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Salicylate inhibits fimbriae mediated HEp-2 cell adherence of and haemagglutination by enteroaggregative *Escherichia coli*

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Abstract

Enteroaggregative *Escherichia coli* (EAggEC) are associated with both acute and persistent diarrhoea in children. Bowel colonisation due to fimbrial adherence factors appears to play a major role in the disease process. In this study, we investigated the effect of sodium salicylate and 5-aminosalicylic acid on adherence of a type strain and 40 clinical isolates of EAggEC to HEp-2 cells and erythrocytes from different species. Growth in the presence of 10 mM salicylate resulted in markedly decreased adherence to tissue culture cells with 33/40 (82.5%) isolates, and was also associated with inhibition of haemagglutination in 20/33 (60.6%) isolates. Complete or partial inhibition of adherence was also seen in two of five isolates showing localised adherence and three of five isolates with diffuse adherence. Decrease in adherence was associated with decreased or absent expression of fimbriae in 28/40 (70%) of the EAggEC isolates, although production of outer membrane proteins was not affected. Salicylates appear to inhibit adherence mediated by fimbrial adhesins.

Keywords: Cell adherence; *Escherichia coli*; Haemagglutination; Fimbriae

1. Introduction

Enteroaggregative *Escherichia coli* (EAggEC) are currently defined as *E. coli* that do not secrete the enterotoxins LT and ST and that adhere to HEp-2 cells in an aggregative pattern [1]. A number of studies from developing countries have supported the association of EAggEC with diarrhoea in children, both in acute and persistent episodes of diarrhoea [1–3]. While available data do not yet permit a full description of EAggEC pathogenesis, it is likely that bowel colonisation due to fimbrial adherence factors would play as major a role in the disease process as it does in diarrhoea due to other categories of diarrhoeagenic *E. coli*. Candidates for factors that may facilitate initial colonisation include the rod-like fimbriae described by Vial et al. [4] the four types of fimbriae, rod, hollow rod, fibrillar and fibrillar bundles described by Knutton et al. [5] and the aggregative adherence fimbriae described by Nataro et al. [6,7]. A 30-kDa outer membrane protein (OMP) which may play a role in adherence of EAggEC has also been identified [8]. In vitro studies on the

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role of adherence of EAggEC in the pathogenesis of diarrhoea have utilised tissue culture cell lines, human intestinal mucosa and erythrocytes from different species [1,4–6].

Previous studies on fimbrial expression and adherence in different classes of *E. coli* have shown that in uropathogenic *E. coli*, certain compounds, such as bismuth salts and salicylates, and growth in hyperosmolar conditions inhibit or modulate the expression of P-fimbriae which mediate adherence to uroepithelial cells, while type I fimbriae and S-fimbriae were not affected [9]. Salicylic acid has also been shown to decrease adherence of *E. coli* to silastic catheters [10]. The current study was undertaken to determine whether salicylates could block adherence of enteroaggregative *E. coli* to HEp-2 cells and erythrocytes.

2. Materials and methods

2.1. Bacterial strains

The strains used were enteroaggregative *E. coli* (EAggEC) strain 17-2 and 40 isolates from children and adults with diarrhoea and controls in earlier studies [3,11]. These were shown to be enteroaggregative in the HEp-2 cell adherence assay, and stored as stab cultures in nutrient agar at 4°C. All isolates were retested for the enteroaggregative phenotype before inclusion in the study, of the 40 isolates, 31 hybridised with the EAgg probe as described earlier [3,12].

2.2. Additives to growth media

For the initial part of the study, strain 17-2 was incubated in appropriate media alone or with 5, 10, 15 and 20 mM final concentrations of sodium salicylate or 5-aminosalicylic acid. Quantitation of bacteria grown in the presence of various concentrations of these compounds was carried out by plating 100 μl of 10^{-5} to 10^{-10} dilutions of 16 h cultures on MacConkey agar plates. All clinical isolates were grown in the presence of 10 mM sodium salicylate, before testing for adherence and haemagglutination in parallel with the same isolate grown without sodium salicylate. The EAggEC were additionally examined for presence or absence of fimbriae by electron microscopy. Reversibility of the effect of salicylates on each isolate was checked for by subculture of the culture in 10 mM salicylate containing medium into media without additives, incubation at 37°C overnight and repetition of the assays.

