

"Beneficial Role of Skim Milk Against Drug Resistant *Escherichia Coli* Associated With Pediatric Diarrhoea"

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Abstract

Background: Antibiotic-resistance in *E. coli* is a global issue affecting humans especially the pediatric population. Antibiotic-resistant *E. coli* is a pathogen frequently pediatric population as well as healthy adults.

Methods: This study aimed to examine the antibiotic resistance of *E. coli* causing pediatric diarrhea and its drug-resistant rates, its adhering abilities to cell line in vitro, and inhibition efficiency of a few selected chemical compounds. Clinical strains were isolated from both the healthy and infected pediatric population of Mizoram.

Results: Adhesion is a significant pathogenic process during bacterial infections, which has been employed for pathotyping of DEC by comparing adhesion efficiency in both normal (CHO-k1) and cancer (HeLa) cell lines. *E. coli* adherent pathotypes were identified by both PCR assay and in-vitro cell adhesion assays; the study also evaluated the adhesion inhibition ability of human skimmed milk, gentamicin, and cephalixin in-vitro. Of all isolates, 20.05% of adherent DEC (EPEC, DAEC, and EIEC) and 11.39 % of non-adherent DEC (STEC and ETEC) were found to be associated with pediatric diarrhoea in Mizoram. Human skimmed milk has a high potential adhesion inhibition against EAEC (50.25/90.90 µg/mL), EPEC (53.42/259.70 µg/mL), and EIEC (59.13/30.30 µg/mL) in both cell lines in comparison with gentamicin and cephalixin.

Conclusion: This study concludes that as a dietary supplement- human skimmed milk has high potential to prevent adhesion of DEC pathotypes in cells in-vitro thus in in-vivo.

Introduction

The infectious diarrheal disease takes the second position as a leading cause of morbidity and mortality in developing countries amongst the pediatric population aged below 5 years with an incidence of 2.3 million annual deaths in Indian children.⁵ Of the bacterial pathogens, diarrheagenic *Escherichia coli* (DEC) is an important etiological agent causing endemic and epidemic diarrhea worldwide and at least six major pathotypes for enteric *E. coli* are now recognized as Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC) and Diffusely adherent *E. coli* (DAEC).²⁷ Although DEC pathotypes cause diarrhea with similar symptoms but show type-specific pathological changes in gut epithelial cells which has been well studied by using Hep2 or HeLa cell lines in-vitro by earlier studies. Earlier studies also report that the pathotypes have never displayed similar adhesion patterns or infection patterns which are host or tissue-specific to their pathogenic counterparts.²⁷ There is no much information is available regarding host tissue-specific adhesion patterns of virulent *E. coli* pathotypes on normal epithelial cell lines. The human cancer cells create a complex heterogeneity environment compared with normal cells for the biochemical interactions between its cell surface and microbes. Hence the present study was intended to analyze adhesion patterns of the pathogenic *E. coli* strains on both cancerous (HeLa) and non-cancerous (CHO-k1) cell lines.

It is important to understand the complex interactions between pathogenic bacteria and human tissues in-vivo for effective treatment by using in-vitro cell lines as models. The human milk contains glycans, glycol-conjugates which act as secretory immunoglobulin A (sIgA), and lactoferrin that has bactericidal properties are having the ability to inhibit gut pathogens in HeLa cell lines.³⁵ Thus the current study formulated with the hypothesis whether the human skimmed milk (1% fat) and buttermilk have a similar effect as the antibiotics in inhibiting the attachment of bacterial pathogens against both normal and cancerous cell lines in-vitro.

Materials And Methods

1. Isolation and Characterization of diarrheal E.coli pathotypes

1. A. Collection of Samples: The faecal samples were collected from the three major Hospitals (Aizawl Civil Hospital, Aizawl Hospital & Research Centre, and Synod Hospital) in Aizawl district, Mizoram Northeast India. The faecal samples were transported in an ice-chamber to the laboratory, plated on MacConkey agar and incubated at 37°C for 24 hours. After incubation, the colonies of selective morphology indicating *E. coli* were selected for further characterization and identification by Standard Bacteriological methods.¹¹ The identified pure isolates were stocked into glycerol and stored at -20°C.

1. B. Serotyping: Serotyping of the *E. coli* isolates based on 'O' antigen was carried out at National Salmonella and Escherichia Centre, Central Research Institute (CRI), Himachal Pradesh, India as per the method of Edwards and Ewing.¹⁶

Molecular detection of Diarrheagenic E.coli pathotypes

2. A. Identification of virulence genes by multiplex PCR: From the overnight cultures grown at 37°C in Luria Bertani broth (HiMedia, India), the genomic DNA extracted using DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA) and plasmid DNA was purified by Genejet Plasmid DNA purification Kit (Thermo Scientific, USA). The isolated genomic and plasmid DNA are subjected to PCR-based screening targeting specific virulence markers of EAEC, EPEC, DAEC, and EIEC by using a different set of primers listed in the table.1. ^{18,37} The multiplex PCR reaction mixture contained 2.5µl of 10X PCR buffer with 1.5 mM of MgCl₂, 1 µl each primer, 2µl of 10mM each of dNTPs, 0.2 µl of 5.0 U Taq DNA polymerase, and 4.0 µl template DNA. The PCR reaction condition includes initial denaturation at 95°C for 5 min, followed by 32 cycles of denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec (for EPEC, EIEC, and EAEC) at 60°C for 45 sec (for DAEC), and extension at 72° C for 1 min followed by a final extension at 72° C for 5 min.

