Int.J.Curr.Microbiol.App.Sci (2017) *6*(1): 979-987

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 1 (2017) pp. 979-987**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2017.601.116

Study of *Bifidobacteria* species and Asthma in Children below Two Years: One Center Experience

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Keywords

Bifidobacteria, PCR, groEL gene

Article Info

Accepted:
28 December 2016
Available Online:
10 January 2017

A B S T R A C T

Bifidobacteria species (spp.) is common component of normal gut flora. In recent years there are evidences that changes in these species may be associated with asthma in children. The aim of the present study was to study the various components of *Bifidobacterium species* by culture and polymerase chain reaction (PCR) from the stool from children with bronchial asthma compared to healthy children. The study included ninety children with asthma ≤2 years and ninety healthy children. Stool samples from all children were subjected to specific culture of *Bifidobacteria* species and biochemical identification. Further confirmation of the isolates was performed by the use of polymerase chain reaction (PCR) with species specific primers targeting housekeeping *groEL* gene. There was statistical significant difference of the isolation of *Bifidobacterium* spp rates between asthmatic children and control regarding *B. pseudocatenulatum*, *B. adolescentis*, *B. catenulatum* group and *B. longum* (P=0.0001 for each). The rates of isolation of *Bifidobacterium*spp increased with age in both groups. However, there was statistically significant higher isolation rates of *B. pseudocatenulatum*, *B. catenulatum group* and *B. longum* (P=0.0001), *B. adolescentis* (P=0.001) at age above 1 year to 2 years in control compared to asthmatic children. There was statistically significant higher rates of isolation of *B. pseudocatenulatum* in children with history of vaginal delivery compared to those with history of cesarean section delivery and in the rates of the isolation of *B. longumin* children with normal breast feeding history compared to formula feed (P=0.02). The present study highlights the difference in the prevalence of certain *Bifidobacterium* spp in children below 2 years between children with asthma and healthy children. *B. pseudocatenulatum*, *B. adolescentis*, *B. catenulatum* group and *B. longum* were significantly reduced in children with asthma. The constituents of *Bifidobacterium* spp differ according to the methods of the delivery, feeding habits and with age. The use of molecular method may be the preferred method for study of *Bifidobacterium* spp. Longituidinal studies with larger numbers of children are required to validate these data. The findings of studies may indicate the usefulness of the use of specific strains of *Bifidobacterium* spp as specific adjuvant therapy.

Introduction

The normal bacterial flora in gastrointestinal tract (GIT) varies significantly among human depending upon individual factors such as pH, the concentration of bile acids, digestion retention time, mucin properties and host defense factors (Budden *et al.*, 2017). However, even with variations with these factors the predominating microbiota in GIT

includes mainly four phyla namely Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. There are rare phyla such as Fusobacteria, Verrucomicrobia and Spirochaetes are also present. These microbiotal comprises up to 14 bacterial genera and 150 bacterial species that not all being identified by bacterial culture. Among *Bacteroides* genus in GIT is *Bifidobacterium* spp. that is linked to humoral immune response in the gut (Budden *et al.,* 2017).

In newly born infant the gut is sterile. Microbiota develops in GIT within one week to reach about $1x10^{14}$ with approximately 500 different species. The normal composition of this microbiotal depends upon several factors like mode of delivery, environmental contact and nutrition (Sommer and Backhed, 2013). Unlike adults microbiota composition, *Bifidobacterium* spp. represents around 95% of normal flora in newborn. (Marsland *et al.,* 2015; Trompette *et al.,* 2014). The functions of *Bifidobacterium* spp. include easy absorption of mil proteins, production of several vitamins like B2, B6m B12, folic acid and nicotinic acid and contribute to normal metabolism of protein (Garssen *et al.,* 2003; Soccol1 *et al.,* 2010).

Besides the functions attributed to *Bifidobacterium* spp. in normal metabolism of vitamins and protein, this species appear to play a vital role in regulation of immune response reflected upon lung. These species produce antimicrobial peptides, secretory immunoglublin A and other pro-inflammatory cytokines. This effect on mucosal immune system appears to be adjust the immune system not only local but at distant location such as lung (Marsland *et al.,* 2015; Trompette *et al.,* 2014). Other suggested theories of the role of microbiota in lung is the translocation of these bacteria to airways as this was noticed in sepsis and changes of microbiota of the respiratory system noticed with digestion of some die try fibers that affect simultaneously both respiratory and GIT microbiota (Budden *et al.,* 2017).

There are some evidences that the change of microbiota composition of GIT in children affected by antibiotics intake may be linked to the development of bronchial asthma in young children between 0-7 years (Trompette *et al.,* 2014).