2.3. Adherence assay

The strains were grown in 1 ml Luria broth for 16 h at 37°C for use in the adherence assay, which was carried out as reported previously [3] and the Giemsa stained slides examined under the oil immersion lens of a light microscope. All experiments were repeated at least three times.

2.4. Haemagglutination assay

Slide haemagglutination was carried out in the absence and presence of mannose as described by Quadri et al. [13]. All tests were repeated at least twice.

2.5. Electron microscopy

Type strain 17-2 and 40 AA isolates were examined for presence or absence of fimbriae by electron microscopy after overnight culture in LB broth and LB broth containing 10 mM salicylate. Eighteen hour cultures of isolates were washed in phosphate buffered saline, pH 7.2 and suspended in distilled water to a concentration of 10^8–10^9 cells ml^{-1}. A 10 μl aliquot of the bacterial suspension was mixed with 10 μl of 2% ammonium molybdate, pH 6.8, and 10 μl of bacitracin (150 μg ml^{-1}). Ten microlitres of the mixture was applied to carbon coated 400-mesh copper grids which had been UV-irradiated for 30 min prior to use. After 2 min, the excess liquid was blotted with a filter paper wick, allowed to air-dry and viewed by transmission electron microscopy at 45 000×, the cells being rated for the presence, decrease in number or absence of fimbriae. At least 30 bacteria were examined on each grid.

2.6. Outer membrane protein preparation

OMPs were isolated as previously described [8] from strain 17-2 and 10 EAggEC isolates grown

overnight at 37°C in 20 ml Luria broth with and without 10 mM sodium salicylate. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 4% stacking gel and an 8% separating gel after the OMP preparations were solubilised for 5 min at 100°C in sample solution (0.05 M Tris-HCl (pH 8.0), 2.5% SDS, 5% 2-mercaptoethanol, 25% glycerol, 0.003% Bromophenol blue). The proteins were visualised after the gels were stained with Coomassie brilliant blue R250 and destained with 30% methanol–10% acetic acid.

2.7. Data analysis

The data were analysed using the Student's *t*-test and χ^2-values for each set of variables.

3. Results

3.1. Effect of salicylates on HEp-2 cell adherence and fimbrial production of strain 17-2

Overnight incubation with sodium salicylate or 5-aminosalicylic acid inhibited adherence of strain 17-2 to HEp-2 cells, with sodium salicylate inhibiting adherence at lower concentrations than 5-aminosalicylic acid (Table 1). Growth in the presence of sodium salicylate resulted in a consistent morphological change in the few adherent bacteria seen, with 50–60% of the bacteria showing elongation to almost twice the length of the bacteria grown without the addition of sodium salicylate. No morphological changes were observed in the bacteria grown in the presence of 5-aminosalicylic acid. Quantitation of bacteria grown with 0–20 mM concentrations of these compounds showed that the decrease or inhibition in adherence was not due to any decrease in the bacterial number.

Electron microscopy of strain 17-2 after growth in Mueller–Hinton broth showed that it had over 100 fimbriae (Fig. 1, top) which were morphologically rods, hollow rods and bundle formimg fimbriae. The aggregative adherence fimbriae were difficult to distinguish from other forms of fimbriae, unless they were actually forming a bundle of at least four to five fimbriae wrapped around each other (Fig. 1, top). Growth in medium containing 10 mM sodium salicylate resulted in a decrease of fimbrial number to less than 10 in strain 17-2 (Fig. 1, bottom).

3.2. Effect of growth in sodium salicylate on HEp-2 cell adherence and fimbrial production of clinical isolates

A total of 33 of 40 (82.5%) clinical isolates showed decrease or inhibition of aggregative adherence after overnight growth in the presence of a 10 mM concentration of sodium salicylate (Table 2). The change in the phenotype occurred with the same frequency

Table 1
Effect of various concentrations of salicylates on adherence of enteroaggregative *E. coli* strain 17-2

Additive and concentration (mM)	Result of HEp-2 cell adherence assay
5-Aminosalicylic acid	
0	AA++
5	AA++
10	AA+
15	Few bacteria show AA
20	Few bacteria show AA
Sodium salicylate	
0	AA++
5	AA±, elongated bacteria
10	NA, few elongated bacteria
15	NA, few elongated bacteria
20	NA, no bacteria

AA++, aggregative adherence to over 75% of HEp-2 cells, no change from original adherence pattern; AA+, aggregative adherence to less than 50% of HEp-2 cells, decrease in adherence, but no change in pattern; DA+, diffuse adherence to less than 50% of HEp-2 cells, change in adherence pattern from aggregative to diffuse; NA, non-adherent, complete inhibition of aggregative adherence.

