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ORIGINAL ARTICLE

Adherence of *Aeromonas hydrophila* strains to human enterocyte-like cells pre-infected with Rotavirus

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Key words

Aeromonas hydrophila adhesiveness • Coinfection

Summary

Introduction. *The interest grown in these years about emerging pathogens in the onset of intestinal disease showed that the pathogenic mechanism is a multifactorial event. Our objective was to evaluate the role of co-infection with rotavirus in the expression of Aeromonas spp adhesiveness.*
Methods. *The rate of co-infection involves contact of Caco-2 cells with the virus, followed by adsorption for 1 and 2 hours. Aliquots of bacterial suspensions were added to tissue-culture plates. After infection, cell monolayers were lysed; serially diluted lysates were plated to determine the number of bound bacteria by performing colony forming units (CFU) counts.*
Results. *Non-adhesive strains were not subject to variations resulting from co-infection, while those who had medium or high adhesiveness gave rise to an increase of the same.*
Discussion. *Infection with rotavirus promotes the Aeromonas ability to adhere to Caco-2 cells and this effect depends on the duration of infection and on the starting adhesiveness of bacteria strain.*

Introduction

In recent in vitro studies it was found that the pathogenicity of the infection of one pathogen can be influenced by the presence of another.
Numerous studies showed that specific virulence determinants (e.g., adhesins, invasins, toxins, capsules, etc.) together with intestinal environment characteristics contribute to unique steps in the pathology of microbes [1-3].
In particular it was highlighted that co-infection by different microorganisms in certain organs or tissues, fosters pathogenic mechanisms for nothing or little detectable in the case of single infection [4].
In addition there is a relationship between invasive bacterial and viral load or time of contact between virus and cells [5].
In this study we analysed the coinfection characteristics of two emerging pathogens, *Aeromonas spp.* and Rotavirus spp., whose etiopathogenetic roles are well known.
Members of the genus *Aeromonas* are implicated in several human pathologies such as diarrheal and extraintestinal infections, including septicemia, wound infections, burn-associated sepsis, and respiratory tract infections [6].
Aeromonas spp. are commonly found in a wide range of aquatic systems and foods and have been isolated from drinking, brackish and waste water and a variety of foods. These species have long been known to cause different infections in poikilothermic animals such as fish, reptiles, and amphibians. *Aeromonas (A.) hydrophila* were characterized for the production of potential virulence determinants, such as production of cytotoxins, cytotonic toxins, hemolysin, and dermonecrotic factors [7].
The mechanisms of the diarrheic action of *Aeromonas* are not completely understood, intestinal pathogens can induce diarrhea by induction of active ion secretion and/or by impairment of the epithelial barrier function of the intestine. *Aeromonas* beta-hemolysin caused a decrease in transepithelial resistance in polarized intestinal epithelial cells, without involvement of additional virulence factors [8].
A study revealed that hemolysin of *Aeromonas sobria* possesses both cytotoxic and enterotoxic activity against mammalian cells [9]. Histopathological examination showed that hemolysin causes diarrhea without damaging the intestinal epithelial cells, showing that aerolysin acts determining the accumulation of fluid in the intestinal lumen without inducing damage to enterocytes.
The presence of this virus is ubiquitous and infection is endemic, although rotavirus is occasionally associated with real epidemics. The infection occurs by fecal-oral transmission through ingestion of contaminated water or food and contact with items or surfaces contaminated too, but it is possible to find transmissions by breathing or by direct contact. Acute episodes are characterized by low grade fever, stomach upset lasted for several days. Almost the whole population is exposed to the virus especially during the first years of life. The incidence is indeed higher in children and infants. The infection, however, is much less frequent in adolescents and in adults and usually shows an unimportant clinical picture or it is asymptomatic, representing, however, a possible source of infection for others [10].

