Journal of
Microbial & Biochemical Technology

Bamola et al., J Microb Biochem Technol 2016, 8:3
http://dx.doi.org/10.4172/1948-5948.1000279

Research Article Open Access

A Non Invasive Technique to Assess Mucosal Immunity in Healthy Population by Measuring Immunoglobulin Receptor Expression on Viable Colonocytes

Bamola VD[1], Sharma N[1], Abhipray Gahlowt[1], Panigrahi P[2] and Chaudhry R[1*]

[1]*Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India*
[2]*University of Nebraska Medical Center and Director, Center for Global Health and Development College of Public Health, Nebraska Medical Center, Omaha, NE, USA*

Abstract

Human gut plays a vital role in the regulation of immune function, mucosal defense and homeostasis. Gut epithelial cells function as an immune cell and express receptors for microbial-associated molecular patterns. The gut epithelium undergoes constant and rapid renewal and some of these cells are exfoliated into the fecal stream. These cells are an important source of macromolecules, which provides a patho-physiological profile of the colonic epithelium. Most of the methods to harvest colonic epithelial cells are highly invasive and involve endoscopy and biopsy. Researchers suggest that studies of gastrointestinal pathophysiology are not feasible by biopsies in neonates and paediatric population. Therefore, isolation of theses exfoliated viable colonocytes from human stool is a non-invasive as well as a highly convenient approach that can be used for diagnostic and research purposes. A very few studies are available across the globe and no study is available from India of using this non-invasive techniques to recovered viable colonocytes in healthy population. For the first time we are reporting the results of the study on healthy Indian population where we recovered viable colonocytes from the stool samples using this non-invasive approach (Cell Sampling Recovery Method) and assessed immunoglobulins (IgA & IgG) receptors expression by Flowcytometry using specific florochrom conjugated antibodies. No study is available which provides the normal reference range of IgA and IgG receptor concentration on viable colonocytes in healthy Indian population. In this study we recruited 25 healthy children and 25 healthy adults from North India and provided the range of IgA and IgG receptor concentration on viable colonocytes for both groups. Results indicated that the difference in the mean IgA and IgG receptor concentration was statistically significant in both groups.

Keywords: Microbial Associated Molecular Patterns (MAMPs); Gut immunity; IgA; IgG; Flowcytometry

Introduction

Gastrointestinal (GI) mucosal surface acts as the primary interface between the host and the physical environment [1,2]. The mucosal surface of the human GI tract is about 200–300 m^2 and is colonized by 10^{13-14} bacteria of different species and subspecies [3,4]. These bacteria which are necessary for health also have potential to contribute to the development of diseases by a variety of mechanisms [2,5,6]. Mucosal immune responses help to confine the microbiota to the gut, avoiding a damaging systemic inflammatory response to the microorganisms present in the healthy gut [4,7].

Mucosal immune system is a highly integrated and regulated system. This sophisticated system may have evolved as a major defense mechanism against mucosal encountered infectious agents. Mucosal surface requires a considerable outflow of lymphoid cells and effector molecules for immunity. Secondary lymphoid tissues in the GI tract facilitate antigen uptake, processing and presentation for induction of mucosal immune responses. These tissues are also known as inductive sites. Gut-associated lymphoreticular tissues (GALT) are major inductive sites. It consists of the Peyer's patches, which are units of lymphoid cells, single lymphocytes scattered in the lamina propria and intraepithelial lymphocytes spread in the intestinal epithelia [8]. The epithelium of the GI tract undergoes constant and rapid renewal [9,10]. In the large bowel rapidly dividing cells provide a constant source of new cells and older cells migrate toward the luminal surface. Some of these cells are exfoliated into the fecal stream [11,12]. These cells are exclusively of colonic origin and representative of entire colon. These cells are an important source of macromolecules, which provides a patho-physiological profile of the colonic epithelium and can be used for clinical investigations and researches.