Fig. 1. A: electron micrograph of a negatively stained preparation of strain 17-2 showing two types of fimbriae, rod-like fimbriae which can be seen individually (arrow) and thin, flexible fimbriae which appear to be forming a bundle (arrowhead). Each bacterial cell has over 100 fimbriae (×61 800). B: electron micrograph of a negatively stained preparation of strain 17-2 after growth in 10 mM salicylate. Occasional rod-like fimbriae are seen (arrow), but no bundle-forming fimbriae are seen. Total number of fimbriae per cell is less than 10 (×61 500).

in the isolates from patients and controls. In eight isolates, growth in the presence of salicylate caused a change in phenotype from an aggregative type to a diffuse type of adherence (Fig. 2A,B), although all these isolates showed adherence to less than 50% of the tissue culture cells. Complete inhibition of adherence was seen in 9 (22.5%) of 40 isolates tested (Fig. 2C). A total of 16 (40%) isolates showed decreased aggregative adherence, with eight isolates of bacteria adhering in small clumps to the HEp-2 cells in a few areas on the slide, and eight isolates adhering to the glass of the slide (Fig. 2D).

The decrease in adherence by isolates grown in salicylate containing media was reversible. Subculture in salicylate free media resulted in aggregative adherence to over 75% of the tissue culture cells by all 40 isolates.

Electron microscopy of the isolates after growth in Mueller–Hinton broth showed that all of the 40 isolates had over 100 fimbriae, similar to those seen in strain 17-2. Growth in medium containing sodium salicylate did not affect expression of fimbriae in 12 (30%) isolates, completely inhibited fimbrial expression in nine (22.5%) and resulted in a decrease of fimbrial number to less than 10 in 19 (47.5%) isolates (Table 2). In the 19 isolates where fimbrial expression was reduced, no bundle-forming fimbriae were seen, the fimbriae seen were hollow rods, often only one or two per cell. All seven isolates which did not show any change in aggregative adherence in the

Fig. 1 (*Continued*).

HEp-2 cell assay also did not have a decrease in fimbrial expression. In five isolates where fimbrial expression was not affected, the adherence pattern remained aggregative, but was reduced to 50% of the tissue culture cells. Complete inhibition of fimbrial expression resulted in lack of HEp-2 cell adherence in seven isolates and diffuse adherence to less than 25% of cells in two isolates. Decrease or absence of fimbrial expression correlated with decrease in aggregative adherence ($P < 0.01$).

Table 2
Comparison of effect of 10 mM sodium salicylate on HEp-2 cell adherence, haemagglutination and fimbrial expression of 40 isolates of enteroaggregative *E. coli*

Adherence pattern change (*n*)	Fimbrial expression			Haemagglutination	
	Unaffected	Decreased	Complete inhibition	Unaffected	Inhibited
AA++ (7)	7	–	–	5	1
AA+ (16)	5	11	–	4	10
DA+ (8)	–	6	2	3	3
NA (9)	–	2	7	1	6

See Table 1 for expansions of terms used. Inhibition of haemagglutination refers to inhibition of mannose-resistant or mannose-sensitive haemagglutination of one or more types of erythrocytes.

