J Exp Clin Med 2014;6(1):16–20

Contents lists available at ScienceDirect

Journal of Experimental and Clinical Medicine

journal homepage: http://www.jecm-online.com

ORIGINAL ARTICLE

Escherichia coli Capsular Polysaccharide Synthesis, Antibiotic Susceptibility, and Red Blood Cell Agglutination

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ARTICLE INFO

Article history:
Received: Oct 31, 2012
Revised: Dec 18, 2013
Accepted: Dec 23, 2013

KEY WORDS:
antibiotic resistance;
human serum bactericidal activity;
red blood cell hemagglutination

Background: *Escherichia coli* is a normal component of the human intestinal flora, but it is also a pathogen particularly in women with urinary tract infections, cystitis, and kidney diseases. A new capsular polysaccharide (CPS)-synthesizing variant strain of *E. coli* was isolated from a culturing bacterial medium containing proteose peptone No. 3 glycerin salt in our previous study. In this study, we further isolated a new *E. coli* variant, which produces capsular polysaccharides (CEF-CPS), using cefazolin (CEF) and examined the pathogenic characteristics of this CEF-CPS strain.

Methods: Polysaccharides produced by CEF-treated *E. coli* were separated and purified using anion exchange resin and gel filtration column chromatographic methods. The red blood cell (RBC) agglutination assay and antibiotic susceptibility test were conducted, and serum bactericidal activity, antibiotic tolerance, and antibiotic uptake were also examined.

Results: (1) Variant strains had greater RBC hemagglutination ability than parental strains. (2) The CEF-CPS variant strain showed a two-fold increase compared to the parental strain. (3) Antibiotic resistance to CEF, ampicillin, and polymyxin B was increased four-fold in the CEF-CPS variant strain. (4) Bacterial cell counts in the parental strain incubated in a medium containing gentamicin were reduced significantly, near to the undetectable level, within 4 hours. By contrast, bacterial counts of the CEF-CPS strain displayed only 50% reduction compared to the original bacterial counts under the same culture environment.

Conclusion: *E. coli* derived from normal human intestinal flora generated a new variant strain along with the production of newly synthesized additional polysaccharides on the cell wall in the presence of CEF. Their productions can contribute to enhancing multiple-drug resistance and pathogenicity.

1. Introduction

Escherichia coli is commonly found in the lower intestine of warm-blooded organisms. Numerous serotypes of diarrheogenic *E. coli* are connected to epidemic or infectious diarrhea in humans and animals.[1] Outside of the intestinal tract, *E. coli* can cause diseases such as urinary tract infections, sepsis, polyserositis, and newborn meningitis.[2–4] Pathogenic substances associated with nonintestinal infections have been researched extensively and include endotoxins [lipopolysaccharide (LPS)], K-antigen, hemolysin, and cytotoxic proteins.[5–7] It remains unclear whether enteropathogenic *E. coli* and nonintestinal *E. coli* are the same strains.

Mucoid and nonmucoid forms of *E. coli* colonies were isolated in a culture medium containing proteose peptone No. 3 glycerin salt (PGS). Although capsular polysaccharide (CPS)-synthesizing strains usually display mucoid-form colonies, they display normal metallic-black-colored colonies when cultured in nonselective or selective media. By contrast, another strain without CPS-synthesizing capability display colonies of dark metallic color under all tested culture media. It has been reported that the CPS-synthesizing strains comprise 44.4% of strains that can be isolated from sputum and 100% of those isolated from central spinal fluid in clinical settings.[1,2] In addition, CPS-synthesizing strains isolated from clinical biopsy or specimens have gained strong resistance to multiple antibiotics. Furthermore, it has also been demonstrated that mice infected with CPS-synthesizing strains have higher mortality rates, as a result of exposure to more enterotoxins. It

Conflicts of interest: All coauthors have provided full disclosure of all biomedical financial interests and specified the nature of all potential conflicts of interest, financial or otherwise, in the manuscript.

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1878-3317/$ – see front matter
http://dx.doi.org/10.1016/j.jecm.2014.01.009

suggests that CPS-synthesizing strains produce more enterotoxins than non-CPS-synthesizing strains.

