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POLYCLONAL ANTI-INTIMIN ANTIBODY: IMMUNOLOGICAL CHARACTERIZATION AND ITS USE IN EPEC AND EHEC DIAGNOSIS

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ABSTRACT

Intimins are outer membrane proteins expressed by enteric bacterial pathogens capable of inducing intestinal attachment-and-effacement lesion (A/E). Through immunoblotting, immunofluorescence, flow citometry and immunogold we observed that the obtained polyclonal antibody against conserved intimin region recognizes the different intimin subtypes and suggests that it can be used as a tool for EPEC and EHEC detection. Besides, *immuno-dot* assay seems to be a possible alternative as a capture method.

Key words: intimin, phenotypical analysis, polyclonal antibody, EPEC, EHEC.

INTRODUCTION

A/E lesion is induced either by EHEC and EPEC, enterohemorrhagic *Escherichia coli* (EHEC), the causative agent of bloody and nobloody diarrhea as well as hemolytic uremic syndrome in humans, is prevalent in developed countries. On the other hand, enteropathogenic *E. coli* (EPEC) is a major cause of acute and persistent infantile diarrhea in developing parts of the world (2). EPEC and EHEC colonize intestinal mucosa by intimate attachment of bacteria to the enterocytes, which is followed by aggregation of the cytoskeleton actin and effacement of microvillus, in which intimin is an involved protein. Until now more than 10 different intimin subtypes have been described, these subtypes are defined by variable region (a, b, g, d, e, h, i, k, x) (1,3,4). The conserved region of intimin molecule is constituted of 388-667 amino acid sequence ($Int_{388-667}$). Thus the aim of this study was the generation a polyclonal intimin antiserum against the conserved region and a phenotypical analysis of isolates from EPEC and EHEC using the IgG-enriched fraction of this antibody and the development of capture method for attaching and effacing *E. coli* detection.

MATERIALS AND METHODS

Recombinant His-$Int_{(388-667)}$ expression and purification

Intimin ($Int_{388-667}$) was obtained from recombinant bacterial strains previously cloned into pET (Novagen) system. The expression and purification were done as manufacturer's recommendations.

Intimin polyclonal antiserum

Rabbits were immunized subcutaneously with 200 μg of intimin in complete Freund's adjuvant. The animals were given booster injection three times using the same protein concentration in incomplete Freund´s adjuvant at intervals of 10 days. The IgG-enriched fractions were obtained from rabbit antiserum after being submitted to caprylic acid and ammonium sulfate precipitation.

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Bacterial strains

Different intimin subtype expressing *E. coli* strains: O127:H6 (E2348/69) (α), O142:H34 (α), O119:H6 (β), O128:H2 (β), O55:H7 (γ), O157:H7 (γ), O86:H34 (δ) e O103:H2 (ε) were used. As negative control strain we used *E. coli* K12. All bacterial strains were grown in LB broth at 37ºC for 18h under constant shaking (180 rpm) at 1OD = 660nm.

Immuno-dot assay

Bacterial growth was boiled at 100ºC for 5 min and diluted 1:100 in 0.05M pH 9.6 carbonate-bicarbonate buffer. Different volumes of bacterial suspension were applied on nitrocellulose membrane by *Slot Blot Filtration Manifolds*, Pharmacia® system. Reaction was done as followed: 4 µg/mL of IgG-enriched fraction of anti-intimin antibody and peroxidase labeled goat anti-rabbit serum (1:10000). This reaction were developed with 4-chloro-α-naphtol (3 mg/mL) plus H_2O_2.

RESULTS

The immunochemical characterization of isolates from EPEC and EHEC using the generated polyclonal antibody against conserved intimin region through immunoblotting, immunofluorescence, flow citometry and immunogold demonstrated that the expression of intimin is recognized for the antibody in the several intimin expressing *E. coli* serotypes, these results are represented in Fig. 1 by immunofluorescence assay using anti-rabbit conjugated to fluorescein (FITC) showing different patterns.

In Fig. 2 we can see immuno-dot standardization for capture assay, in which we used to detect intimin-expressing strains. Bacterial growth (1DO) was centrifuged for 5 min in 5000x g, supernatants were discarded and remaining pellet was ressuspended in 100 µL 0.5M pH 6.8 Tris/HCl buffer and boiled at 100ºC for 5 min, followed by a 1:100 dilution in 0.05M pH 9.6 carbonate/bicarbonate buffer. Between different tested volumes 200 µL was defined as ideal amount of protein and reaction was done using 4 µg/mL of IgG-enriched fraction of anti-intimin antibody and peroxidase labeled goat anti-rabbit serum (1:10000) as ideal concentration of first and second antibodies, respectively.