2. B. Bacterial culture: The selected bacterial strains were inoculated in the LB broth and incubated for 18 to 24 hr at 37°C. A total of 10 representative *E. coli* strains (2 isolates from each adherent pathotypes) were selected based on their virulence factor, cell adhesion properties, and the drug-resistance profile, which was subjected to bacterial quantitative adhesion assays on HeLa and CHO-k1 monolayer as per previously described protocols. ³³

2. Cell lines and culture medium: HeLa and CHO-k1 were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with trypsin solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks, and all experiments were carried out in 96 microtiter plates (Tarsons India Pvt. Ltd., Kolkata, India).

HeLa and CHO-k1 cells (1x10⁶cells/ml) were seeded with DMEM supplemented with 1% mannose (DMEM-mannose) without FBS on 6 well plates containing sterile poly L-lysine coated glass cover slips (Tarsons, India). The cells were incubated at 37°C with 5% CO₂. An amount of 100 µl of overnight bacterial cultures of selected *E. coli* strains to contain 3 x 10⁸bacteria/mL was added to the wells and incubated for 3 hours. After incubation, the unattached bacteria were removed by washing with 1x PBS, the cells were fixed with 4% paraformaldehyde and stained with 10% Giemsa stain for 15 minutes. At least 100 fields were observed under a microscope to detect the number of bacteria adhered to the cells and counted. The strains that adhered to the monolayers were recorded as adhering in localized (LA), localized-like (LAL), diffuse (DA), or aggregative (AA) patterns. ³³ All tests were repeated at least two times as the duplicates helped to eliminate the potential technical errors, the bacterial adherence was calculated for each preparation by the standard error of mean using SPSS 17 software.

3. Enumeration of adherent bacteria

HeLa and CHO-k1 cells were co-inoculated with bacterial culture as the method motioned above and incubated at 37°C with 5% CO₂ for 3 hours. After incubation, unattached bacteria were removed by washing with 1xPBS, the OD values were detected to compare with OD values of blank media taken before inoculation and calculated the percentage of adherence to find out the level of adherence by a particular *E. coli* pathotypes. For further confirmation to find the level of adherence the cells were also plated in MacConkey agar and incubated at 37°C for 18h for the colony counting. Data were expressed as Log CFU per well. Adhesion was measured as several bacterial cells adhering to monolayers of HeLa and CHO K1 cells. Bacterial strains were designed as non-adhesive (Adh-) when fewer than five bacterial CFU; as an adhesive (Adh) when six to 40 bacterial CFU and strongly adhesive (Adh+), when more than 40 CFU were detected after the mentioned incubation time. ¹²

Bacterial Inhibition Assay

HeLa and CHO-k1 cells were co-inoculated with bacterial culture as the method motioned above and incubated at 37°C with 5% CO₂ for 3 hours for cell adhesion. The cells were washed thrice with 1X PBS (pH 7.4) for the removal of non- adherent bacterial cells. The HeLa and CHO-k1 cells containing adherent bacterial cells were incubated with the different inhibitory concentrations (20,40,60,80and100µg/ml) of cephalixin (CN), Buttermilk, and Human skimmed milk (20, 40, 60, 80, and 100%) for 3 h at 37°C and control (without antimicrobial agents) wells were also included for each inhibitory agents. After incubation, the adherent bacterial cells remain in the cell lines were extracted and plated on MacConkey agar medium. The CFU was calculated for each concentration. The percentage inhibition of cell adhesion for each concentration was calculated and compared with control. ⁴⁰

4. Statistical Analysis: The prevalence of diarrheagenic *E. coli* in patient and control samples were compared by a two-tailed X² test with Yates correction and Fisher's exact test. When analyzing the adherence to HeLa and CHO-k1 cell lines with different dilutions, and to detect the minimal inhibitory concentration of antibiotics in cell lines were expressed as means of SD and SEM. Statistical significance was tested by the two-tailed non-paired Student's t-test (SPSS.17). A P value of < 0.05 was considered significant among the values of tests.

Results

1. Molecular detection of Diarrheogenic *E. coli* pathotypes: A total of 334 bacterial isolates were recovered from the faecal samples of pediatric diarrheal patients. The genomic and plasmid DNA isolated from 334 diarrheogenic *E. coli* isolates were subjected to the PCR based virulent marker detection assays. Among the isolates, 31.44% (105 of 334) isolates carried pathogenic virulent genes and 68.56% (229 of 334) isolates were non-pathogenic which does not carry the selected virulent genes confirmed by multiplex PCR using gene-specific primers (Table-1). Pathogenic isolates were further classified into two types based on the virulence and cytological effects on cell monolayers such as non-toxicogenic adherent pathotypes (20.05%) which include EAEC, EPEC, EIEC, and DAEC and adherent toxigenic pathotypes (11.37%) which included the strains of STEC, ETEC, and EHEC.

In this study, we have used four different types of adherent pathogenic isolates which were classified based on the virulent genes and adherence pattern on the cell monolayer. The first type isolates are EPEC 14.97% (50 of 334) which have both *eeA* and *bfpA* genes containing EAF plasmid; this EPEC has been further classified into two subtypes such as typical EPEC (tEPEC) 8.68% (29 of 334), which carried both *eeA* and *bfpA* genes and atypical EPEC (aEPEC) 6.28%, (21 of 334) which carried the *eeA* gene alone. The Second type was EIEC 2.40% (8 of 334) which carried virulent gene *ial* which was a part of the plasmid of invasion (*plnV*). The third and fourth types were DAEC 0.90% (3 of 334) and EAEC 1.80% (6 of 334) which carried the virulent genes *daaE* for diffuse adherence and *pCVD432* occurred in DAEC and EAEC respectively (Fig. 1A).