Capsular antigens of *E. coli* are acidic polysaccharides derived from specific oligosaccharides. Surface antigens include fimbriae and K-antigen; these acidic polysaccharides are associated with nonintestinal *E. coli* infections and are also producing pathogenic factors related to numerous bacterial infections, leading to decreased phagocytic effectiveness and neutralization of antibiotics. In addition, they are also crucial target molecules for developing vaccines against bacteria expressing CPS. In this study, we intended to investigate the effects of CEF-CPS strain on the virulence, drug resistance, and human RBC agglutination.

2. Methods

2.1. Isolation of parental stains and induction of CPS-synthesizing variant stains

Non-CPS-synthesizing *E. coli* was isolated from stool samples of healthy adults and cultivated on PGS plates. The colonies were metallic and characterized as non-CPS-synthesizing parental strains.[8] The parental strains were cultured in PGS plates[9,10] at 37 °C for 1 week, and the colonies with mucus on the surface were isolated and further cultured in PGS plates. They were recognized as cefazolin-capsular polysaccharide (CEF-CPS) variants.

2.2. Production and purification of CEF-CPS strain

CEF-CPS strain was first cultured in PGS, and then further cultured on PGS plates at 37°C for 48 hours without perturbation. CEF-CPS was purified by gel filtration using a Sepharose CL-4B column (Pharmacel Biotech, Uppsala, Sweden). The lyophilate from the *diethylaminoethyl* (DEAE) Sephacel column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was dissolved in 5 mL deionized distilled water and then passed through the column at a flow rate of 20 drops/minute (calibrated by a microtube pump).[11] Each fraction was collected, and its uronic acid and protein contents were determined by the carbazole test[12] and Lowry method,[13] respectively. Endotoxins were determined using an LAL assay kit (Sigma-Aldrich, St. Louis, Missouri, USA).

2.3. Hemagglutination test

Bacterial suspension was placed on a glass slide, and one drop of a 2% suspension of guinea pig red blood cells (RBCs) or human A-, B-, AB-, and O-type RBCs was added to it. A hemagglutination (HA) value[14,15] was defined as the minimal concentration necessary for agglutination.

2.4. Human serum bactericidal activity

The parental and CEF-CPS strain broths were placed in 1.5 mL centrifuge tubes, and human serum was added and stirred to produce a uniform mixture. The tubes were placed in an incubator at 37°C for 30 minutes and 60 minutes. The mixture was evenly mixed and diluted with sterile normal saline. The diluted mixture was spread on Trypticase soy broth (TSB) agar plates (BD Difco, Franklin Lakes, New Jersey, USA) and cultured at 37°C for 18–24 hours, and the colonies were counted.[16]

2.5. Antibiotic susceptibility

Antibiotics were diluted with sterile normal saline to produce a working solution. These solutions were mixed with a sterile heart-infusion (HI) agar broth that had been warmed to 48°C; the broth was poured into plastic culture plates, cooled, and air dried. The parental and CEF-CPS strains were cultured on PGS, HI, and Mueller Hinton (MH) media at 37°C for 18–24 hours. The antibiotics used were chloramphenicol (Chunghwa Chemical, New Taipei City, Taiwan), gentamicin (Schering, Kenilworth, New Jersey, USA), cefazolin (CEF; Fujisawa, Taipei, Taiwan), carbenicillin, clindamycin (Upjohn, Kalamazoo, Michigan, USA), trimethoprim/sulfamethoxazole (Glaxo Wellcome, London, UK), ampicillin, polymyxin B, and tetracycline HCl (Sigma-Aldrich, St Louis, MO, USA). The bacterial HI broth [3×10^8 colony-forming units (CFU)/mL] was inoculated onto agar plates containing antibiotics of various concentrations,[17] and inspected to determine the minimal inhibitory concentration (MIC).

Table 1 Antibiotic susceptibility of the parental strain and CEF-CPS-synthesizing variant

Antibiotics	Minimum inhibitory concentration (μg/mL)	
	Parental strain	CEF-CPS variant
Cefazolin	16	64*
Carbenicillin	16	16
Ampicillin	2	8*
Polymyxin B	1	4*
Gentamicin	2	2
Tobramycin	2	2
Amikacin	16	16
Chloramphenicol	128	128
Tetracycline	1	1

*Antibiotic resistance in the CEF-CPS variant strain was increased four-fold only in cefazolin, ampicillin, and polymyxin B.
CEF-CPS = cefazolin-capsular polysaccharide.