Stool is a heterogeneous mixture consists of undigested food residues, microflora, endogenous secretions, and circular cellular components exfoliated from walls of GI tract [12]. These circular components are rich source of viable intact cells. The circular components are made up of mostly terminally differentiated colonic epithelial cells. Studies have suggested that these exfoliated colonocytes can be used in diagnosis and researches. A study examined the utility of live colonocytes in stool in studying cellular markers during early neonatal life [13]. In this study Expression of IgA, IgG, cluster of differentiation-45 cells (CD45), and toll-like receptors-2 and 4 (TLR2 and TLR4) were analysed. Colonocyte RNA extracts were used in quantitative real-time PCR (qRT-PCR) to examine the expression of cytokeratin-19, ribosomal protein-24, and tight-junction (Tj) protein zonula occludens-1 (ZO-1) [13]. A study suggested that that the diagnosis (endoscopy, and biopsy) and continued clinical management of Inflammatory Bowel Disease (IBD) [14] remain highly invasive, expensive, and inconvenient for the paediatric patient. Nair et al conducted a study to see if colonocytes obtained from stools of subjects with IBD and normal controls would

***Corresponding author:** R Chaudhry, Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India, Tel: 011-26594795; Fax: 011-25488641; E-mail: drramach@gmail.com

Received January 23, 2016; **Accepted** March 24, 2016; **Published** March 31, 2016

Citation: Bamola VD, Sharma N, Gahlowt A, Panigrahi P, Chaudhry R (2016) A Non Invasive Technique to Assess Mucosal Immunity in Healthy Population by Measuring Immunoglobulin Receptor Expression on Viable Colonocytes. J Microb Biochem Technol 8: 161-165. doi: 10.4172/1948-5948.1000279

demonstrate higher levels of inflammatory markers (Cox 2 in CD45+ and CD45− cells) and if the inflammatory process and treatment effects would be reflected in an altered cytokine expression in the subjects compared to controls [14]. A study on associations between markers of colorectal cancer stem cells and adenomas among ethnic groups by also reported the use of Somatic Cell Sampling Recovery (SCSR) method to isolate viable colonocytes [15].

In the present study for the first time from India, we have reported the use of this noble non-invasive approach to recover viable colonocytes from stool and application of these viable colonocytes for the measurement of immunoglobulin (IgA & IgG) receptor expression using specific florochrom conjugate antibodies and flowcytometry. For the first time we have reported the normal range of IgA and IgG receptor concentration on viable colonocytes for healthy Indian population which can be used as reference range for future studies. This is a pioneer study from India for Indian population in the subject area and it will also encourage further intensive study with diverse parameters and components using this non-invasive approach in different demographic segments. This non-invasive technique can be exploited further for the detection, evaluation and management of different GI diseases including IBD and Colon Cancer.

Materials and Methods

Collection of sample

The study conducted at All India Institute of medical Sciences, New Delhi, India. Ethical approval for the study was obtained from the 'Institute Ethics Committee' of All India Institute of Medical Sciences, New Delhi, India. Before enrollment the detail participant information sheet of the study was provided to the adult participants and parents of the child participants. Objective and expected outcomes of the study were also explained. After receiving written participant information consent stool samples were collected from 50 subjects including 25 children and 25 adults. For the collection of fresh stool sample a sterile stool collection vial was provided to the participants. Parents of the child participant were explained and instructed for proper collection of sample to maintain maximum sterile condition. It was ensured that the collected stool sample to be transported to the lab in the minimum possible duration in ice cooled box to maintain 4-8°C. After receiving in laboratory the aliquots of stool samples (0.5-1.0 gm) were transferred in a collection vial containing nontoxic preservative solution (SCSR transport medium, provided in the kit) and processed further to isolate viable colonocytes and immuno-phenotyping.

