

*Full Length Research Paper*

# Molecular cloning of human dendritic cell associated lectin-1 (DECTIN-1) isoform genes, expression and localization as a green fluorescent protein (GFP) fusion in caco-2 cell line

Esayas Gelaye<sup>1\*</sup>, Jan Roosien<sup>2</sup> and Arjen Schots<sup>2</sup>

<sup>1</sup>National Veterinary Institute, P.O.Box:19, Debre zeit, Ethiopia.

<sup>2</sup>Laboratory of Molecular Recognition and Antibody Technology, Wageningen University and Research. The Netherlands.

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The human Dectin-1 molecule known as a  $\beta$ -glucan receptor is an immune cell surface receptor implicated in the immunological defense against fungal and other pathogens. Dectin-1 is a type II transmembrane receptor with a single extracellular carbohydrate recognition domain (CRD), a stalk region which separates the CRD from the membrane and an immunoreceptor tyrosine activation motif in its cytoplasmic tail. The objectives of the present study were isolation of the Dectin-1 genes from the human monocyte complementary deoxyribonucleic acid (cDNA), cloning of the isolated human Dectin-1 isoform transcripts into the mammalian expression vector, make a green fluorescent protein (GFP) fusion, expression and sub-cellular localization of the GFP fusion in the mammalian cell line. Reverse transcription-polymerase chain reaction (RT-PCR) and Vector NTI sequence analysis revealed six transcripts from a human monocyte cDNA. Basic local alignment search tool (BLAST) analysis showed the transcripts are member of the human Dectin-1 gene family. Sequence alignment analysis using contig express and ClustalW revealed a 100% sequence similarity with the human Dectin-1 isoform A, B and F with a size of 741, 603 and 232 bp, respectively and another one transcript (193 bp) which do not homologous with the six isoforms. Cloning of the four isolated human Dectin-1 isoform transcripts into the mammalian expression vector at the 3'end of cytomegalovirus promoter (CMVp) and the GFP was ligated at the 3'end of the cloned Dectin-1 gene. The ligation experiment was proven by restriction enzyme digestion. Transient transfection of the plasmid deoxyribonucleic acid (DNA's) that contain the chimeric human Dectin-1 isoform-GFP fusion transcripts into a Caco-2 cells were conducted and after 24 h of nucleofection. The tissue culture plate cells were examined by fluorescent microscope and all the tested samples showed green fluorescence signal. Transfection efficiency of 40.2 to 58.0% and 70.7 to 82.8% cell viability were recorded using flow cytometry assay at 48 h of post nucleofection on cells that were cultured on tissue culture plate. Localization of fused GFP was assessed using a laser scanning confocal microscopy after 24 h of transfection on cells that were cultured on a chambered tissue culture coverglass and revealed the GFP is localized on the cell membrane.

**Key words:** Cloning, GFP, human DECTIN-1 isoforms, localization, RT-PCR, transfection

## INTRODUCTION

The innate immune system is a universal defense against infection and it is the first defense line against microbes

and mediates the activation of the adaptive immune response (Janeway et al., 2002; Brown et al., 2003). The innate immune recognition relies on a limited number of germline encoded receptors and these receptors evolved to recognize conserved products produced by microbial pathogens (Brown et al., 2003; Daba and Ezeronye,

\*Corresponding author. E-mail: [egelaye@yahoo.com](mailto:egelaye@yahoo.com).