2.6. Antibiotic tolerance

The parental and CEF-CPS strains were cultured in an HI broth and diluted with sterile normal saline to a suspension of approximately 10^9 CFU/mL. Gentamicin was prepared at a concentration of 100× the MIC for the parental and CEF-CPS strains, and introduced into the bacterial suspension. Observations were made at 0, 15, 30, and 60 minutes.[18] The broth was diluted with normal saline and centrifuged, and the pellet was washed three times using saline. A saline suspension was inoculated onto nutrient agar plates and cultured at 37°C for 18–24 hours. Colonies were then counted.

2.7. Antibiotic uptake

The parental and CEF-CPS variant strains were cultured in liquid PGS. The antibiotic strength was calibrated using *Staphylococcus aureus* ATCC 25923, which was cultured in a HI broth for 18 hours and then prepared as described above. Gentamicin and tobramycin were diluted with normal saline, and introduced into a suspension of CFP-CPS strain. In the control group, the antibiotic was replaced using normal saline. This study used the cylinder plate method.[19]

2.8. Data analysis

Analyses in this study were performed using SigmaPlot 5.5/PC (SSPS, Inc., Chicago, Illinois, USA), and all the tests described in the study were analyzed by paired *t* tests. A *p*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Isolation and purification of CEF-CPS strain

The parental strain displayed limited growth rate in PGS agar. After culturing for 48 hours, metallic-black colonies were observed,

measuring on average 2–3 mm in diameter. Among CPS-synthesizing variants, only the CEF-induced CEF-CPS strain was used in this study. The mucoid lawn derived from the CEF-CPS strain after culturing on PGS agar plates for 2 days was washed with sterile normal saline and then rough CPS was collected in the saline. The rough CPS was purified by ion-exchange chromatography (DEAE Sephacel) and gel filtration (Sepharose CL-4B), and was then frozen. After performing carbazole tests to determine the maximum quantity and purity of CEF-CPS, there were extremely low levels of LPS and protein in CEF-CPS.

3.2. Biochemical characteristics and antibiotic susceptibility of the CEF-CPS strain

We found that the uptake efficiency of carbohydrates in the CEF-CPS strain was similar to that of the parental strains (data not shown). However, the antibiotic susceptibility between the CEF-CPS and parental strains was different, as shown in Table 1. Resistance of the CEF-CPS strain to CEF was substantially higher than that of the parental strains (with MIC values of 64 μg/mL and 16 μg/mL, respectively). Moreover, MICs of the CEF-CPS strain for ampicillin and polymyxin-B were four times higher than the parental strains.

3.3. Serum bactericidal activity

As shown in Figure 1, the CEF-CPS strain had reasonably high resistance to the bactericidal effects of human serum. The growth curve and CFU counts were obviously higher than those of the parental strain, and the difference in CFUs was statistically significant ($p < 0.05$).

3.4. HA activity

As shown in Table 2, the CEF-CPS strain triggered agglutination at 1:128, which was 16 times lower than the 1:8 threshold for the parental strain. Various concentrations of the CEF-CPS strain were added to 2% guinea pig RBCs, and then the parental strain was added. Under these conditions, as shown in Figure 2, the CEF-CPS strain caused a two-fold increase in agglutination sensitivity. No further changes were observed when the concentration of the CEF-CPS strain was increased to > 0.25 mg/mL, which may be the optimal bacterial concentration for agglutination. Therefore, it is clear that the presence of the CEF-CPS strain was linked to agglutination. For blood types A, B, and AB, both the parental and the CEF-CPS strains caused agglutination at 2:2. However, for type O blood, neither strain caused an agglutination reaction (Table 2).