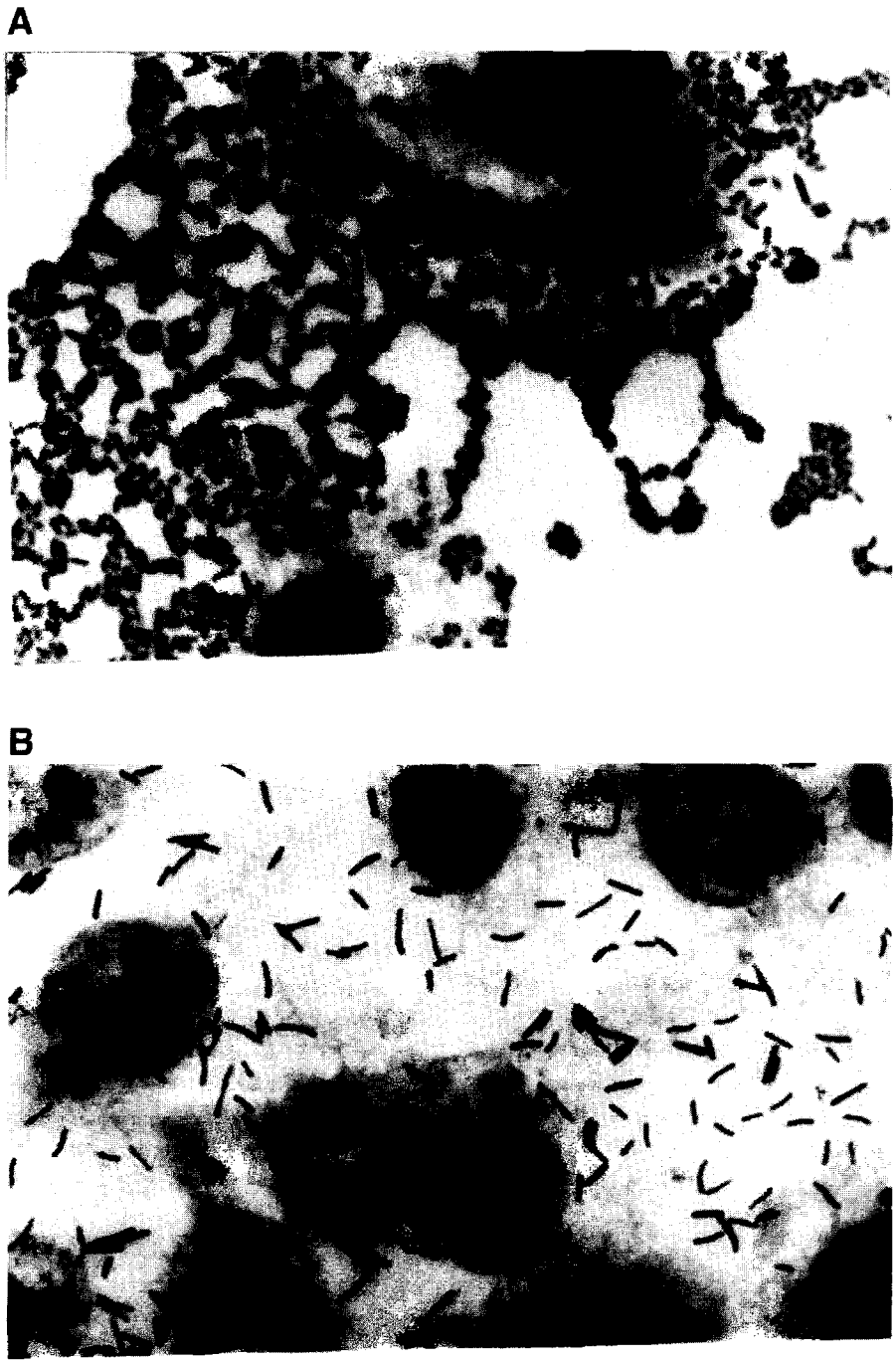

Fig. 2. A: Adherence assay using HEp-2 cells showing typical aggregative adherence of *E. coli* before growth in 10 mM salicylate (×3240). B: Adherence assay using HEp-2 cells incubated with EAggEC grown in 10 mM salicylate. A change in pattern to the diffuse type of adherence is seen. Bacteria are markedly elongated (×3420). C: Adherence assay using HEp-2 cells incubated with EAggEC grown in 10 mM salicylate. Inhibition of adherence is almost complete, with occasional adherent bacteria seen (×3240). D: Adherence assay using HEp-2 cells incubated with EAggEC grown in 10 mM salicylate. A decrease in aggregative adherence is seen, along with an elongation of bacteria (×3330).