Rotavirus gastroenteritis are also associated with neurological involvements [11].
The mechanism of rotavirus diarrhea may involve a generalized inhibition of Na+-solute symport systems of water reabsorption.
Rotavirus causes a reduced capacity for intestinal Na-Cl and nonelectrolyte absorption. Both D-glucose and L-alanine absorption are impaired, although that of L-alanine appears to be affected only partially [12].
Despite the superficial location, rotavirus induces both a local and systemic immune response. The primary infection induces specific immunological memory sufficient to prevent reinfection, but reduces its severity. Repeated exposure to the virus during childhood helps to strengthen immunity to infection and this explains the rarity of clinical manifestations in adults and adolescents [13].
Rotavirus infection makes Caco-2 cells more susceptible to *Y. enterocolitica* and *Y. pseudotuberculosis* invasion by favouring the early bacteria-cell interaction of attachment and internalization required to initiate infection. In contrast, the superinfection with bacteria resulted in decreased viral antigen synthesis maybe caused by damage of intracytoplasmic organelles produced by intracellular bacterial multiplication [14]. In cells infected with rotavirus there was an increase of *L. monocytogenes* internalisation, whereas the preinfection with poliovirus had only a slight interfering effect on bacterial invasiveness [15].
The purpose of this study was to assess if rotavirus preinfection of Caco-2 cells, which are highly sensitive to aerolysin not only in the apical zone but also in the basolateral portion [16], leading to an increase of *A. hydrophila* adhesiveness.

Methods

Bacterial strains

Fourteen *A. hydrophila* strains were used. These strains were isolated originally from seafood both raw and ready to eat and frozen [17], they were cultured in Brain Hearth Infusion broth (BHI; Oxoid) and subcultured on Tryptone Soya Agar (TSA; Oxoid).

Cell cultures and adhesion assays

Caco-2 cells are derived from human colon adenocarcinoma. They are characterized by mature enterocytes of the intestinal epithelium with tight junctions and brush border microvilli with associated enzymes [18]. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Md) and they were grown in Eagle's Minimum Essential Medium (MEM) containing sodium pyruvate (1 mM) and Fetal Calf Serum (FCS) (15%) (SIGMA-ALDRICH).
Semi-confluent monolayers of these cell lines were grown without antibiotics for 48 h, in 24-well plates (Corning, New York. USA). Cell monolayers were washed twice with 1 ml phosphate buffered saline (PBS), before the addition of bacterial cells and covered with 1 ml of fresh MEM containing 10% FCS without antibiotics.
Test bacterial strains were inoculated into BHI broth and incubated overnight at 37°C. For adhesion assay the bacterial inoculum was estimated by the measurement of optical density (OD) at λ = 560 nm using UV-9200 spectrophotometer.
The bacterial cultures were centrifuged at 4000 rpm for 10 min and resuspended in cell culture Medium (MEM containing sodium pyruvate 1 mM and 10% FCS).
Aliquots of 100 µl bacterial suspensions, containing 10^8 colony forming units (CFU) ml^{-1} were added to tissue-culture plates and incubated in 5% CO_2 atmosphere at 37°C for 90 min.
After infection, cell monolayers were washed twice with PBS to remove unattached bacteria, lysed at room temperature for 15 min with cold 0.1% Triton X-100. Serially diluted lysates in 0.1% Maximum Recovery Diluent (Oxoid) were plated on TSA to determine the number of bound bacteria by performing CFU counts. The plates were incubated at 37°C for 24 h and adherence was expressed as the number of associated bacteria /100 Caco-2 cells.
Experiments were performed in duplicate.

Rotavirus and infection conditions

The RRV stock used was generated in MA104 cells after preincubating the cells for 3 h in a serum-free culture medium [19]. Viruses were activated by treatment with 20 µg/ml trypsin at 37°C for 30 min, and MA104 cell monolayers were infected at a multiplicity of infection of 0.01. After being left to adsorb for 1 h at 37°C, the inoculum was removed and the infected cells were incubated in serum-free medium containing 5 µg/ml trypsin. Viral lysates were harvested when substantial cytopatic effect was observed by freezing and thawing three times. The lysates were cleared by centrifugation at 3000 rpm at 4°C for 10 min. Virus titers were determined in monolayers of Caco-2 cells by a Focus Forming Unit (FFU) assay as described elsewhere [20], and aliquots of this lysate were stored at -80°C.