DISCUSSION

Although the excellent immune response obtained in each immunized rabbit with conserved intimin ($Int_{388-667}$) the antibody reactivity or its IgG-enriched fraction showed some inespecificity with other *E. coli* protein when tested by immunoblotting assay, which is solved by sera adsorption with *E. coli* K12. Through immunoblotting we could observe the reactivity of IgG-enriched fraction of anti-intimin serum with different bacterial serotypes that expresses intimin. This reactivity was confirmed by

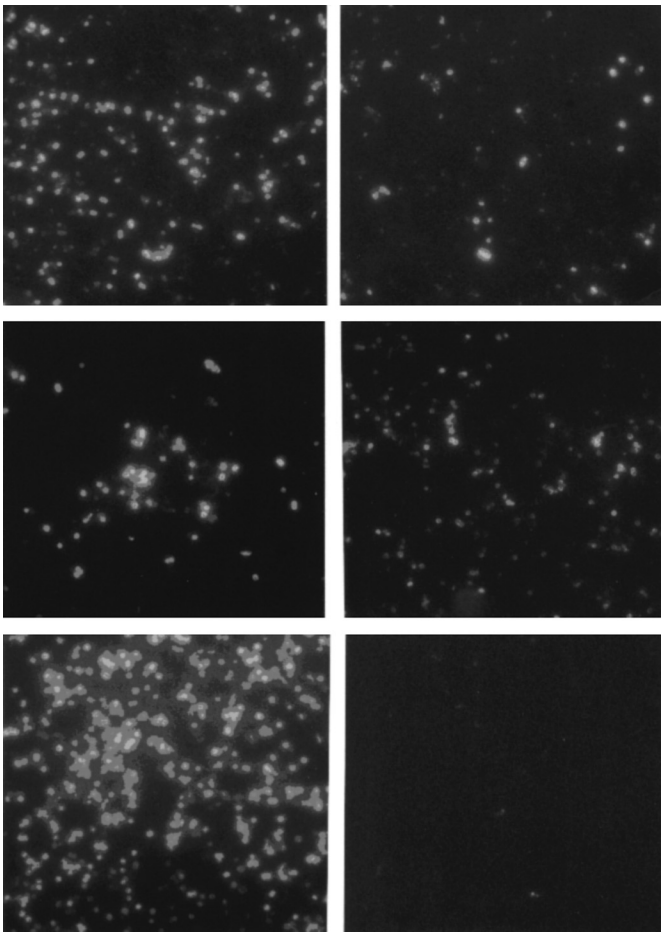

Figure 1. Immunofluorescence of *E. coli* serotypes expressing intimin subtypes. A. O127:H6 (α), B. O128:H2 (β), C. O157:H7 (γ), D. O86:H34 (δ), E. O103:H2 (ε) e F. *E.coli* K12 (negative control). Bacterial were incubated with 4µg/mL of IgG-enriched fraction of polyclonal anti-intimin antibody and FITC anti-rabbit (1:400).

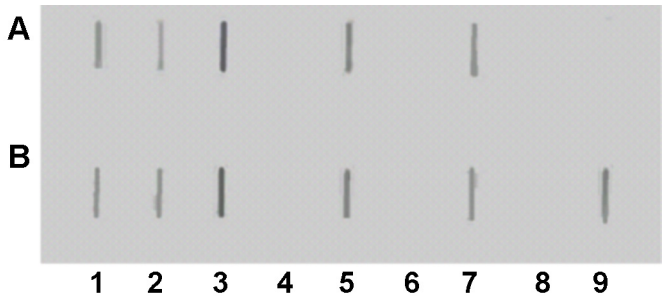

Figure 2. Immuno-dot. Different bacterial serotypes: 1. O127:H6 (α), 2. O142:H34 (α), 3. O103:H2 (ε), 4. O55:H7 (γ), 5. O157:H7 (γ), 6. *E. coli* K12 (negative control), 7. O128:H2 (β), 8. O119:H6 (β), 9. O86:H34 (δ). Tested volumes: A. 100 µL, B. 200 µL. Reaction was done as described in material and methods.

immunofluorescence, flow citometry and immunogold labeling and showed differences in bacterial strains expressing intimin subtypes. permeabilization or denaturation of protein allows this antibody to recognize intimin in bacterial strains. These results lead us to immuno-dot assay, testing the ideal pattern of capture assay, boiling bacterial growth and with the slot-blot filtration manifolds intimin could be detected by IgG-enriched fraction of anti-intimin antibody, thus our results suggest that Permeabilization or denaturation of protein from bacterial samples allow the antibody to recognize several subtypes of intimin and immuno-dot assay seems to be a possible alternative as a capture method.

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RESUMO

Anticorpo anti-intimina: caracterização imunológica e diagnóstico de amostras de EPEC e EHEC

EPEC e EHEC constituem um risco significativo para a saúde pública em diferentes partes do mundo. Ambas colonizam a mucosa intestinal e subvertem as funções celulares do epitélio intestinal ao produzirem uma lesão histopatológica característica, conhecida por lesão A/E (*attaching-and-effacing*), na qual a intimina é uma das proteínas envolvidas. A família das intiminas apresenta também uma região conservada, que compreende os aminoácidos de 388 a 667 (Int $_{388-667}$). O objetivo do presente trabalho foi a obtenção de um anticorpo policlonal contra a região conservada de intimina. A caracterização fenotípica das amostras de EPEC e EHEC utilizando este anticorpo permitiu observar-se a maneira variável que ele reconhece os diversos subtipos de intimina e sugere que ele seja uma ferramenta para detecção destes patógenos, sendo o ensaio de *immuno-dot* o método de captura de escolha.

Palavras-chave: intimina, análise fenotípica, anticorpo policlonal, EPEC, EHEC.

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