2. Screening of pathological interaction between diarrheogenic *E. coli* pathotypes and cell monolayers: In cell monolayer adhesion assay, all the adherent pathogenic isolates showed strong adhesion against HeLa and CHO-k1 cell lines. The pattern of isolates adherence to monolayers of human cancer (HeLa) and non-cancerous (CHO-k1) cells were classified into four types such as localized (LA), localized-like (LAL), diffuse (DA), or aggregative (AA) patterns (Table-2) (Fig-1A). The AA pattern was observed from 8.96% (6 of 67) of EAEC isolates on HeLa and CHO-k1 monolayer and 1.49% (1 of 67) DAEC on HeLa monolayer only. The LAL pattern was observed from both atypical and typical EPEC. The atypical EPEC on HeLa monolayer was 32.8% (22 of 67 isolates) and the CHO-k1 monolayer was 31.3% (21 of 67 isolates). The typical EPEC on HeLa monolayer was (4.47%, 3 of 67 isolates) and the CHO-k1 monolayer was 1.49%, (1 of 67 isolates). Similarly, the LA pattern was also observed from both atypical, typical EPEC, and EIEC. The atypical EPEC observed at 10.4% (7 of 67 isolates), typical EPEC was detected at 26.8% (18 of 67 isolates), and EIEC (4.47%, 3 of 67 isolates) on HeLa monolayer. Atypical EPEC at 11.9% (8 of 67 isolates), typical EPEC at 29.8% (20 of 67 isolates), and EIEC (1.49%, 1 of 67 isolates) on the CHO-k1 monolayer were detected with LA pattern. The DA pattern was observed from 2.98% (2 of 67) of DAEC isolates, 7.46% (5 of 67) of EIEC isolates on HeLa monolayer and 4.47% (3 of 67) of DAEC isolates, 10.4% (7 of 67) EIEC isolates were observed on CHO monolayer (Fig. 1B).

3. Serogrouping of the pathogenic *E. coli* isolates based on 'O' antigen: The diarrheogenic *E. coli* of this study mostly belongs to the following serotypes including O22, O141, O124, O128, O86, O5, O96, and UT. The O antigen serotypes of all the strains included in this study with their virulence profile, origin, antibiotic resistance profile are shown in table- 2.

4. Enumeration of Bacterial adhesion: The percentage of an adherent pattern of different pathotypes varies from cancerous (HeLa) and non-cancerous (CHO-k1) cell monolayers in in-vitro adhesion assay. The bundle forming pilus containing typical EPEC isolates showed stronger adhesion (Adh++) in both HeLa and CHO-k1 cell lines but the adherent percentage was high in cancerous HeLa cells (60%) when compared with normal CHO-K1 cells (20%). Similarly, the *pCVD432* gene carried EAEC isolates showed an adhesive adhesion (Adh+) in both HeLa and CHO-k1 cell lines but the adherent percentage was high in cancerous HeLa cells (20%) when compared with normal CHO-K1 cells (10%). DAEC and EIEC isolates were showed non-adhesion (Adh-) on HeLa monolayer and CHO-k1 monolayers (Fig. 3) (Table-3).

5. Effect of Minimum inhibitory concentration of antibiotics against bacterial adhesion: Adhesion inhibition of different pathotypes was observed in two different methods of inhibition in-vitro that by cell monolayers and Luria Bertani broth dilution assay. In this study, we used a different concentration of antibiotics, buttermilk, and skimmed milk powder. The adhesion inhibition of these antibiotics, buttermilk, and skimmed milk was dose-dependent in in-vitro cell monolayer adhesion assay and Luria Bertani broth dilution assay but the MIC of antibiotics, buttermilk, and skimmed milk was varied depending on cell types and adhesion pathotypes. The adhesion inhibition (MIC) of Buttermilk in HeLa cell monolayer containing EAEC, EPEC, DAEC, and EIEC was 69.5, 73.7, 87.0 and 83.7 µg/ml, respectively and CHO-k1 cell monolayer containing EAEC, EPEC, DAEC, and EIEC was 69.5, 73.7, 87.0 and 83.7 µg/ml, respectively. Similarly, the adhesion inhibition (MIC) of cephalexin in HeLa cell monolayer containing EAEC, EPEC, DAEC, and EIEC was 67.7, 67.5, 69.2, and 68.9 µg/ml, respectively, and CHO-k1 cell monolayer containing EAEC, EPEC, DAEC, and EIEC were 75.3, 72.5, 79.9 and 84.8 µg/ml, respectively. In Luria Bertani broth dilution assay, MIC of buttermilk on EAEC, EPEC, DAEC, and EIEC was 4, 8, 8, and 4 µg/ml respectively which is very low in comparison with the results got from the cell lines. Similarly, MIC of cephalexin on EAEC, EPEC, DAEC, and EIEC was 0.75, 8, 8, and 8 µg/ml respectively and MIC of human skimmed milk on EAEC, EPEC, DAEC, and EIEC was 0.5, 16, 12, and 24 µg/ml respectively.

In adhesion inhibition (MIC) of human skimmed milk on HeLa cell monolayer containing EAEC, EPEC, DAEC, and EIEC was 50.2, 53.4, 72.7, and 59.1 µg/ml, respectively and on CHO-k1 cell monolayer containing EAEC, EPEC, DAEC and EIEC were 190.9, 259.7, 130.3 and 83.8 µg/ml

respectively. As a whole, the result revealed that the minimal inhibitory concentration required by all the components was greatly lower in broth medium as compared to cell lines especially against the normal cell lines (CHO-k1 monolayers).

Further, this study revealed that human skimmed milk has a lethal effect on adhesion inhibition of EPEC pathotypes in both CHO-k1 and HeLa cell lines. Followed by cephalixin and buttermilk showed adhesion inhibition with higher concentrations in both cell lines. Further, this study revealed that the immune/chemical components of human skimmed milk have the maximum potential to inhibit EAEC pathotypes with 50.25 µg/ml followed by cephalixin (67.7µg/ml) and buttermilk (69.6µg/ml) in both cell lines with minor concentration differences.