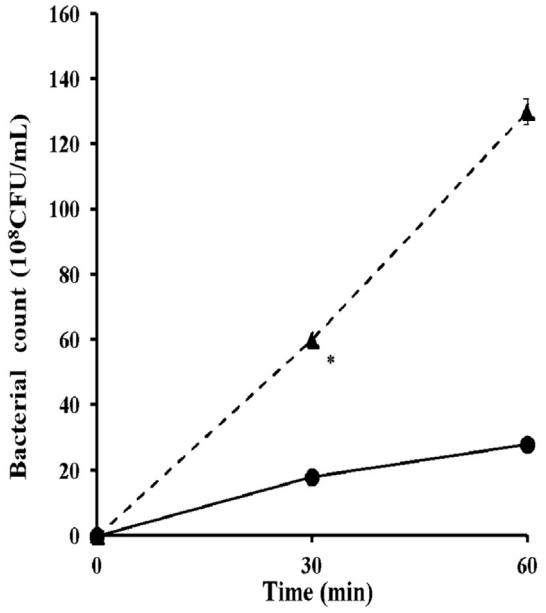

Figure 1 Parental and CEF-CPS variant strains to the bactericidal activity of pooled adult human serum. One-tenth milliliter of bacterial suspension was added to 5% pooled human serum in an HI broth, and the number of viable bacteria remaining was determined. The bars represent standard error of the means. The -●- line represents the parental strain and –▲– the CEF-CPS strain. *$p < 0.05$ indicates a significant difference from the parental strain. CEF-CPS = cefazolin-capsular polysaccharide; CFU = colony-forming unit; HI = heart infusion.

Table 2 Effect on hemagglutination of the parental and CEF-CPS-synthesizing strains

Strain	CEF-CPS concentration (mg/mL)*				Human 2% RBC type (minimum inhibitory concentration)			
	0	0.1	0.25	0.5	A	B	AB	O
Parental	8	8	16	16	2	2	2	–
CEF-CPS	128>	128>	128>	128	2	2	2	–

*A suspension of 2% guinea pig RBCs.
CEF-CPS = cefazolin-capsular polysaccharide; RBC = red blood cell; – = negative.

3.5. Effects of the CEF-CPS strain on antibiotic susceptibility

The resistance to gentamicin is shown in Figure 3. The CEF-CPS strain was more resistant than the parental strain to the antibiotic effect. This resistance may have been a function of CEF-CPS production. The same effect is shown in Figure 4A, when bacteria were exposed to gentamicin for 10 minutes and then washed in phosphate-buffered saline. The CFU count of the CEF-CPS strain was consistently higher than that of the parental strain; results are shown in Figure 4A and B. Gentamicin levels were equal, suggesting that the parental strain absorbed all the antibiotics. However, tobramycin levels were not equal.

3.6. Suppression of the antibiotic effect by CEF-CPS

CEF-CPS was prepared across a range of concentrations in phosphate-buffered saline and mixed with tobramycin at a

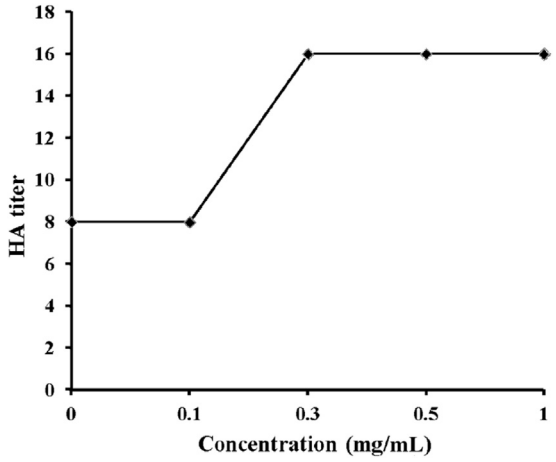

Figure 2 Effect of CEF-CPS on hemagglutination by the parental strain. Suitable volume of various concentrations of CPS in PBS were coincubated with the same volume of 2% washed guinea pig RBC at room temperature for 30 minutes. Positive results showed the maximum dilution. CEF-CPS = cefazolin-capsular polysaccharide; HA = heart infusion; PBS = phosphate-buffered saline; RBC = red blood cell.