2003; Abbas and Lichtman, 2005). Recognition of these molecular structures allows the immune system to distinguish infectious nonself from noninfectious self. The innate immune system uses a variety of pattern recognition receptors that can be expressed on the cell surface, in intracellular components, or secreted into the blood-stream and tissue fluids (Daba et al., 2003; Abbas and Lichtman, 2005). Many C-type lectins belong to the large family of pattern recognition receptors (PRRs) that detect conserved pathogen associated molecular patterns (PAMPs), helping to distinguish self from non-self and modulating immune responses against pathogens. C-type lectins (CTLs) are expressed by a broad spectrum of cells, including dendritic cells (DCs) and other antigen-presenting cells (APC) (Willcocks et al., 2006). Dendritic cells are the key players in the regulation of cell-mediated immunity and represent the most potent APC capable of sensitizing naive T cells to novel antigens and incubation of primary immune responses (Geijtenbeek et al., 2002; Grunebach et al., 2002; Peiser et al., 2002). Dectin-1 (Dendritic cell associated lectin-1) is a C-type lectin-like receptor (Herre et al., 2004a; Brown et al., 2006, 2007). Dectin-1 was originally thought to be a dendritic cell (DC)-specific receptor, from which its name 'dendritic-cell-associated C-type lectin-1' was derived, but later on confirmed that the receptor can also be expressed by many other cell types, including macrophages, monocytes, neutrophils and a subset of T cells, although it is not expressed on these cells in all tissues (Brown et al., 2006; Willcocks et al., 2006). Dectin-1 was first identified in mice (mDectin-1) as a receptor for  $\beta$ -glucan, a common component of fungal cell walls (Yokota et al., 2001; Herre et al., 2004a; Willcocks et al., 2006). Hernanz-Falco et al. (2001) indicated that the full length of human Dectin-1 isoform A showed 60% sequence identity and 71% sequence homology to mDectin-1.

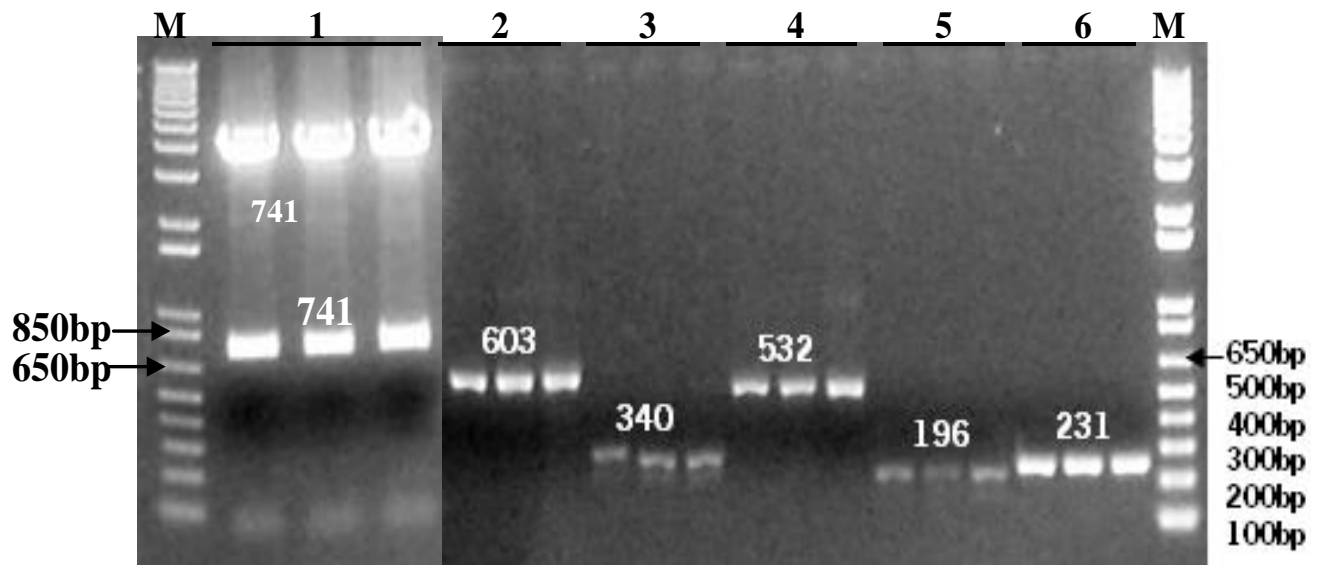
In general, hDectin-1 differs from mDectin-1 in several respects: it produces two major messenger ribonucleic acid (mRNA) transcripts, is expressed predominantly by peripheral blood leukocytes and is a less glycosylated protein than mouse Dectin-1 (Yokota et al., 2001). Using Northern blotting, Herre et al. (2004a) found that Dectin-1 was widely expressed in the liver, lung, thymus, stomach, spleen, small intestine, kidney and heart. Analysis of the amino acid sequence of human Dectin-1 isoform A reveals a type II membrane receptor with a cytoplasmic tail domain of 44aa (with two tyrosines in positions 3 and 15) containing an unpaired immunoreceptor tyrosine-based activation motif (ITAM), a putative 21aa transmembrane domain (spanning leucine 45 to glycine 65), the extracellular part of 182aa is composed of a stalk region of 54aa that connects the transmembrane domain to a single C-type lectin-like domain (CTLD) with N-glycosylation sites in the COOH-terminus that spans the last 128aa as shown in Figure 1 and Annex 2 (Hernanz-Falco et al., 2001; Heinsbroek et al., 2006). The Dectin-1 gene is localized in the natural killer gene complex on