Discussion

Diarrhea remains an important health issue for the pediatric population worldwide. Adhesion to the host cell is the prime process in any infection for colonization and subsequent consequences of the disease. In infectious diarrhea, after adhesion, the pathogens invade the intestinal tissues which lead to the penetration of inner organs and cause systemic infections. Of all the bacterial pathogens causing infectious diarrhoea, *E. coli* predominates in infantile diarrhoea, so the different adherent pathotypes of diarrheagenic *E. coli* prevalent among children of Mizoram is taken for the current study. Earlier some studies carried out to investigate the adhesion mechanisms of *E. coli* in different cell lines and inhibition of adhesion by different components in-vitro. Similarly, this current study with few changes in the protocols checks the adherence pattern of all the different *E. coli* pathotypes and in both normal (CO-k1) and cancer (HeLa) cell lines. This study also used food components such as buttermilk and commercial human skim milk powder to check its efficiency inhibiting the adhesion process of *E. coli* pathotypes in comparison with the customary antibiotics i.e. cephalixin. Diarrheagenic *E. coli* causes mild to severe diarrhoea in children and infants, efficient therapy against DEC infections are lacking, as *E. coli* are neglected in stool cultures as it belonged to a normal gut flora of human. Current treatment is based on fluid replacement and supportive care; however, increasing knowledge on DEC virulence factors and mechanism of infections has contributed to the development of new treatment policies including inhibition of quorum sensing, use of strain-specific bacteriocins, and inhibition of strain binding to their host receptor with antibodies or ligands.³⁰

In this study, we tested the efficiency of food supplements as an alternative approach to interfere with DEC infections which may avoid the complications which are seen while using antibiotics during therapy. EPEC are those strains belongs to specific *E. coli* serogroups (O groups) and produce a characteristic LA or LAL adherence pattern in tissue culture and also based on the carriage of adherent cum virulent markers (eaeA and bfpA) which are then classified as atypical (aEPEC) and typical (tEPEC).^{7,19} Clonal analyses of the classical EPEC serogroups (O26, O55, O86, O126, and O128) were detected in this present study and corroborates with previous reports.^{15,32}

The previous studies reported that EAEC pathotypes have an aggregative adhesion (AA) on HEP-2 cell lines which is induced by a specific gene (pCVD) carrying plasmid (Fig. 2).^{6,9} This study confirmed the correlation of the virulent marker pCVD with the pattern of adherence in cell lines. EIEC strains cause watery diarrhoea and dysentery in humans; the lack of epidemiological attention to EIEC is related to the low incidence of this pathogen as a cause of diarrhoea concerning other strains of diarrheal *E. coli*.³⁹

Despite earlier reports, this current study detects a significant correlation between DAEC isolates and diarrhoea; however, most of the children in the present study carried DAEC were between the ages of 12 to 36 months, which suggested that this particular age group may be a vulnerable one for the DAEC pathotypes.^{2,23} This data also found that the non-breastfeeding infants were at high risk for the infection with DAEC strains which may be connected to deprived innate immunity. These findings led us to check the efficiency of milk-based food supplements against DEC.

Many studies have investigated the mechanism of adhesion of *E. coli* in different cell lines in-vitro extensively. Studies proved the significance of bacterial adhesion to the epithelial cells of the mucosal surface to initiate the enteric disease, and also reported a large number of different colonization factors for *E. coli*.^{14,22,34} Also analyzed the inflammatory response of the host in-vitro as a defence against diarrheagenic *E. coli* pathotypes in cell infection models.³¹

Therefore, to prevent enteric infections, it is a must to stop the colonization and adhesion of pathogens to the gut epithelium by either killing or by blocking the pathogens colonization factors. Of all the inhibitors studied extensively earlier, reports regarding food supplements efficiency against all the different pathotypes of *E. coli* are not found. Hence, the current study investigates the efficiency of buttermilk and skim milk powder (commercial) against all the pathotypes of *E. coli* in the in-vitro model.

Buttermilk contains water-soluble components such as milk protein, lactose, minerals, and also milk fat globule membrane (MFGM) derived from milk. Skim milk powder contains very little fat (< 0.5%) compared with buttermilk (> 5%) which may be due to the presence of MFGM fractions, small milk fat globules, and free lipids that were not extracted during the churning process.³⁶ Earlier studies found that the immunoglobulins found in buttermilk are more reactive than the ones found in skimmed milk powder, which is also evident here in this study as in both the cell lines more MIC was required for skim milk powder to inhibit the adhesion of pathogens (table-4). Studies proved that although the concentration

of proteins decreased after fermentation the immune-reactive IgE was detected in buttermilk, but the concentration of whey proteins was more in skim milk compared to buttermilk.

Earlier studies reported that the casein found in skim milk also caused milk allergy in children.^{13,25} These two qualities of the buttermilk make it a novel dairy ingredient, especially in the formulation of low-pH food and good food supplement for diarrhoea management. Many epidemiological studies of diarrhoea have shown that breast-feeding protects infants from intestinal and respiratory infections.^{20,24,29}

Milk contains maternal antibodies triggered by infection lead to an active immune response that is relevant to a particular infection and subsequently will protect her infant. Studies proved the role of immunoglobulin and non-immunoglobulin elements present in human milk in protecting the host from the diarrheal pathogens.^{1,28,17} Studies also reported human colostrum, milk, and oligosaccharides efficiency inhibiting the localized adherence of EPEC to cultured cells.³⁵ Such findings verify the interaction between milk elements and bacterial pathogens, their role in inhibiting the adherence to cultured cells. This current study also confirmed the reports of similar earlier studies that have pointed out the significant role of human skimmed milk and buttermilk antimicrobial activity against diarrheal pathogens; hence this could be used to boost the innate immunity in children and adults.^{10,26,28}

Although skim milk and buttermilk are in use as a food supplement in pediatric nutrition, complications or irreversible reactions in humans are not reported. The foremost benefit of its use would be that does not encourage any resistance in bacterial strains as an antibiotic does which is the major issue in the medical field. The effects of inhibitory concentrations of antimicrobials on bacterial growth were studied by comparing the number of colony-forming units after growth in the absence or presence of antibiotics (data not shown).