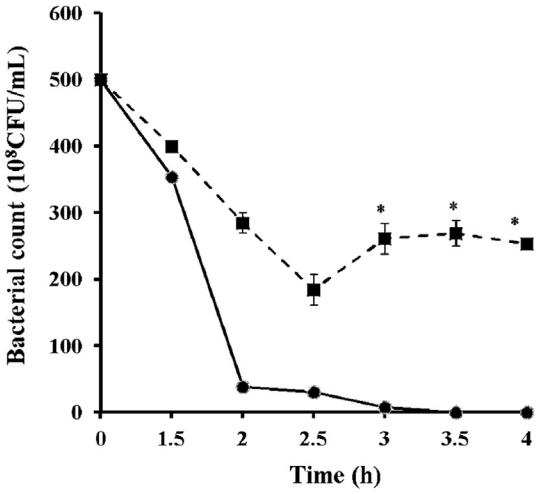

Figure 3 *In vitro* effect of gentamicin tolerance of the parental and CEF-CPS variant strains. Test strains in the late logarithmic growth phase in TS broth were coincubated with a TS broth containing gentamicin (10 times the MICs). The cell pellets were suspended in a fresh, antibiotic-free TS broth, and the number of viable bacteria remaining was counted. The –●– line represents the parental strain and –■– the CEF-CPS variant strain. CEF-CPS = cefazolin-capsular polysaccharide; CFU = colony-forming unit; MIC = minimal inhibitory concentration; TS = trypticase soy. $^{*}p < 0.05$

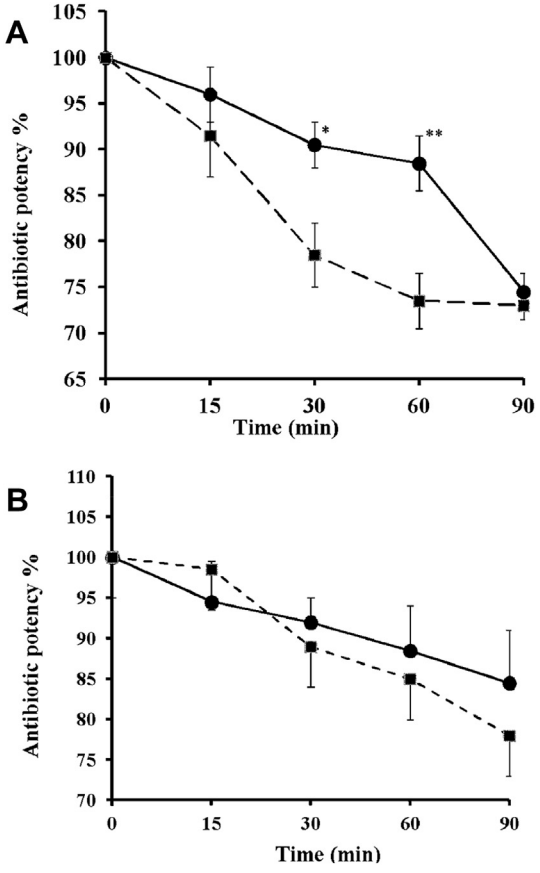

Figure 4 Time courses of (A) gentamicin and (B) tobramycin uptake by the parental and CEF-CPS variant strains. The tested strains were grown to logarithmic phase earlier in a BHI broth and then added to fresh gentamicin or a tobramycin-containing BHI broth. The supernatant was sampled, and the samples were stored at −70°C for determination of residual gentamicin and tobramycin concentration by bioassay (*Staphylococcus aureus* ATCC25923), and percent uptake of gentamicin and tobramycin was expressed. The -●- line represents the parental strain and –■– the CEF-CPS strain. BHI = brain–heart infusion; CEF-CPS = cefazolin-capsular polysaccharide.

concentration 10-times the MIC. The resulting CFU counts are shown in Figure 5. CEF-CPS interfered with the antibiotic effect of tobramycin by absorbing tobramycin in the broth. These effects increased with the concentration of CEF-CPS.

4. Discussion

The R^{1+} R^{2+} gene controls CEF-CPS production in *E. coli*. Extensive research into mucoid *E. coli* showed that the R^{1+} gene controls the production of several enzymes, including PHI, UDP-D-galactose-4-epimerase, GDP-D-mannose dihydronase, and GDP-L-fucose synthetase. The R^{2+} gene may control GDP-L-fucose synthetase.[20,21] The allosteric protein regulates R^{1+} R^{2+} gene expression. According to Tseng,[20] when DL-p-fluorophenylalanine is added to the culture medium, it induces the growth of mucoid colonies, because it is incorporated into the repressor protein produced by the R^{1+} gene. Therefore, the repressor is inoperative, and enzymatic activity continues. This is the primary cause of mucoid colony growth.