human chromosome 12 p12.3-p13.2 (Hernanz-Falco et al., 2001). The natural killer (NK) receptor gene complex (NKC) encodes a large number of C-type lectin-like receptors, which are expressed on NK and other immune-related cells (Hao et al., 2006). As stated by Herre et al., (2004b) in contrast to the murine receptor, hDectin-1 has only one predicted N-linked glycosylation site and the significance of these glycosylation differences has yet to be determined. The presence or absence of the stalk region in the two major isoforms A and B, respectively did not significantly affect their carbohydrate specificity and pattern recognition function (Herre et al., 2004b). The full length of human Dectin-1 gene has six exons due to alternative splicing six different isoforms are produced which have an approximate coding sequence base pair length of isoform A=744, B=606, C=570, D=432, E=507 and F=234 bp (NCBI, 2007). Whereas Xie et al., (2006) indicated that the full gene of human Dectin-1 is alternatively spliced generating eight isoforms (Yokota et al., 2001; Willment et al., 2005). Dectin-1 is the main receptor on leukocytes that mediates innate immunity to fungal pathogens (Daba and Ezeronye, 2003; Reid et al., 2004; Palma et al., 2006). It is now known to be expressed at the surface of monocytes and neutrophils, and at low levels on a sub-population of T-cells (Palma et al., 2006). There are evidence that the function of Dectin-1 may strongly interact with the TLR2 signaling pathway as co-expression of dectin-1 with TLR2 increases the production of Tumor necrosis factor (TNF) and IL-2 (Gantner et al., 2003; Willcocks et al., 2006; Yadav and Schorey, 2006). Research on the protein interaction of Dectin-1 gene with the different pathogens of fungal, parasitic, bacterial or viral origin and the importance of the receptor in autoimmune disease is not yet well studied. Thus, cloning of human Dectin-1 immune receptor genes and fusion with GFP, a reporter gene, will help in studying the presence and level of protein-protein interactions with other proteins and to assess its therapeutic or preventive properties to pathogenic organisms. The objective of the research project was isolation of the different Dectin-1 transcripts from the human monocyte cDNA library, cloning of the isolated Dectin-1 isoform transcripts, make a recombinant fusion construct with the green fluorescent protein and detect the expression and sub-cellular localization of the GFP fusion in Caco-2 cell line.

## MATERIALS AND METHODS

### Gene isolation

To amplify and isolate the Dectin-1 gene from the human monocyte cDNA library (Spring Bioscience) a Polymerase Chain Reaction (PCR) (Yokota et al., 2001; Glover and Hames, 1995; Michael et al., 2006) was applied using gene-specific primers for each different isoform transcripts. To design the primers the sequence of each human Dectin-1 isoforms was used from the database. Each forward and reverse primer was designed containing NheI sequence at the



**Figure 1.** Band pattern of a gel picture showing the PCR result of the different Dectin transcripts amplified from the cDNA. Lane M is a 1 Kb plus DNA ladder; lane 1 (741bp); lane 2 (603 bp); lane 3 (340 bp); lane 4 (532 bp); lane 5 (196 bp) and lane 6 (231 bp) (the same PCR product was loaded on three consecutive lanes).