Cell numbers were decreased by about 20% in a few cultures but were not affected in most cases. Cephalexin has also produced such similar effects but with higher MIC concentrations; such findings were also observed by Vidya et al., against the *E. coli* of urine sample origin.³⁸ This effect has a clinical significance as the anti-adhesive effect of a cephalosporin with low concentrations effectively reducing the adherence; a higher dosage must be having a killing effect and avoid the colonizing ability of these pathogens in vivo.

Our findings are similar to the observations in previous studies.^{3,4} Interestingly, our work detected that the significant role of food supplements to block the adhesion of all *E. coli* pathotypes including the multidrug-resistant strains carrying β -lactam enzymes onto both normal and cancerous cells. The malignant epithelial cells exhibit biochemical heterogeneity which is reflected in their cell surface composition, enzyme levels, immunogenicity and response to microbial interaction, and so on.^{21,31}

Despite the extensive interest in the possible association of *E. coli* pathotypes between malignant and non-malignant epithelial cells, also related knowledge of ours remains inadequate. CHO-k1 may express receptors similar to those in the human intestine, as both cells belong to the normal epithelial cell lines and are derived from non-cancerous hosts. Furthermore, an adhesive *E. coli* has been shown to have a similar degree of adhesion for CHO-k1 and fetal enterocytes.⁸ These data are suggesting that the CHO-k1 may be particularly suitable for use in the study of *E. coli* which was thought to have properties of intestinal epithelial tissues.

Conclusion

In conclusion, the cell lines are a great model to prove the bacterial pathologic mechanism and effect of antimicrobials on adhesion or inhibition of pathogens. Human skimmed milk and buttermilk show a great effect to inhibit the adherence of *E. coli* pathotypes on both the cell lines with small concentrations in comparison with antibiotics used for the treatment of diarrheal pathogens in hospital settings. The main advantage of human skim milk and buttermilk is not inducing any allergic reactions or complications as it is seen with most antibiotics used in infants and children. It is a good dietary supplement next to breast milk and this supplement can be used in both children and adults for the treatment or prevention of diarrheagenic *E. coli*. This will bring a change in empirical therapy of pediatric diarrheal diseases, as it can be used along with antibiotics without interacting to cause contradictory effects; and never inducing antibiotic resistance among the pediatric diarrheal pathogens.

Declarations

Ethics approval and consent to participate: Ethics approval is received from the concern hospitals and patients to participate for sample collection.

Consent for publication: It is to certify that all authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

Availability of data and materials: All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests: "The authors declare that they have no competing interests"

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Authors' contributions: All the authors have contributed equally in bringing out the data and compilation of this research article. **(A). Conception:** KC, TKD, NSK, SS and IS; **(B). Design of Work:** CK, LR, SM, NSK, SS; **(C). Acquisition and analysis:** SS, SM, TKD, IS and LR; **(D). Interpretation of data:** KC, SS, SM, LR, NSK, TKD and IS; **(E). Drafting and Revision:** TKD, LR, IS and SM.

All the authors have approved the submitted version (and any substantially modified version that involves the author's contribution to the study); And also have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Authors' information: "Given in the Title page".

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Tables

Table-1 List of Primers used in this study to detect diarrheagenic *E. coli* (DEC) pathotypes

Pathotypes	Virulent markers (product size-bp)		Sequence (5'- 3')	Annealing temp.	References
EPEC	eaeA (229)	F	TGATAAGCTGCAGTCGAATCC	57°C	Hegde et al. 2012
		R	CTGAACCAGATCGTAACGGC		
	bfpA (450)	F	CACCGTTACCGCAGGTGTGA		
		R	GTTGCCGCTTCAGCAGGAGT		
EAEC	CVD432 (630)	F	CTGGCGAAAGACTGTATCAT	55°C	
		R	CAATGTATAGAAATCCGCTGTT		
EIEC	ial (320)	F	CTGGTAGGTATGGTGAGG	57°C	
		R	CCAGGCCAACAAATTATTTCC		
DAEC	daaE (542)	F	GAACGTTGGTTAATGTGG GGTA	60°C	Vidal et. al., 2005
		R	TATTCACCGGTCGGTTATCAG T		
EHEC	hlyA (534)	F	GCATCATCAAGCGTACGTTCC	55°C	Hegde et al. 2012
		R	AATGAGCCAAGCTGGTTAAGCT		
STEC	Stx ₁ (180)	F	ACACTGGATGAATCTCAGTGG	55°C	Shetty et al., 2012
		R	CTGAATCCCCTCCATTATG		
	Stx ₂ (225)	F	CCATGACAACGGACAGCAGTT		
		R	CCTGTCAACTGAGCAGCACTTTG		
ETEC	elt (322)	F	CTCTATGTGCACACGGAGC	55°C	Hegde et al., 2012
		R	CCATACTGATTGCCCAAT		
	Stla (170)	F	TCTTTCCCCTCTTTTAGTCAGTC		
		R	CCGCACAGGCAGGATTAC		