Isolation of viable colonocytes

Viable colonocytes from stool were isolated by Somatic Cell Sampling Recovery, (SCSR) method (Non-invasive Technology, USA). This is a globally most accepted commercial available kit method for the isolation of viable colonocytes isolation from stool samples. In this study aliquots of stool samples (0.5-1.0 gm) were collected in a collection vial containing nontoxic preservative solution (SCSR transport medium, provided in the kit). Samples were adequately mixed by vortex for 15-30 sec. After proper mixing, the dispersed sample was passed through a 330 µm nylon mesh to remove large particles. For further removal of undesired large particles, the filtrate was again passed through a second filter cap (40 µm, BD). The final filtrate was collected in a fresh falcon tube (50 ml). The final suspension under laid with 10 ml of cushion solution (SCSR cushion medium, provided in kit). After adding cushion medium the sample was centrifuged at 200 X g for 10 minutes in refrigerated centrifuge. After centrifugation the light cells moved to interface, and heavy cells migrate into the cushion. Each fraction was collected separately. The light cells were aspirated from the interface and the heavy cells were recovered from the pellet. The collected fraction containing cell population was washed three times with phosphate buffer saline.

Sample preparation, immunoglobulin receptor staining, acquisition in flowcytometer and analysis

After adequate washing cell pellets were suspended in 200 µl PBS. A wet preparation of the final cell suspension when viewed under a phase-contrast microscope shows a large numbers of cells. Cells were stained with trypan blue (0.4%) to distinguish viable cells from necrotic cells and observed in phase contrast microscope (PH 2, Nikon, Japan). Simultaneously the cells were counted in a Coulter counter (Z2 Coulter Particle Count and Size Analyzer, Beckman Coulter) to give size distribution and calculate cell numbers. The SCSR procedure generally yields 20-40 million cells per gram of stool and size distribution histograms show the existence of 2 distinct populations, one between 2-5 µ and another between 5-8 µ [10,16]. In our all assay a total count of 3×10^3 cells were taken in each tube for further staining with florchrom conjugate antibody. To measure the IgA & IgG receptor concentration on viable colonocytes we used IgA-FITC conjugated (Sigma) & IgG-PE conjugated (Sigma). The cells were incubated with specific antibody using a standard protocol and acquired in Flowcytometer (CyAn ADP, Beckman coulter). Acquired data were analyzed with specific software (Summit Software-Beckman Coulter).

Results

In this study, we recruited 25 healthy children and 25 healthy adults. The mean age of the children group was 13.6 months (range from 01 month to 48 months). Among children male: female ratio was 12: 13 (male 48%: female 52%). The mean age of adult group was 34.12 year (range from 18 year to 58 year). Among adults male: female ratio was 13: 12 (male 52%: female 48%).

The range of IgA receptor on viable colonocytes isolated from stool of children varied from 64.2% - 86.5% with the mean concentration of 71.30 ± 0.97% and median value of 70.2%. The range of IgG receptor on viable colonocytes isolated from stool of children varied from 56.5% - 76.6% with the mean concentration of 65.73 ± 1.13% and median value of 66.5% (results summarized in Table 1 and Figure 1). The difference in the mean IgA and IgG receptor concentration was statistically significant in this group (P, 0.0005). All the statistical analysis was carried out by using Microsoft excel 2007 and Graph Pad Prism Software.

The range of IgA receptor on viable colonocytes isolated from stool of adults varied from 50.5% - 78.2% with the mean concentration of 66.82 ± 1.26% and median value of 66.8%. The range of IgG receptor on viable colonocytes isolated from stool of adults varied from 45.5% - 70.4% with the mean concentration of 62.71 ± 1.32% and median value of 66.5% (results summarized in Table 1 and Figure 1). The difference in the mean IgA and IgG receptor concentration was statistically significant in this group (P, 0.0292). Further when the IgA receptor concentrations were compared between children and adult groups it showed the difference was statistically significant (P, 0.0072). And when the IgG receptor concentrations were compared between children and adult groups it showed the difference was not statistically significant (P, 0.0894).