5' end and BamHI sequence at the 3' end. Internal primers were also designed to isolate the full isoform gene using a PCR overlap extension method (Wurch et al., 1998; Lu et al., 2005). The DNA of each PCR product was run on a 1% Ethidium Bromide (EtBr) agarose gel. Purification of the DNA from the gel was conducted by using the DNA extraction kit (Marligen Bioscience) and the concentration of the purified DNA was measured by NanoDrop (NanoDrop Technologies) and Gel electrophoresis (using  $\lambda$  DNA as a marker).

#### Cloning of genes in pCR2.1 TOPO vector

The isolated genes from the PCR product were cloned into the pCR2.1 TOPO vector by using the standard TOPO cloning technique (Invitrogen Life Technologies). Transformation of the cloned gene into the bacterial strain of chemically competent Top10 *Escherichia coli* was carried out and cultured on solid agar media which contains Ampicillin, X-gal and isopropyl-beta-D-thiogalactopyranoside (IPTG) incubated overnight at 37°C (Bruce et al., 1997; Christopher, 2007; Invitrogen). The transformed colonies were again cultured on Luria-Bertani (LB) media containing Ampicillin and incubated at 37°C with 250 rpm overnight. Purification of the plasmid DNA from the liquid culture was conducted using the plus Midipreps DNA purification system (Promega).

#### Sequencing and Identification of the hDectin-1 isoforms

The purified plasmid DNA was sequenced in the department of Molecular Biology, Wageningen University. The sequences were analyzed using Vector NTI software (Invitrogen Vector NTI Advance™10, Life Technologies). The sequences were subjected to a homology search using the BLAST algorithm to check whether they are members of the human Dectin-1 gene family (Grunebach et al., 2002). Based on the sequence analysis the sequences were clustered with the different isoforms of Dectin-1 transcripts using the Contig express programme (Invitrogen Vector NTI Advance™10).

#### Cloning of Dectin-1 isoforms in pEYFPn-mC2Y vector

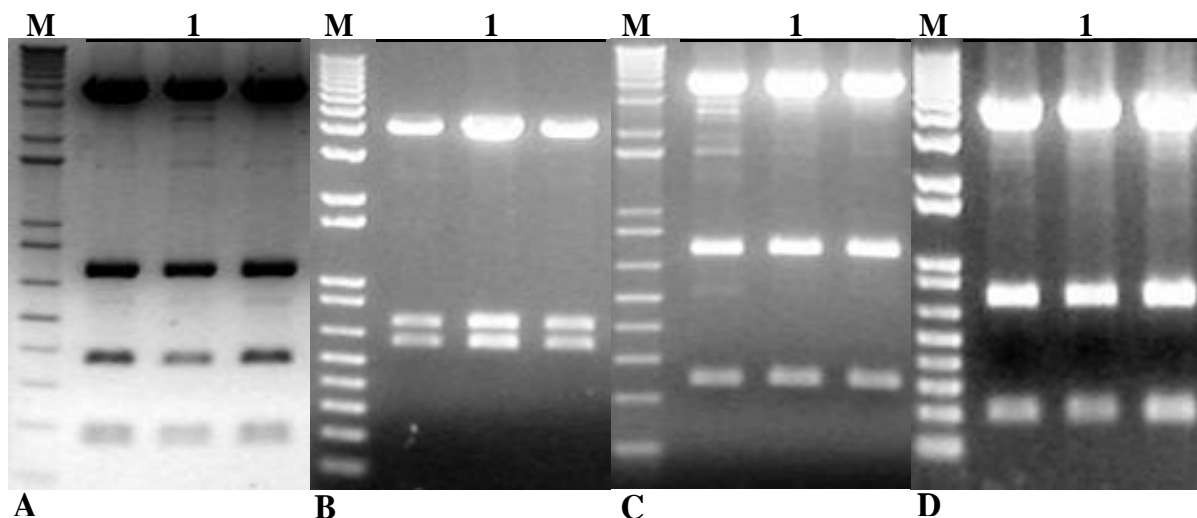
The Dectin-1 isoform transcripts were cloned into a mammalian expression pEYFPn-mC2Y vector that contains the red and yellow fluorescence protein sequences (Clontech). The cyan fluorescence protein (CFP) sequence was digested with the unique NheI/BamHI enzymes and replaced by the sequence of Dectin-1 isoform transcripts, since CFP was not important for the study. Electro-competent Top10 *E. coli* cell was used for transformation and cultured on kanamycin containing solid and liquid media.