Coinfection Aeromonas-Rotavirus

A virus inoculum was activated for 30 min at 37°C by exposure to 20 µg/ml trypsin. Caco-2 cells cultured without antibiotics for 48 h were infected with an inoculum of activated RRV for 1 h at 37°C. The inoculum was then removed washing with PBS and fresh medium without antibiotics was added for different time intervals before adding bacterial suspensions following adhesion and invasion assay.
In T0 the bacterial suspensions were added immediately after virus removal, in T1 and T2 bacterial suspensions were added after 1 and 2 hours at 37°C respectively.
Experiments were performed in duplicate.

Statistical analysis

Statistical significance of Aeromonas adhesion values respect controls, was assessed by a Student t test.
The results were also evaluated using the Fisher F test (ANOVA).

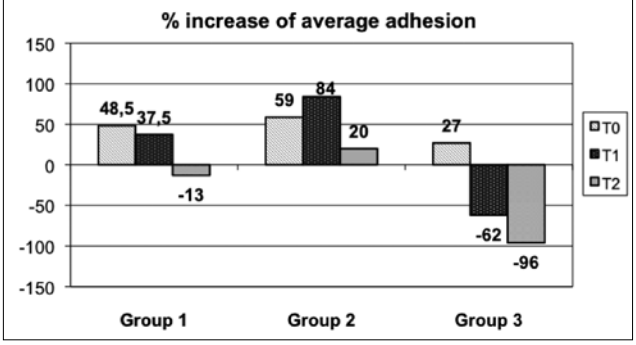
Results and discussion

The overall average values (Tab. I) indicate a general increase in adhesion of the T0 strains (total delta % 0,96) and T1 strains (total delta % 1,38) than K, while in T2 is evident a strong decline.
Because of the sample inhomogeneity shown by calculating the standard deviation, it was necessary to subdivide Aeromonas strains in three different groups.
The first group consists of strains A4, A6, A9 and A14, it has a high starting average adherence and the standard deviation equal to 25, strains A1, A5, A8, A11 and A12 show an intermediate adherence and a deviation standard of 7.4, while the last group consists of strains A2, A3, A7, A10 and A13 presenting low adherence and a standard deviation of 5.6.
How can we see in Figure 1, after one hour of contact with rotavirus (T0) the first group showed an increase of average adhesion of 48.5%, the second group had a percentage increase of 59% while the average adhesion of the third group strains increased by 27%.
In T1 the average adhesion of the first group strains decreased slightly compared to T0, but still increased compared to control (37.5%), the average adhesion of the second group strains on the contrary, further increases in T1 (84%) while average adhesion of the third group collapses (-62%).
In T2 the first group shows a decrease of average adherence by 13% compared to K, even the average adhesion of the strains of the second group decreases dramatically but still shows an increase of 20% compared to K, the average adhesion of the third group underwent a further decrease of 96%.

Tab. I. Average values of Aeromonas hydrophila strains adhesion to Caco-2 cells without Rotavirus (K) and at different times of contact with the virus (T0, T1 and T2), furthermore the table shows the relative standard deviations.