Discussion

Human stool is a heterogeneous mixture of undigested food residues,

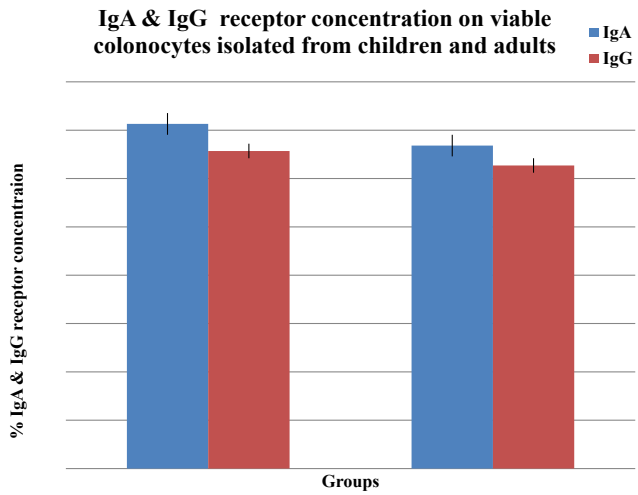

Figure 1: IgA & IgG receptor concentration on viable colonocytes isolated from stool samples of children and adult subjects.

	Children		Adult	
	% IgA	% IgG	% IgA	% IgG
Average ± SE	71.30 ± 0.97	65.73 ± 1.13	66.82 ± 1.26	62.71 ± 1.32
Range (Min –Max)	64.2 – 86.5	56.5 – 76.5	50.5 – 78.2	45.5 – 70.4
Median	70.2	66.5	66.8	64.5

Table 1: IgA and IgG receptor concentration on viable colonocytes isolated from stool samples of children and adult group.

microflora, endogenous secretions and cellular components exfoliated from GI tract. These exfoliated epithelial cells are exclusively of colonic origin and representative of entire colon. Their life span is of 3 to 4 days and a mean generation time of about 1 day accounts for the rapid turnover of this cell population [17,18]. It is estimated that the normal colonic epithelium contains 5×10^{10} cells and that one-sixth to one-third of these cells are shed every 24 h, which exfoliated every day [19,20]. Most of the diagnosis and researches which requires colonic epithelial cells are highly invasive and involve endoscopy and biopsy. Therefore, isolation of theses exfoliated viable colonocytes from human stool is a non-invasive as well as a highly convenient approach that can be used for diagnostic and research purposes. Our study highlights this non-invasive approach for isolation of viable colonocytes from stool which can be used for the detection of the immunoglobulin receptors and other surface antigens. It is also believed that this approach can be useful in research and diagnosis of various metabolic and clinical conditions in non invasive manner.

Chandel et al. [13] suggested that use of stool colonocytes can be a valuable non-invasive approach for studying gut patho-physiology in the neonatal period as studies of gastrointestinal patho-physiology are not feasible by biopsies in human neonates and pediatric population. Chandel et al. [13] examined the utility of live colonocytes in stool in studying cellular markers during early neonatal life. In this study Expression of IgA, IgG, cluster of differentiation-45 cells (CD45), and toll-like receptors-2 and 4 (TLR2 and TLR4) were analysed. Colonocyte RNA extracts were used in quantitative real-time PCR (qRT-PCR) to examine the expression of cytokeratin-19, ribosomal protein-24, and tight-junction (Tj) protein zonula occludens-1 (ZO-1).

Nair et al. [14] also suggested that that the diagnosis (endoscopy, and biopsy) and continued clinical management of IBD remain highly invasive, expensive, and inconvenient for the paediatric patient. Nair et al. [14] conducted a study to see if colonocytes obtained from stools of subjects with IBD and normal controls would demonstrate higher levels of inflammatory markers (Cox 2 in CD45+ and CD45– cells) and if the inflammatory process and treatment effects would be reflected in an altered cytokine expression in the subjects compared to controls. A study on associations between markers of colorectal cancer stem cells and adenomas among ethnic groups by Leavell et al. [15] also reported the use of Somatic Cell Sampling Recovery (SCSR) method to isolate viable colonocytes.