#### Fusion of GFP gene

The GFP gene was isolated from the plant vector using the unique BamHI/XbaI enzymes. The sequence of yellow fluorescent protein (YFP) was removed by using BamHI/XbaI enzymes and replaced by the GFP gene at the 3' end of the cloned Dectin-1 isoform gene (Herre et al., 2004a). Mutant strain of 190 *E. coli* was used for transformation since it does not contain the methylation activity at XbaI site. Finally, the plasmid DNA that contain the cloned genes of Dectin-1 isoform and GFP was digested by NheI/BamHI/SstI restriction enzymes to confirm the cloning experiments.

#### Cell line and culture

The Caco-2 cell line, a human colon adenocarcinoma epithelial cell (Sambuy et al., 2005), was maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 50IU/mL penicillin and 50 mg/mL streptomycin (Invitrogen). It is an adherent cell that grows as a monolayer cell and it has the phenotype of normal intestinal epithelial cells *in vivo*. Cells were grown at 37°C in 5% CO<sub>2</sub> (William et al., 2004). Confluent Caco-2 cells were trypsinized (0.25% trypsin with 0.1% ethylenediaminetetraacetic acid (EDTA)-Invitrogen) and subcultured for 3 days before transfection experiment at a density of 5x10<sup>5</sup> cells in a 25 cm<sup>2</sup> tissue culture flasks and maintained with a complete DMEM.



**Figure 2.** A gel photo explaining the band pattern of the digestion result of the Dectin-1 isoform GFP gene fusions construct. Lane M is 1Kb plus DNA ladder and lane 1 is the digested plasmid DNA for the different constructs. **A-** the pEYFPn-A-GFP plasmid DNA digested using NheI/KpnI/BamHI/SstI revealed the expected band size of 3.9Kb, 770, 374, 202 and 175 bp. **B-** the pEYFPn-B-GFP plasmid DNA digested using NheI/BamHI/SstI showed the expected band size of 4Kb, 770 and 617 bp. **C-** the pEYFPn-F-GFP plasmid DNA digested with NheI/BamHI/SstI revealed the band size of 4.4 Kb, 770 and 245 bp. **D-** the pEYFPn-U-GFP plasmid DNA digested with NheI/BamHI/SstI revealed a band of 4.4Kb, 770 and 206 bp. Therefore, the ligation experiment of the different isolated Dectin-1 isoforms and GFP fusion genes that were ligated in the mammalian expression vector was confirmed using restriction enzyme digestion.

### Transfection of plasmid DNA

The chimeric plasmid DNA containing the human Dectin-1 isoform and GFP genes were purified based on the manufacturer's instruction with the Endofree plasmid purification kit (QIAGEN). Caco-2 cells were nucleofected using the Cell Line Nucleofector Kit T, program B-24 and 2  $\mu$ g of the purified plasmid DNA containing the different Dectin-GFP constructs were transiently transfected into the Caco-2 cells following the instructions of the manufacturer (Distler et al., 2005; Jacobsen et al., 2006). The transfected cells were cultured in a six well tissue culture plates and on a chambered tissue culture coverglass (Invitrogen Life Technologies).