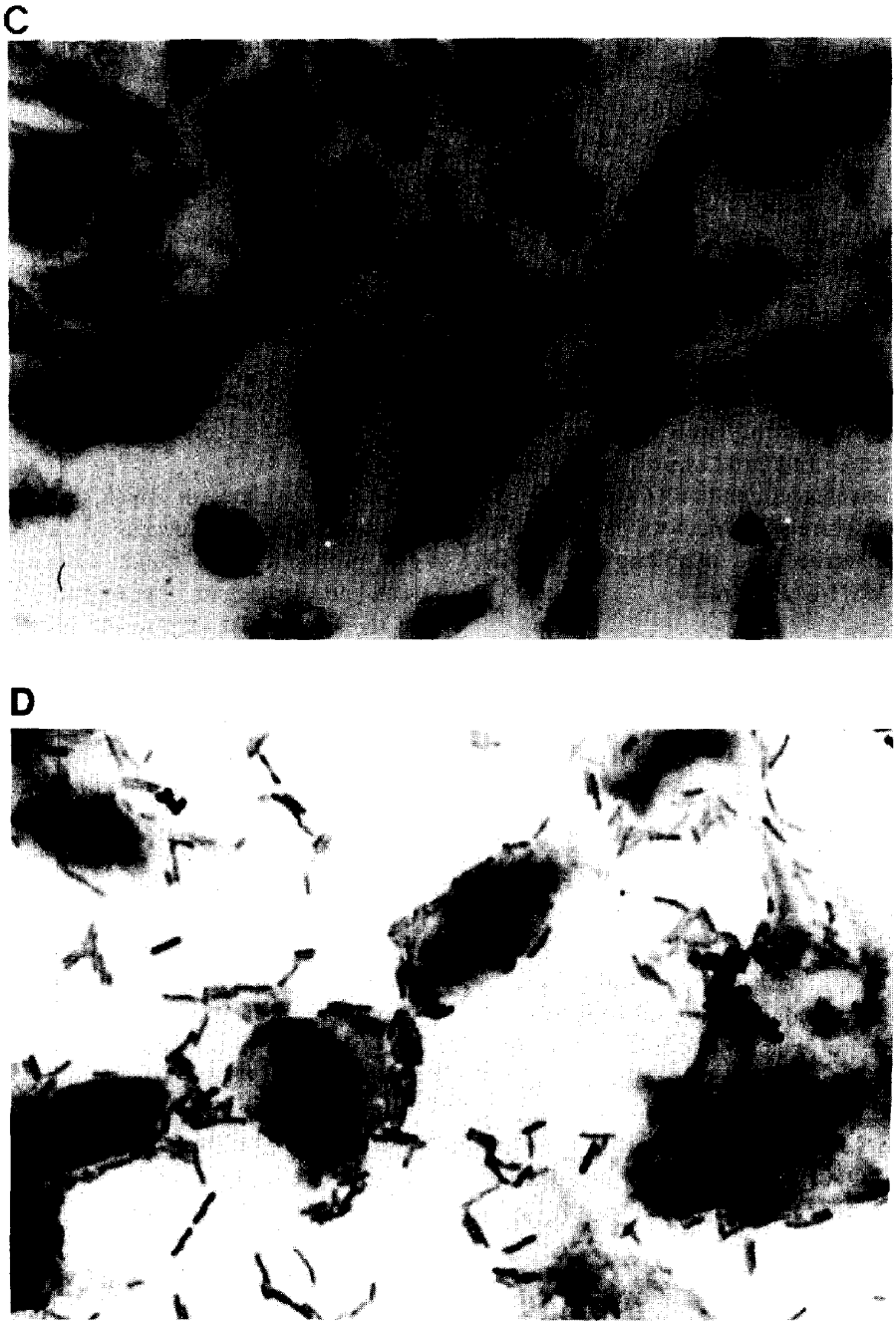

Fig. 2 (*Continued*).

Subculture of the isolates in salicylate free media resulted in restoration of fimbrial expression in all isolates, which was paralleled by aggregative adherence in the adherence assay.

3.3. *Effect of growth in sodium salicylate on haemagglutination by clinical isolates of EAggEC*

Of the 40 isolates included in this study, seven did

not agglutinate human, rat or sheep erythrocytes. Mannose-resistant haemagglutination (MRHA) alone of one or more types of erythrocytes tested was seen in 20 isolates. An additional 11 isolates showed both MRHA and mannose-sensitive haemagglutination (MSHA) and two isolates showed only MSHA of one or more of the three types of erythrocytes used in this study. After growth in 10 mM sodium salicylate, inhibition of MRHA was seen in 18/31 (58%) and inhibition of MSHA was seen 7/13 (53.7%) of the isolates. Cumulatively, of the 33 isolates which showed haemagglutination, 20 (60.6%) showed inhibition of haemagglutination after growth in sodium salicylate containing media. Decrease or absence of fimbrial expression correlated with decrease in haemagglutination ($P < 0.04$). There was a significant correlation between inhibition of tissue culture adherence and inhibition of MRHA ($P < 0.01$) and MSHA ($P < 0.04$).