Osborn and Ahlquist [21] reported that assay of molecular markers in stool represents a promising noninvasive approach to screen colorectal cancer. They have suggested that neoplasms exfoliate abundantly into the lumen and that DNA recovered from stool can be assayed with sensitive techniques, there is a strong biologic rationale to pursue this emerging technology. Further they have emphasized that the molecular heterogeneity of cancer, no single marker has yielded perfect sensitivity. Several combinations of markers in early stool assays have produced high detection rates of both colorectal cancer and advanced adenomas in selected patient groups, but observations from large representative populations are lacking at present. Further marker discovery and technologic refinements should translate into improved test performance and fuel a continued evolution with this screening approach.

Ahmed et al. [22] reported quantitative stem-loop reverse transcriptase followed by TaqMan PCR expression analysis on stool and tissue samples using fifteen human (Homo sapiens, hsa) micro(mi) RNA genes. They were able to monitor changes at various stages of CRC, allowing for reliable diagnostic screening of colon cancer particularly at the early, pre-malignant stages, and for difficult-to-treat active ulcerative colitis (UC). Although the expression of some of the miRNA genes tested in tissue showed less variability in CRC or UC patients than in stool, the stool by itself appears well-suited to screening.

Matsushita et al. [23] have developed a new methodology for isolating colonocytes from feces that describes a promising procedure for future clinical evaluations and the early detection of colorectal cancers, including right-side colon cancer. They have isolated colonocytes exfoliated into feces from 116 patients with colorectal cancer and 83 healthy volunteers. Part of the exfoliated colonocytes was examined cytologically, whereas the remainder was subjected to DNA analysis. The extracted DNA was examined for mutations of the APC, K-*ras*, and p53 genes using direct sequence analysis and was also subjected to microsatellite instability (MSI) analysis.

Wu et al. [24] demonstrated that the detection of molecular markers in stool samples is a potential strategy for colorectal cancer (CRC) screening by evaluating the feasibility of detecting miR-21 and miR-92a in stool samples of patients with CRC or polyps. They have detected MiRNA levels in CRC tissues and stool samples by real-time quantitative reverse transcription PCR. Stool miR-21 and miR-92a levels were compared before and after the removal of tumour or advanced adenoma. The study demonstrated that stool-based miRNA were stable with highly reproducible detection. The expression of miR-21 and miR-92a was significantly higher in CRC tissues compared with their adjacent normal tissues ($p<0.0001$). Patients with CRC had a significantly higher stool miR-21 level ($p<0.01$) and miR-92a level ($p<0.0001$) compared with normal controls. Stool miR-92a, but not miR-21, was significantly higher in patients with polyps than in controls ($p<0.0001$). At a cut-off value of 435 copies/ng of stool RNA, miR-92a had a sensitivity of 71.6% and 56.1% for CRC and polyp, respectively, and a specificity of 73.3%.

In addition, the stool miR-92a level demonstrated a higher sensitivity for distal CRC than proximal CRC ($p<0.05$), and a higher sensitivity for advanced adenoma than minor polyps ($p<0.05$) [24].

We applied this non invasive approach to assess gut immune system in healthy pediatric and adult population. We recovered significant number of viable colonocytes from fresh stool samples and measured IgA and IgG receptor expression on theses viable colonocytes. The range of IgA receptor on viable colonocytes isolated from stool of children varied from 64.2% - 86.5% with the mean concentration of 71.30 ± 0.97%. The range of IgG receptor on viable colonocytes isolated from stool of children varied from 56.5% - 76.6% with the mean concentration of 65.73 ± 1.13%. The range of IgA receptor on viable colonocytes isolated from stool of adults varied from 50.5% - 78.2% with the mean concentration of 66.82 ± 1.26%. The range of IgG receptor on viable colonocytes isolated from stool of adults varied from 45.5% - 70.4%.