### Fluorescent microscopy

24 h post nucleofection the cells that were cultured on the six well plates (transfected, positive and negative control cells) were examined for the presence of GFP signals using a fluorescent microscope (Pertaya et al., 2007).

### Flow cytometry

After 48 h of nucleofection the six well plate cells were trypsinized and processed separately. The percentage of transfection efficiency and viable cells for each tested constructs were measured by Fluorescence-activated cell sorting (FACS) system (Beavis and Kalejta, 1999; Herzenberg et al., 2002; Trudi et al., 2007).

### Localization of chimeric genes

Sub-cellular localization of the chimeric Dectin-1 and GFP fusion genes was examined using the laser scanning confocal microscope (Chalfie et al., 1994; Kain et al., 1995; Rizzuto et al., 1995; Martinez

and Miledi, 2001; Xia et al., 2005) on those Caco-2 cells that were transiently transfected and cultured for 24 h at 37°C on a chambered tissue culture removable coverglass (Invitrogen Life Technologies).

## RESULTS

### Polymerase chain reaction

RT-PCR experiment was conducted using the expanded high fidelity enzyme to isolate the Dectin transcripts from the PCR ready human cDNA monocyte. As shown in Figure 2, six different transcripts having different band sizes were identified in a 1% EtBr agarose gel electrophoresis. The DNA of each PCR product from the three slots was mixed and purified from the gel using the DNA extraction kit.

### Cloning of Dectin gene into pCR2.1 TOPO vector

Selected DNA fragments from each PCR product were cloned into a pCR2.1TOPO vector using chemical transformation and cultured on solid agar containing Ampicillin, X-gal and IPTG. The transformed colonies were again cultured on LB media. The plasmid DNA of each cloned genes was purified from the overnight liquid culture and 6  $\mu$ l with a concentration of around 200 ng/ $\mu$ l was adjusted for each samples and kept at -20°C until submission for sequencing.

### Sequence analysis and identification of isoforms

Different selected clones from the six different transcripts were sequenced in Molecular Biology Department, Wageningen University and Research. The sequences were analyzed by the Vector NTI software programme. All the analyzed sequences were blasted on the National Center for Biotechnology Information (NCBI) website and it was confirmed that all the sequences were members of the human Dectin-1 gene family. Clustering of the analyzed sequences with the sequences of the six human Dectin-1 isoform transcripts which are found in the Gene bank were done by the Contig express programme and the sequence analysis revealed the presence of four different transcripts with a size of 741, 603, 231 and 196 bp and another two transcripts with a size of 532 and 340 bp which have a stop codon. The human Dectin-1 isoform A (741 bp), B (603 bp), F (231 bp) and another one gene (196 bp) which is not homologous with any of the six isoforms were isolated and cloned for further experiments. The full gene of human Dectin-1 isoform A (741bp) was isolated using the internal primers of overlap extension method. The BLAST analysis result of the isolated Dectin-1 transcripts in the human genome data base confirmed that the isolated transcripts have a hit and are located in the human (*Homo sapiens*) genome of chromosome number 12 at 12p12.2- p13.2.

### Cloning of Dectin-1 isoform genes into pEYFPn-mC2Y vector

Since the human Dectin-1 gene is a type II transmembrane protein, the GFP gene was fused at the 3' end of the Dectin-1 gene and this is because this fusion arrangement is important to study the interaction between the Dectin-1 genes with any other protein compounds. The pEYFPn-mC2Y vector and the pCR2.1TOPO vector that contain the inserted human Dectin-1 isoform B gene were digested by NheI/BamHI restriction enzymes, thus, the CFP gene and the cloned isoform-B gene were removed from the two vectors, respectively. The two vectors pEYFPn-mC2Y and pCR2.1TOPO were also digested with NheI/BamHI. The sequence of isoform-B and the cloned isoform F and U gene fragments were removed from the two vectors, respectively. The full length of hDectin-1 isoform A that was cloned in pCR2.1TOPO vector was digested with NheI/BamHI and revealed a clear band of approximately 741bp after the digested product was run on a 1% EtBr agarose gel.