Asthma is defined as chronic inflammatory diseases due to bronchial hypersensitivity. There are various mediators of inflammation that is associated with this disease (Sears *et al.,* 2002).

The aim of the present study was to study the various components of *Bifidobacterium species* by culture and polymerase chain reaction (PCR) from the stool from children with bronchial asthma compared to healthy children.

Materials and Methods

The study was performed on Mansoura University Children hospital, Egypt from January 2016 till October 2016. The study included ninety children below or at 2 years old with asthma that was diagnosed clinically by repeated attacks of wheezy chest without fever or signs of infections that respond to bronchodilators.

In addition, ninety healthy children with similar age were included as control group. The children with previous antibiotics therapy in the last two weeks were excluded from the study.

The study was approved by Mansoura Faculty of Medicine ethical committee and informed consents were obtained from the parents of each child. The children were subjected to full history taking from the parents with special

attention to the method of delivery, the feeding habits of the child whether breast feeding or formula.

From each child stool sample was collected in clean plastic container and transported to the laboratory within half an hour.

Stool Samples

One stool sample was collected from each child and transported to the laboratory in clean plastic container. In the laboratory around one gram of stool sample was incubated on tubes with prepared liquid Tripticase Phyton Yeast (TPY) medium (Becton Dicknson).

The tubes were incubated 48 hours at 37ºC under anaerobic condition in anaerobic jar with the use of gas packs AnaeroGen (Oxoid and Mitsubishi Gas Company), After 48 hours subcultures were performed on selective media Bifidobacterium Medium (BD, BD Diagnostic Systems Europe Becton Dickinson France SA) which is based on modified Columbia medium. The cultured plates were incubated under complete anaerobic conditions for furthure 72 hours at 37ºC. The colonies were identified by gram stain and biochemical identification by the use of API20 A (BioMerieux, Marci L'etoile, France).

Molecular Identification of Isolated Bifidobacterium

DNA Extraction of Pure Colonies

Two pure colonies of the culture were dissolved in 0.5 milliliter of sterile phosphate buffer solution and was used for DNA extraction by Qiagen extraction kit according to the manufacturer protocol. The extracted DNA was kept frozen at -20^{0}C for further amplification procedures.

PCR for Identification of Bifidobacterium species

PCR was performed to identify 7 common species of Bifidobacterium isolates with specific target of housekeeping *groEL* gene. The sequences of the primers were listed in table 1 (Matsuki *et al.*, 2003; Junick and Blaut, 2012).

The amplification was performed by the use of Qiagen kit according to the previous method described by (Junick and Blaut, 2012). After amplification the products were subjected to electrophoresis by the use of 1.7% gel stained with ethidium bromide for 20 minutes. The products were visualized by UV in comparison with ladder marker and positive control strains.

Statistical Analysis

Statistical analysis was performed by the use of SPPS24. The numerical data were recorded as number and percentages for categorical variables. Chi-square test was used to compare between the numerical results and the results were considered significant at P≤ 0.05.

Results and Discussion

The study included 90 child with asthma they were mainly males\(57.8%). The mean age± SD of the children with asthma was 16.00± 4.3 months and the mean age± SD of the healthy children was 14.5± 4.2 with statistically significant difference P=0.04. There was statistically insignificant difference between patients and control as regards method of delivery and the type of feeding methods (P=0.3, P=0.2 respectively), table 2.

The main species of *Bifidobacterium* spp isolated from asthmatic children was *B. longum* (31.1%), *B. bifidum* (23.3%), and *B.*

Pseudocatenulatum and *B. breve* (22.2% for each). In healthy children the main isolated *Bifidobacterium* spp. was *B. catenulatum* group (73.3%), *B. pseudocatenulatum* (66.7%) and *B. longum* (65.6%). Meanwhile, there was statistical significant difference of the isolation of *Bifidobacterium* spp rates between asthmatic children and control regarding *B. pseudocatenulatum*, *B. adolescentis*, *B. catenulatum* group and *B. longum* (P=0.0001 for each), table 3.

Figure 1 demonstrated positive PCR for B. longum and figure 2 was for positive culture.

The rates of isolation of *Bifidobacterium* spp increased with age in both groups. However, there was statistically significant higher isolation rates of *B. pseudocatenulatum, B. catenulatum group* and *B. longum* (P=0.0001), *B. adolescentis* (P=0.001) at age above 1 year to 2 years in control compared to asthmatic children, table 4.

There was statistically significant higher rates of isolation of *B. pseudocatenulatum* in children with history of vaginal delivery compared to those with history of cesarean section delivery and in the rates of the isolation of *B. longumin* children with normal breast feeding history compared to formula feed (P=0.02), table 5.