Chandel et al. [13] reported colonocyte yield varied between 5×10^4 to 2×10^6 cells/g of stool. Meconium samples yielded a highly enriched population of viable cells. Although low, all samples showed CD45-positive cells during the initial weeks of life. Starting as early as d 2, IgA expression was observed in 69% of the cells. Low to moderate expression of IgG was observed with a linear increase as the infants grew. There was an almost total lack of TLR2 staining; however, >55% of the colonocytes showed TLR4 expression. Although high levels of IgA in gut cells may serve as a natural protectant during neonatal period, increased TLR4 may provide a niche for lipopolysaccharide (LPS)-mediated epithelial damage.

The major human antibody is secretory IgA (sIgA), which comprises approximately 80% of all human immunoglobulin and is present in all exocrine secretions. It prevents microbes from reaching mucosal membranes and causing infections [25,26]. After exposure with microbes the lymphoid cells migrate from Peyer's patches to various mucosal membranes and exocrine glands where they settle and produce the sIgA antibody. Secretory IgA neutralizes infectious agents while at the same time limiting the damaging effects of tissue inflammation that can occur with other antibody types. B-lymphocytes give specific immunity by the production of specific antibodies against different antigens. These antibodies inactivate infectious organisms. Acquired antibodies from the mother contribute to a decreased risk of infection in infants. Neonatal B-cells produce primarily IgM and limited amounts of IgA and IgG. IgM production can occur in the fetus in response to an intrauterine infection [27]. The mean concentrations of IgG in infants have been reported as 60 mg/dL at 25 to 28 weeks of gestation, 104 mg/dL at 29 to 32 weeks of gestation, and over 400 mg/dL after 38 weeks gestational age [28,29].

Mucosal IgA serve a variety of functions including a first line of immune defense at mucosal surfaces. High-affinity IgA antibodies emerging from T cell-dependent pathways are thought to protect intestinal mucosal surfaces against colonization and invasion by pathogenic microorganisms [30,31]. At birth mucosal IgA producing cells are either absent or extremely rare but IgM and IgG producing cells are present in the intestine along with macrophages, neutrophils and dendritic cells. Therefore, immune system eliminates infectious agents and minimizes the damage they cause. It is believed that the induction of the tolerance of the immune system occur primarily in the gut and is facilitated by the specialized B and T cells failure of which is hypothesized to contribute to food-related allergy, autoimmunity and inflammatory bowel disorders [32,33]. Evidence indicates that IgA uses a high-affinity binding system to neutralize microbial toxins and pathogens, and a low-affinity binding system to prevent commensal bacteria from breaching the mucosal surface. IgA responses are highly dependent on intestinal colonization by commensal microorganisms.

IgG provides essential host defense and immuno-regulatory functions at the mucosal surfaces. The overall amount of serum IgG in full-term infants at birth is equal to or slightly greater than IgG levels in the mother because of the active transport across the placenta [34,35]. During birth the offspring becomes exposed to microbes of maternal origin. This microflora is the least hostile, because the mother provides defense against these microbes primarily via breast-feeding and transferred IgG antibodies which provide tissue defence that is pro-inflammatory by activating the complement system and phagocytes [26]. In the present study, for the first time, we have reported the use of a noble non-invasive approach to recover viable colonocytes from stool and application of these viable colonocytes for the measurement of immunoglobulin (IgA & IgG) receptors expression using specific florochrom conjugate antibodies and flowcytometry. This is a pioneer study in the subject area and it will also encourage further intensive study with diverse parameters and components using this non-invasive approach in different demographic segments. This non-invasive technique can be exploited further for the detection, evaluation and management of different GI diseases including IBD and Colon Cancer.

Acknowledgment

This study was conducted with financial support from Department of Biotechnology, Government of India.

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