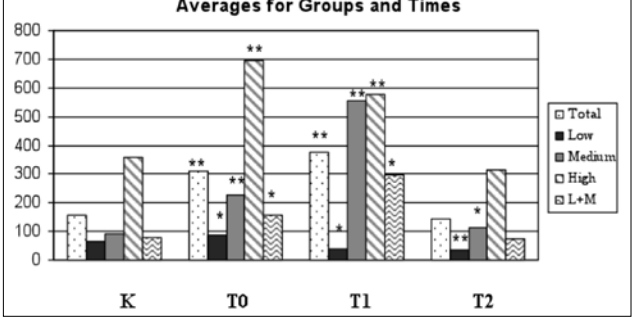
Aeromonas strains	K	T0	T1	T2
1	93.7	241.0	558.3	117.3
2	64.7	93.7	39.3	37.7
3	63.3	105.7	46.3	35.0
4	385.9	670.0	576.0	314.3
5	104.5	223.3	550.3	109.7
6	343.5	683.7	569.7	293.7
7	72.0	85.3	32.3	36.3
8	88.3	206.7	562.7	132.3
9	368.1	669.3	563.3	333.7
10	70.8	89.3	43.7	34.3
11	86.4	253.7	550.7	117.0
12	88.2	201.3	559.7	99.3
13	58.3	74.0	42.0	24.7
14	330.3	752.0	596.3	321.7
Total average	158.4	310.6	377.9	143.4
Median	90.8	226.5	545.5	110.0
Standard deviations	130.0	257.5	255.8	117.3

Fig. 1. Percentage increase of average adhesion of the three different groups in T0, T1 and T2.



From these data it appears that the one-hour contact with the virus promotes the *Aeromonas* adhesion to Caco-2 cells while delaying the addition of the bacteria there is an adhesion decrease perhaps due to the cytotoxic effect of Rotavirus on the cells.
We also noticed that higher is the starting adhesion of the strain more early is its increase, in fact, in strains with high adhesion its increase takes place in T0 while those with medium starting adhesion will have a greater increase in T1.
The contact with the virus does not increase the adhesion of these strains that in T0 adhere to cells with a low percentage indeed they suffer a strong decrease already at T1.
Changes in Aeromonas strains adhesion relative to the control in different conditions were evaluated considering the average of the amount and of the groups (low, medium and high adherence) using the t Student test respect to K.
From Figure 2 we can see a highly significant difference between K and the values to those at T0 and T1 although in this case, with regard to the low adherence, the significance is due to the sharp drop in values.
Whereas the amount it is not significant the difference between K and the values at T2. Considering the single group consisting of low+medium adherence, we have a high significance in T0 ($p < 0.01$), in T1 the significance is low ($p < 0.05$), while no significant difference is K/T2.
The results were also evaluated using the Fisher F test (ANOVA) and this showed that T0 and T1 represent the best times because probably cells are more damaged in T2.
For the single group low + medium adhesion there is a very

Fig. 2. Averanges of the groups low, medium, high and low + medium adherences using the t Student test respect to K, in the different times of virus absorption.



high significance in all three times but we must consider that the test assesses all the numbers and not the medium.

Conclusions

In these years the interest about the role of emerging pathogens in the intestinal disorders development has grown, showing that more than one virulence determinant is typically involved in pathogenesis. Adhesion of bacteria to mucosal surfaces has been recognised as an important process in the pathogenesis of many infections in man and animals [21].
Viruses of the intestinal tract can induce cellular modifications that may modulate the ability of other pathogens to adhere to cells and to favour more severe infections in man [22].
From our data it appears that the one-hour contact with the virus promotes the Aeromonas adhesiveness to Caco-2 cells while delaying the addition of the bacteria, thus allowing greater absorption of the virus, there is an adhesiveness decrease perhaps due to the cytotoxic effect of Rotavirus on the cells.
We also noticed that higher is the starting adhesiveness of the strain more early is its increase, in fact, in strains with high adhesion its increase takes place in T0, then immediately after the time of contact with the virus, while those with average starting adhesion will have a greater increase in T1, that is after an hour of virus absorption at 37°C.
The contact with the virus does not increase the adhesiveness of these strains that in T0 adhere to cells with a low percentage indeed they suffer a strong decrease already at T1.
In conclusion in this study we found that a preinfection of human enterocyte-like cells with Rotavirus can influence some *Aeromonas* strains increasing their capacity of adhesion to cells and this effect varies with the duration of viral infection. Therefore we can say that, where the social and economic conditions conducive to the simultaneous exposure of the population to Aeromonas and Rotavirus, the action of such emerging pathogens could be potentiated.

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