Previous studies hypothesized a pivotal role played by intestinal microbiota in the pathogenesis of allergic diseases in children such as bronchial asthma, allergic rhinitis and eczema. Both the composition and the diversity of the microbiota are associated with this hypothesis. There were various aspects for this hypothesis implicating microbiota in the development of allergic conditions. First, it is reported that adhesive properties to mucosa of gastrointestinal tract of certain biof species may be reduced than other species leading to less modulation of the immune system system (O'Halloran *et al.,* 1998). Second factor it is claimed that some species of biofido have good inducing capacity of interleukin 10 with efficient inhibiton of Th2 related cytokines IL5 and IL13 claimed to be associated with allergic conditions (He *et al.,* 2001).

The composition of *Bifidobacterium* spp differs according to the age and geographical regions of the studies. In the present study we attempt to identify common species of *Bifidobacterium* spp among children ≤2 years old with and without asthma. The studied species are the common species found in previous studies to constitute the microbiota of children at this age (Peirotén *et al.,* 2018).

The main species isolated from all children were more or less similar with *B. longum* and *B. pseudocatenulatum* most prevalent isolates. The gut flora in infants rapidly grows and differentiates to the adults predominating species within 5 days after birth. The composition of its species depends mainly upon the method of delivery, the feedings regimen and the environment (He *et al.,* 2001).

In the present study the methods of delivery and the feedings methods did not show significant difference between children with asthma and those without. Therefore, the species were more or less similar in the types. However, there was statistical significant decrease in the isolation rates of *Bifidobacterium* spp. rates in the asthmatic children compared to the control regarding B. pseudocatenulatum, B. adolescentis, B. catenulatum group and B. longum (P=0.0001 for each). These species were considered in previous studies as an important constituent of *Bifidobacterium* spp that were reduced the allergic disorders. These isolates had many mechanisms in modulating the immune system.

Table.1 Bifidobacterium species specific primers sequences used in the study

Bifidobacterium species	Sequence	bp
Bifidobacterium longum	5/-TTCCAGTTGATCGCATGGTC-/3 5/-GGGAAGCCGTATCTCTACGA-/3	831
Bifidobacterium adolescentis	5/-CTCCAGTTGGATGCATGTC-/3 5/-CGAAGGCTTGCTCCCAGT-/3	279
Bifidobacterium breve	5/-CCGGATGCTCCATCACAC-/3 5/-ACAAAGTGCCTTGCTCCCT-/3	288
Bifidobacterium bifidum	5/-CCACATGATCGCATGTGATTG-/3 5/-CCGAAGGCTTGCTCCCAAA-/3	278
Bifidobacterium infantis	5/-TTCCAGTTGATCGCATGGTC-/3 5/-GGAAACCCCATCTCTGGGAT-/3	828
Bifidobacterium catenulatum	5/-CGGATGCTCCGACTCCT-/3 5/-CGAAGGCTTGCTCCCGAT-/3	285
Bifidobacterium pseudocatenulatum	5/-AGCCATCGTCAAGGAGCTTATCGCAG-/3 5/-CACGACGTCCTGCTGAGAGCTCAC-/3	325

Table.2 Demographic Data of the studied children

	Patients No. %	Control No. %	P
Gender Male Female	 52 57.8% 38 42.2%	 58 64.4% 32 35.6%	 0.2
Age (months)	16.00± 4.3	14.5± 4.2	0.04
Method of delivery CS Vaginal	 42 46.7% 48 53.3%	 52 57.8% 38 42.2%	 0.3
Feed Breast Feed Formula	 39 43.3% 51 56.7%	 45 50% 45 50%	 0.2

Table.3 Comparison of *Bifidobacterium* spp isolated from asthmatic Children versus healthy children

	Patients N0. %	Control No. %	P
B. pseudocatenulatum	20 22.2%	60 66.7%	P=0.0001
B. adolescentis	8 8.9%	28 31.1%	P=0.0001
B. catenulatum group	17 18.9%	66 73.3%	P=0.0001
B. breve	20 22.2%	28 31.1%	P=0.1
B. bifidum	21 23.3%	26 28.9%	P=0.2
B. infantis	20 22.2%	27 30.0%	P=0.2
B. longum	28 31.1%	59 65.6%	P=0.0001

Bifidobacterium: B.

