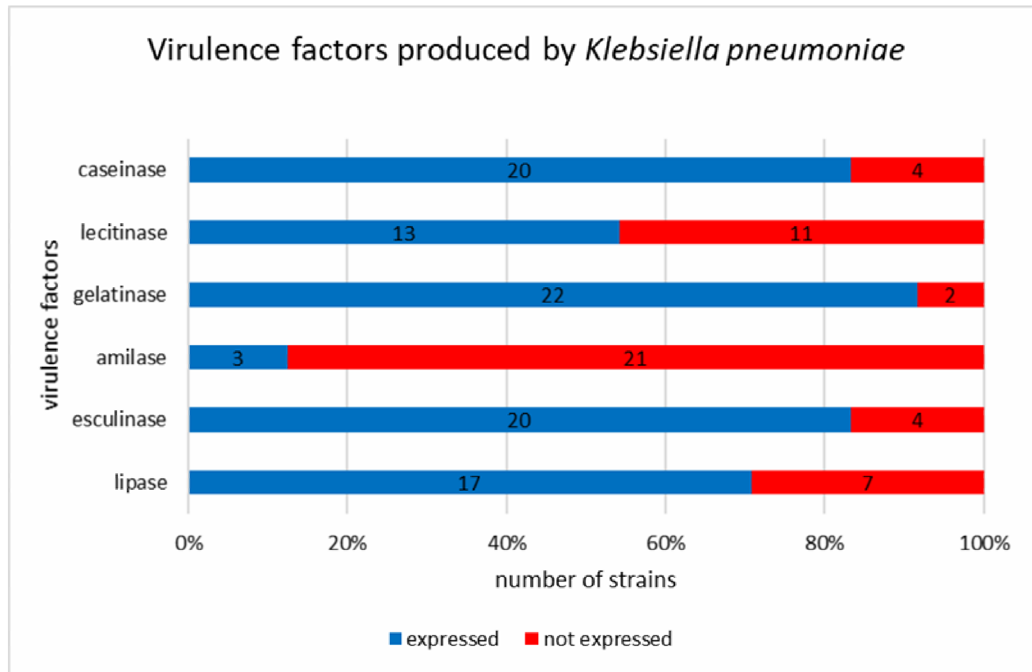

Figura 3. Soluble virulence factors produced by *K. pneumoniae*.

CONCLUSION

The strains included in this study produced multiple virulence factors; most of them produced more than three. *Klebsiella pneumoniae* was observed to produce more virulence factors than *Escherichia coli.* The high number of produced virulence factors may be one of the reasons which contributed to the persistence of the infection and the difficulty to treat it. The characterization of the produced virulence factors might help to improve the treatment approach.

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