Table-2 Cellular adherence and Molecular profiles of *E. coli* pathotypes

Strain No.	E. coli pathotypes	Serotype	Seasonal	Adherence pattern		Genetic profile	Resistance profile
				HeLa cell line	CHO-k1 cell line		
D-21	EAEC	O55	Winter	AA	AA	CVD432+	bla _{TEM} , bla _{CMY2}
D-54	EAEC	O141	Summer	AA	AA	CVD432+	bla _{TEM} , bla _{CTX-M-15}
D-29	EPEC (t)	O22	Summer	LA	LA	eaeA + bfpA+	bla _{TEM}
D-36	EPEC (t)	O22	Summer	LA	LA	eaeA + bfpA+	bla _{TEM}
D-59	EPEC (a)	O86	Summer	LAL	LAL	eaeA + bfpA-	bla _{CTX-M-15}
LR-141	DAEC	UT	Autumn/Rainy	DA	DA	daaE+	bla _{CTX-M-15}
LR-142	DAEC	O128	Autumn/Rainy	DA	DA	daaE+	bla _{CTX-M-15}
LR-143	DAEC	O141	Autumn/Rainy	AA	DA	daaE+	-Nil-
D-12	EIEC	O96	Summer	LA	DA	ial+	bla _{SHV}
LR-128	EIEC	O124	Monsoon	DA	DA	ial+	bla _{TEM}

EAEC – Enteraggregative Escherichia coli, EPEC-Enteropathogenic Escherichia coli, DAEC-Diffusely adherent Escherichia coli, EIEC-Enteroinvasive Escherichia coli, LA- localized adherence, LAL- localized-like adherence, DA- diffuse adherence, AA- aggregative adherence patterns.

Table-3 Quantitative adhesion of bacterial isolates on HeLa and CHO-k1 cell lines

S. No	Strains	Adhesion on HeLa cells		Adhesion on CHO-k1 cells	
		(%)	Level	(%)	Level
1.	D-21	42.6 ± 0.9	+	70.0 ± 0.5	++
2.	D-54	44.7 ± 0.6	+	39.4 ± 0.3	+
3.	D-29	90.0 ± 9.3	++	60.7 ± 53.0	++
4.	D-36	58.3 ± 2.4	++	32.5 ± 1.4	+
5.	D-59	59.3 ± 3.2	++	60.7 ± 2.3	++
6.	LR-141	13.7 ± 1.3	-	55.5 ± 0.7	++
7.	LR-142	16.1 ± 14.9	-	90.0 ± 8.7	++
8.	LR-143	26.7 ± 0.2	-	24.7 ± 0.2	-
9.	D-12	27.7 ± 0.4	-	26.4 ± 0.2	-
10.	LR-128	33.3 ± 0.6	+	34.8 ± 0.3	+

(++) strong adhesive; (+) adhesive; (-) non adhesive

Table-4 Inhibitory concentrations of antimicrobial agents on HeLa and CHO-k1 cell lines against diarrheal *E. coli* pathotypes

Pathotypes	MIC on HeLa cell lines			MIC on CHO-k1 cell lines			MIC on nutrient broth (Broth dilution method)		
	Gentamicin (µg/ml)	Cephalexin (µg/ml)	Skimmed milk fragments (µg/ml)	Gentamicin (µg/ml)	Cephalexin (µg/ml)	Skimmed milk fragments (µg/ml)	Gentamicin (µg/ml)	Cephalexin (µg/ml)	Skimmed milk fragments (µg/ml)
EAEC	69.56*	67.71*	50.25 ⁺	69.57*	75.39	190.90 [#]	4.0	0.75 ⁺	0.5 ⁺
EPEC	73.70 ⁺	67.53	53.42 ⁺	73.70 ⁺	72.55	259.70 [#]	8.0*	8.0	16.0 ⁺
DAEC	87.05	69.20*	72.73*	87.05	79.92	130.30 [#]	8.0*	8.0*	12.0 [#]
EIEC	83.71	68.95*	59.13*	83.71	84.82	83.88 [#]	4.0	8.0*	24.0 [#]

P < 0.05 significant * p < 0.05; +p < 0.01; # p < 0.001

Figures

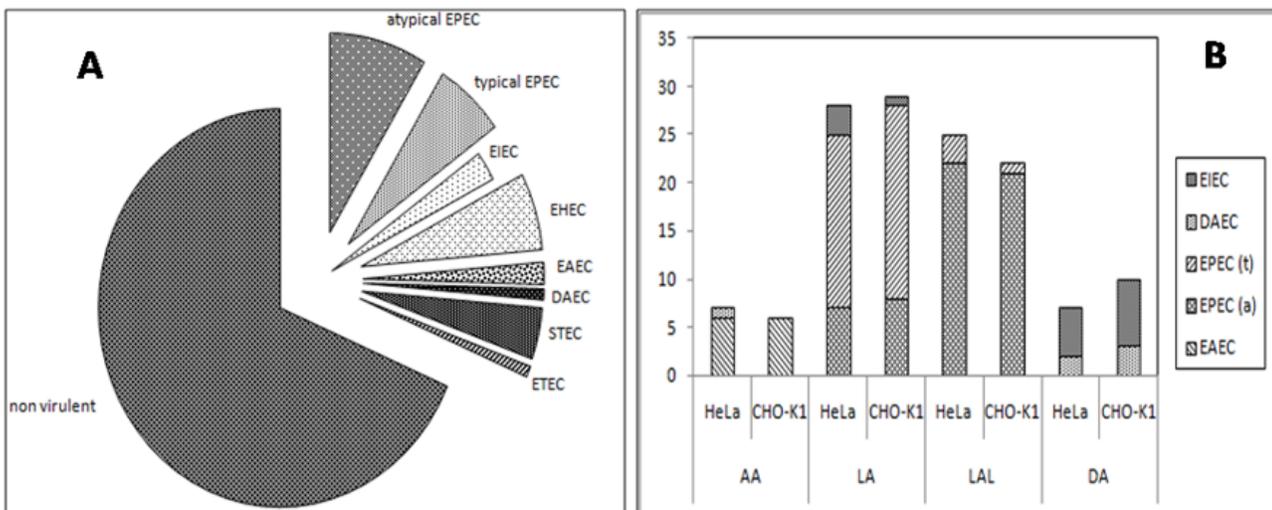


Figure 1

Distribution and adhesion pattern of *E. coli* pathotypes associated with pediatric diarrhoea in Mizo children A. Distribution of diarrheagenic *E. coli* isolates. B. Adhesion pattern of *E. coli* pathotypes on HeLa and CHO-k1.