Several studies on CPS- and non-CPS-synthesizing strains isolated from clinical cases revealed that the CPS-synthesizing strains have substantially higher antibiotic tolerance (as measured by the MIC) and drug resistance.[22] The CEF-CPS strains displayed the highest resistance to antibiotics, such as ampicillin and CEF, disrupting the formation of cell walls. No differences in other antibiotics resistances of the CEF-CPS and parental strains was observed. In addition, the growth on selective media and biochemical characteristics of the CEF-CPS strains were similar to those of the parental strains. This result is in contrast to the findings of past research on CMr-CPS and oxytetracycline OTCr-CPS strains that exhibit differences in biochemical characteristics, growth on selective media, carbohydrate uptake, and so on.[9] These differences may have been related to the mechanism of induction.

For the CEF-CPS strain, CPSs on the cell wall affect its antibiotic tolerance and uptake, although it is unknown whether changes within the cytoplasm affect cell-wall formation. CPSs produced by the CEF-CPS strain improved the antibiotic resistance and uptake of bacteria, demonstrating that the heightened antibiotic resistance of the CEF-CPS strain is connected to its production of CPSs.

CPS-synthesizing strains resulted in higher mouse mortality, and their rate of growth in media was substantially different from that of parental strains. Mouse lethality, adrenal cells, and reversed

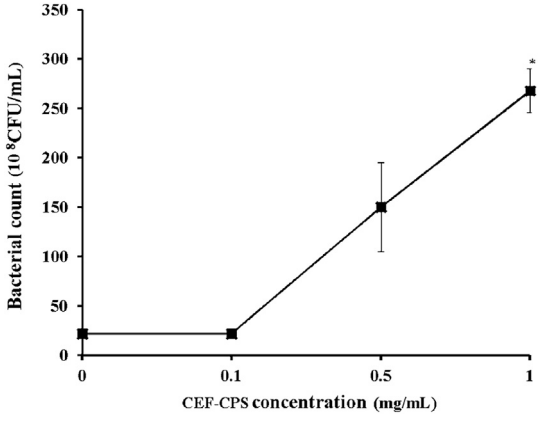

Figure 5 Influence of CEF-CPS on the antibiotic activity of tobramycin. The parental strain in the logarithmic growth phase in a BHI broth was diluted 1:10 using a fresh BHI broth to achieve a final concentration of -10^{7} CFU/mL and a final drug concentration of 10-times the MIC for tobramycin containing various concentrations of CEF-CPS. After incubation at 4°C for 1 hour, the antibiotic was removed by centrifugation of the organism at 15,000*g* for 10 minutes. BHI = brain–heart infusion; CEF-CPS = cefazolin-capsular polysaccharide; CFU = colony-forming unit; MIC = minimum inhibitory concentration. $^{*}p < 0.05$

passive latex agglutination assays have been demonstrated that CPS-synthesizing strains release endotoxins and enterotoxins.[23–26] Acidic CPSs are crucial molecules for resistance of a host's immune responses, including opsonophagocytosis and the serum complement system.[4,27] Gram-negative bacteria typically have a negative charge, hydrophilic fimbrial antigens, capsular antigens (e.g., K antigen, additional slime polysaccharides, and the O antigen), and a smooth LPS. Outer-membrane lipids of RBCs include lecithin, sphingomyelin, and glycolipids. Therefore, the outer membrane possesses electronegative properties that cause RBCs in the blood to present a dispersed state. Furthermore, a reduction in the electronegativity causes RBCs in the blood to agglutinate. In this study, we collected CEF-CPS by extraction and purification of variant strains.

CEF-CPS is an electropositive acidic polysaccharide formed by a group of oligosaccharides. Therefore, we processed the parental strain with 0.3 mg/mL of CEF-CPS and obtained a guinea pig RBC HA titer of 16. Thereafter, we compared HA titers of variant strains of human A-, B-, AB-, and O-type RBCs with the parental strain and found that HA titers of the variant strains of A-, B-, and AB-type RBCs all were greater than those of the parental strain. By contrast, both the parental and the variant strains for O-type RBCs were negative. According to numerous reports, enterohemorrhagic *E. coli* (EHEC) cell membrane surface possesses various adhesion molecules (i.e., type 1 fimbria, Curli, Ag43, Cah, and EhaA),[28,29] and these molecules might have caused the comparative differences between O-type RBCs and other RBC types in this study. However, further research are required to determine which surface molecules of CEF-CPS are responsible for the adhesive properties in RBC agglutination.

Acknowledgment

This study was supported by a grant TMC87-Y05-A201 from Taipei Medical University.

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