3.4. *Effect of salicylates on OMP production of EAggEC*

Strain 17-2 and all 10 isolates tested did not show any difference in OMP profiles when grown in media with and without 10 mM salicylate.

4. Discussion

Growth of the type strain 17-2 and clinical isolates of EAggEC in media containing salicylates resulted in significant decrease in aggregative adherence, fimbrial expression and haemagglutination. This was a reversible phenomenon, with restoration of the original adherence characteristics with one subculture in salicylate-free medium. This inhibition of HEp-2 cell adherence and HA occurred in both pathogenic and non-pathogenic EAggEC.

There was a significant correlation between decrease in fimbrial expression and inhibition of aggregative adherence and haemagglutination. In addition to the decrease in fimbrial production, an alteration in the morphology of the bacteria was also noted, with elongation of the cells. Studies on the adherence of EAggEC carried out on animal models and explants of intestinal tissue indicate that adherence to tissue culture cells and human erythrocytes is mediated by 2–3 nm diameter, flexible, bundle-forming fimbriae called aggregative adherence fimbriae 1 and 2 (AAF/I, AAF/II) [1,7]. Formation of these fimbriae was inhibited in 28 of the 40 strains by growth in salicylate containing media. In two isolates, complete inhibition of fimbrial production was associated with change in the phenotype of adherence to the diffuse pattern, indicating that adherence to epithelial cells may be due to many adhesins, not all of which are fimbrial. In a further seven isolates in which the change in phenotype was seen, only rod type fimbriae were identified, indicating that the rod type of fimbriae may mediate some degree of HEp-2 cell adherence, but not the aggregative pattern.

In eight isolates in which decrease of adherence was accompanied by decreased, but not absent, fimbrial production, no bundle-forming fimbriae were seen and the bacteria appeared to adhere to the glass of the slide rather than to the epithelial cell, in contrast to the non-salicylate grown bacteria, although both were arranged in parallel rows. This pattern has been described by Nataro et al. as non-specific aggregative adherence in their study on transposon mutagenesis of an aggregative adherence clone [6].

The decrease in adherence to tissue culture cells was paralleled by a decrease in both mannose-sensitive and mannose-resistant haemagglutination. It has been shown that the AAF/I fimbriae mediate haemagglutination of human RBCs [6], and the decrease in MRHA and MSHA of all types of RBCs with inhibition of fimbrial expression indicates that fimbriae may be responsible for haemagglutination of other types of RBCs as well.

It is also possible that, in addition to fimbriae, outer membrane proteins may also be involved in adherence of EAggEC to mammalian cells, as in enteropathogenic *E. coli*. Antibodies raised against a 30-kDa OMP have been shown to interrupt adherence of EAggEC to HEp-2 cells and inhibit haemagglutination by isolates that express antigenically related OMPs of molecular weights between 30 and 43 kDa [8]. However, in this study, we have shown that inhibition of EAggEC adherence to tissue culture cells and erythocytes by salicylate is not due to lack of expression of outer membrane proteins.

In addition to inhibiting P-fimbriae of uropathogenic *E. coli* and CFA fimbriae of enterotoxigenic *E.*

coli and blocking the expression of OmpF porin [9], salicylate also inhibits expression of porins in other Gram-negative organisms like *Serratia marcesens* and the production of capsular polysaccharide in *Klebsiella pneumoniae* and potentiates the effect of aminoglycoside antibiotics against these bacteria [14,15]. A recent study has shown that salicylate, in addition to inhibiting fimbrial synthesis, also inhibits flagellin synthesis in *E. coli*, *Proteus*, *Providencia* and *Pseudomonas* [16]. The exact mechanism by which salicylate exerts its effect is not known, but the results presented here suggest that it interferes with the formation of aggregative adherence fimbriae.

Acknowledgments

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