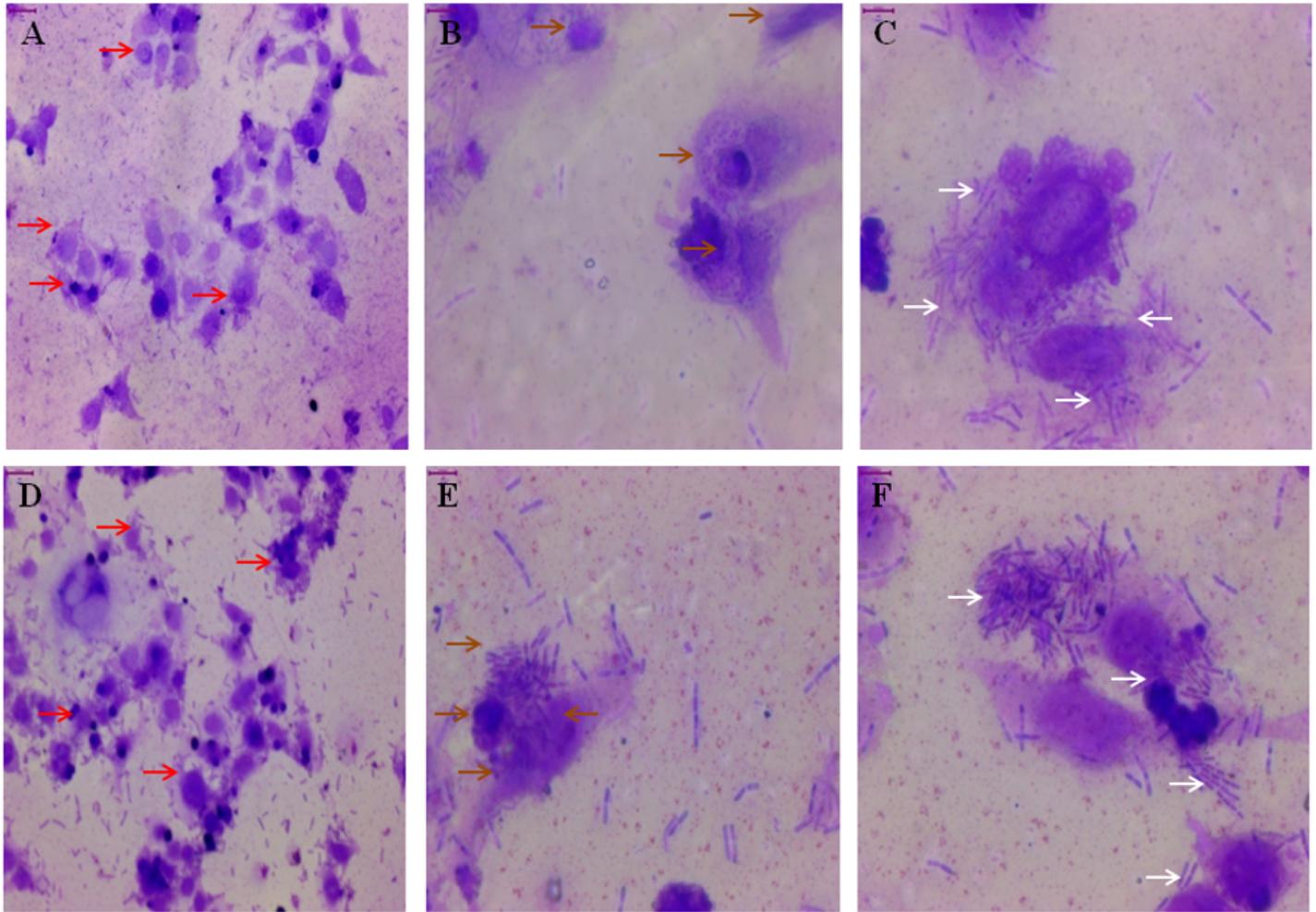


Figure 2

Giemsa stained images of Co-cultures of Bacterial strains with HeLa and CHO cell lines Upper panel: HeLa cell line (A, B- Localized Adherence (LA) and Localized Like adherence (LAL) pattern of Adhesion and C-Aggregating Adherence (AA) adhesion pattern of bacterial strains). Lower panel: CHO K1 cell line (D, E- Localized Adherence (LA) and Localized Like adherence (LAL) pattern of Adhesion and F- Aggregating Adherence (AA) adhesion pattern of bacterial strains).