Table.4 Distribution of *Bifidobacterium* spp as regard age in asthmatic Children and normal children

	Patients (n=90) No. %	Control (n=90) No. %	P
B. pseudocatenulatum 0-1 year 1-2 year	 0 0% 20 22.2%	 9 10% 50 55.6%	 P=0.1 P=0.0001
B. infantis 0-1 year 1-2 year	 2 2.2% 18 20%	 2 2.2% 20 22.2%	 P=0.3 P=0.6
B. catenulatum group 0-1 year 1-2 year	 0 0% 17 18.9%	 9 10% 50 55.6%	 P=0.1 P=0.0001
B. adolescentis 0-1 year 1-2 year	 2 2.2% 6 13.2%	 6 13.2% 53 58.9%	 P=0.1 P=0.001
B.breve 0-1 year 1-2 year	 1 1.1% 19 21.1%	 3 3.3% 24 26.7%	 P=0.1 P=0.2
B. bifidum 0-1 year 1-2 year	 0 0% 21 23.3%	 4 4.4% 24 26.7%	 P=0.1 P=0.2
B. longum 0-1 year 1-2 year	 1 1.1% 27 30%	 8 8.8% 48 53.3%	 P=0.1 P=0.0001

Table.5 Comparison of the isolation rates of *Bifidobacterium* spp in relation to methods of delivery, feeding methods and gender in asthmatic children (n=90)

	Delivery Vaginal CS	Feeding Breast Feed Formula	Gender Male Female
B. pseudocatenulatum	16 4 P=0.005	14 6 P=0.1	9 11 P=0.4
B. infantis	11 9 P=0.4	7 10 P=0.2	13 7 P=0.6
B. catenulatum group	8 9 P=0.4	7 10 P=0.2	9 8 P=0.7
B. adolescentis	7 1 P=0.1	5 3 P=0.4	6 2 P=0.4
B.breve	12 8 P=0.6	11 9 P=0.8	12 8 P=0.5
B. bifidum	13 8 P=0.4	12 9 P=0.3	12 9 P=0.6
B. longum	18 10 P=0.2	19 9 P=0.02	15 13 P=0.4

Caesarean section: CS

These species modulate Th2 immune response and produce a short-chain fatty acid that reduce the risk of asthma (Arrieta *et al.,* 2015). Other mechanisms also are associated with these specific species such as good adhesive properties to intestinal mucosa associated with B. adolescentis leading to immune system modulation (O'Halloran *et al.,* 1998).

Moreover, B. longum B. pseudocatenulatum and B. catenulatum group, a major components of VSL#3 with high probiotic potency and designated for treatment of certain gastrointestinal disorders such ulcerative colitis and are now being tried experimentally in mice as a therapeutic adjuvants for treatment of asthma (Mendes *et al.,* 2017).

The present study demonstrated an increase of the isolation rates of *Bifidobacterium* spp in patients and control. This finding is online with previous studies that have shown that the *Bifidobacterium* spp in human gut has dynamic changes with age. In early two years of life the changes occur with transition of feeding from milk to solid food (Avershina *et al.,* 2014; Odamaki *et al.,* 2016).

The interesting finding of the present study that the rates of increase in asthmatic children declined compared to control and there was However, there was statistically significant higher isolation rates of *B. pseudocatenulatum, B. catenulatum group* and *B. longum* (P=0.0001), *B. adolescentis* (P=0.001) at age above 1 year to 2 years in control compared to asthmatic children. Again, this finding supports the hypothesis that different species of *Bifidobacterium* are linked to the presence of asthma in children. However, if this is a causal relationship involved in the pathogenesis of asthma or the changes of the constitution of *Bifidobacterium* spp is due to asthma and other environmental immunogenes and genetic factors interaction remains as a question that need to be verified (Conlon and Bird, 2014).

The vaginal delivery and breast feeding appears to be a dominant factors in young children in the composition of *Bifidobacterium* spp. This was found in the present study as There was statistically significant higher rates of isolation of B. pseudocatenulatum in children with history of vaginal delivery the rates of the isolation of B. longum in children with normal breast feeding history (P=0.02). These findings online with previous studies (He *et al.,* 2001).

The laboratory study of *Bifidobacterium* to the species level is a fundamental research field that is needed to study the pathogenesis of many disorders and for future attempt to their use in a treatment protocol. However, the study is tedious when culture techniques only is used due to the fastidious nature of *Bifidobacterium* spp and the need for specific culture medium (Apajalahti *et al.,* 2003). The use of molecular method in the present study had proven to be associated with accurate identification of the species by the use of PCR and specific primers sequences with the use of housekeeping *groEL* (Ventura *et al.,* 2004; Junick and Blaut, 2012). Future studies are needed to evaluate the use of this molecular method directly on stool samples by passing the need for culture method.

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How to cite this article:

Maysaa El Sayed Zaki, Mohamed Anies Rizk and Ali Sobh. 2017. Study of *Bifidobacteria* species and Asthma in Children below Two Years: One Center Experience. *Int.J.Curr.Microbiol.App.Sci.* 6(1): 979-987. doi: http://dx.doi.org/10.20546/ijcmas.2017.601.116