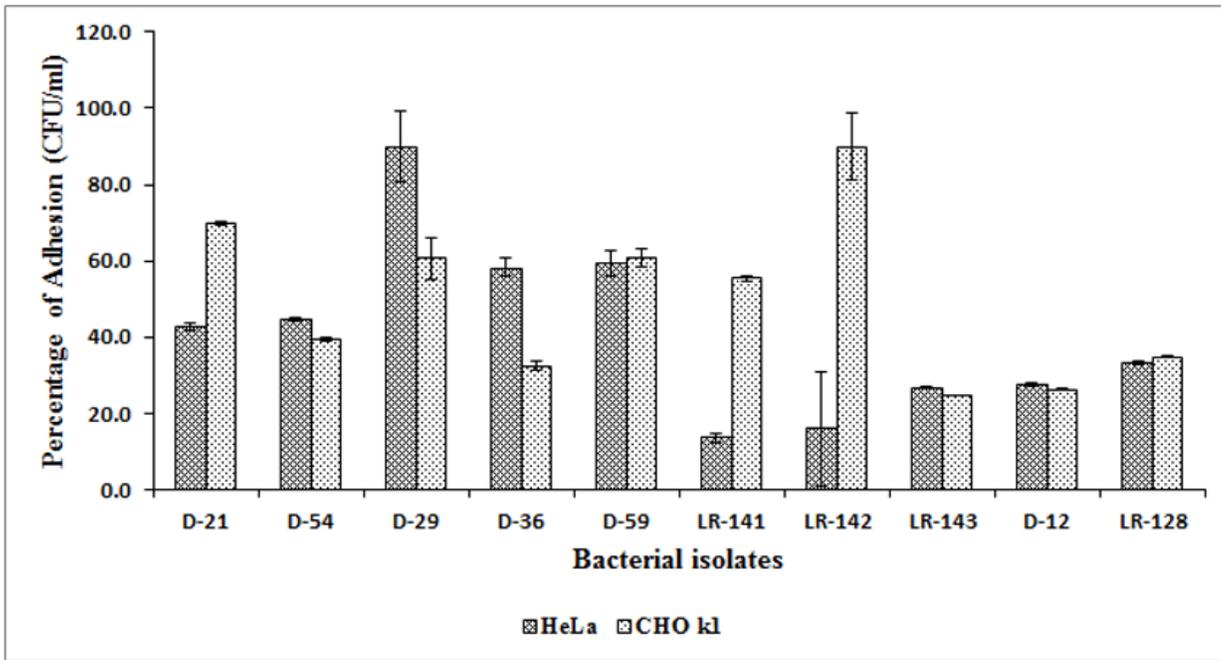


Figure 3

Percentage adhesions of pathotypes in HeLa and CHO-k1 cell lines

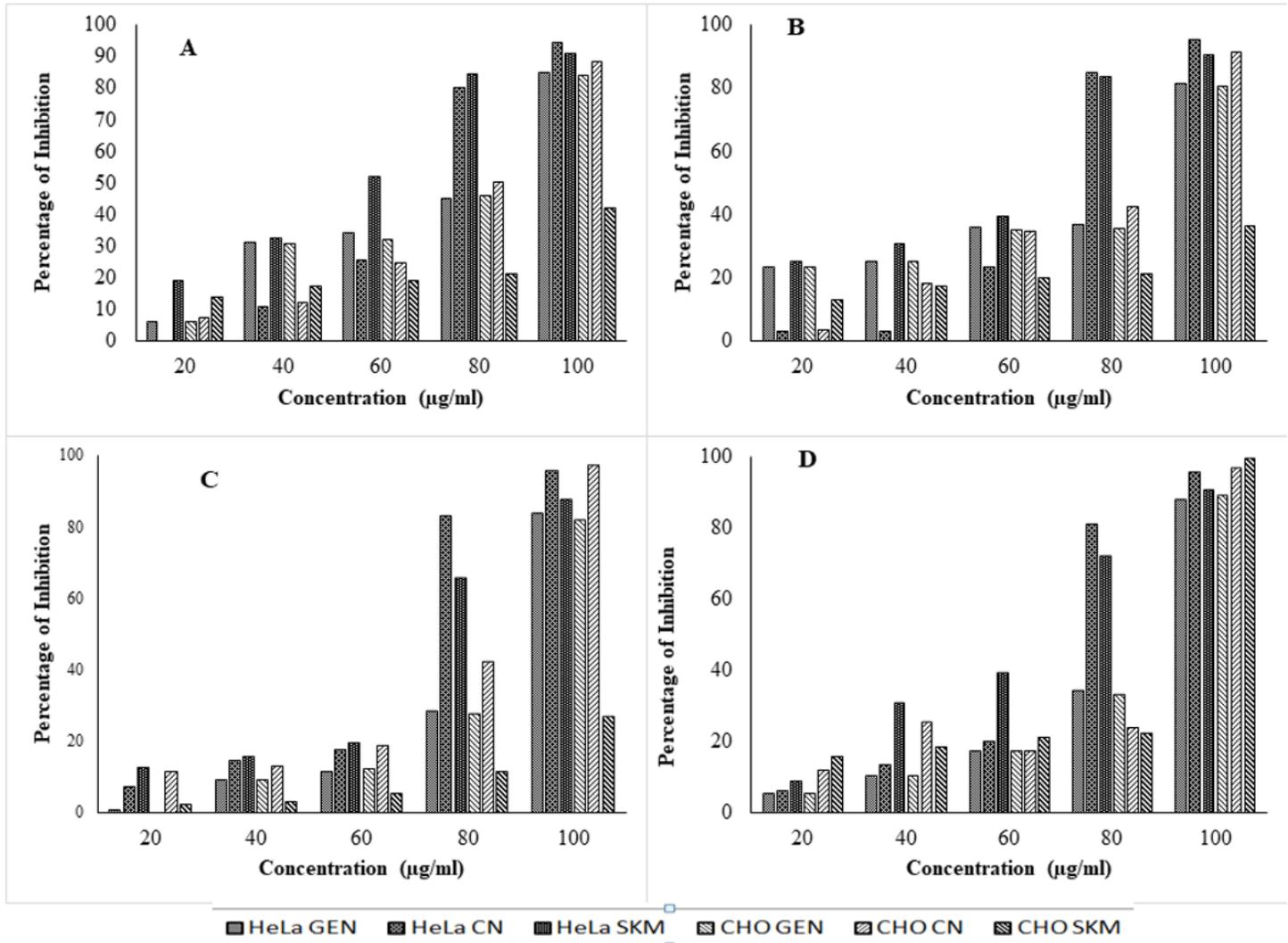


Figure 4

MIC (minimum inhibitory concentration) values and percentage of inhibition of *E. coli* pathotypes in HeLa and CHO cell lines. A- inhibition against EAEC, B- inhibition against EPEC, C- inhibition against DAEC, and D- inhibition against EIEC