### Ligation and transformation of pEYFPn vector and isoform fragments

The pEYFPn vector and the isoform A, B, F and U gene fragments were ligated based on the equimolar principle and transformed on electro-competent *E.coli* cells. A

colony PCR was conducted for some of the grown colonies and those colonies that revealed the PCR amplification of the isoform transcripts were cultured on LB media.

### Digestion of pEYFPn-B-YFP and pRAB-CBPGFPmyctag

The plasmid DNA and the vector were digested with BamHI/XbaI to cut the YFP and GFP gene fragments from the pEYFPn-B-YFP and pRAB-CBPGFPmyctag, respectively. The digested product was run on a 1% EtBr agarose gel and the digestion process revealed a clear band of YFP and GFP gene fragments.

### Digestion of pRAB-N-GFP and pEYFPn-B-GFP

Digestion was conducted with NheI/SstI to get the GFP and pEYFPn gene fragments from the pRAB-N-GFP plant vector and pEYFPn-B-GFP (contain isoform-B and GFP) plasmid DNA, respectively. The digestion product showed the clear bands of GFP and pEYFPn.

### Ligation and transformation of pEYFPn and GFP fragments

The isolated and purified pEYFPn and GFP fragments from the gel were ligated and transformed into the electro-competent *E.coli* cells and cultured on agar plates containing kanamycin and incubated at 37°C overnight.

### Fusion of GFP gene

The plasmid DNA that contains the cloned gene of the different Dectin-1 isoforms which was digested with BamHI/XbaI to remove the YFP gene was again ligated with the GFP gene. The ligation and transformation experiment was proved by digestion of the purified plasmid DNA using restriction enzymes NheI/BamHI/SstI. SstI is the unique site for GFP, thus digestion of the plasmid DNA using this enzyme confirm the proper cloning of GFP gene. The pEYFPn-GFP plasmid DNA was digested with NheI/SstI to get the GFP gene which will be used for transfection.

### Endotoxin free purification of plasmid DNA

For transfection experiment, the plasmid DNA's that have the correct chimeric clone construct of the different human Dectin-1 (Table 1) isoform and GFP genes were purified with the EndoFree plasmid purification kit following the manufacturer's instructions. The concentration of the purified DNA was measured by NanoDrop.

## Transfection

A 3 days old of around 90% confluent grown Caco-2 cells of passage 5 were trypsinized and nucleofection was conducted for the different isoform-GFP fusion constructs and positive control. The cells were cultured on the six well tissue culture plates and on chambered tissue culture coverglass and incubated at 37°C/5% CO<sub>2</sub>.

## Fluorescence test

After 24 h of transient nucleofection, the incubated six well plate cells was examined under the fluorescent microscope. The GFP signal was observed in all the different GFP constructs and positive control culture plates under the fluorescence microscopy.

## Flow cytometry assays

After 48 h of nucleofection of the plasmid DNA, the transfected cells for each sample (GFP fusion constructs, positive control and negative control) were trypsinized and processed. The percentage of GFP positive (45 to 60%) and viable cells (72 to 85%) was determined using the fluorescence-activated cell sorting system.

## Localization of chimeric GFP

Sub-cellular localization of the chimeric fusion constructs of the plasmid DNA that contains the recombinant cloned genes of human Dectin-1 isoform A and GFP genes was examined using the confocal laser scanning microscope at 24 h of post nucleofection on those Caco-2 cells that were cultured on chambered tissue cultured coverglass at 37°C/5%CO<sub>2</sub>. The GFP that was fused with the gene of human Dectin-1 isoform A is localized on the cell membrane (Figure 4).

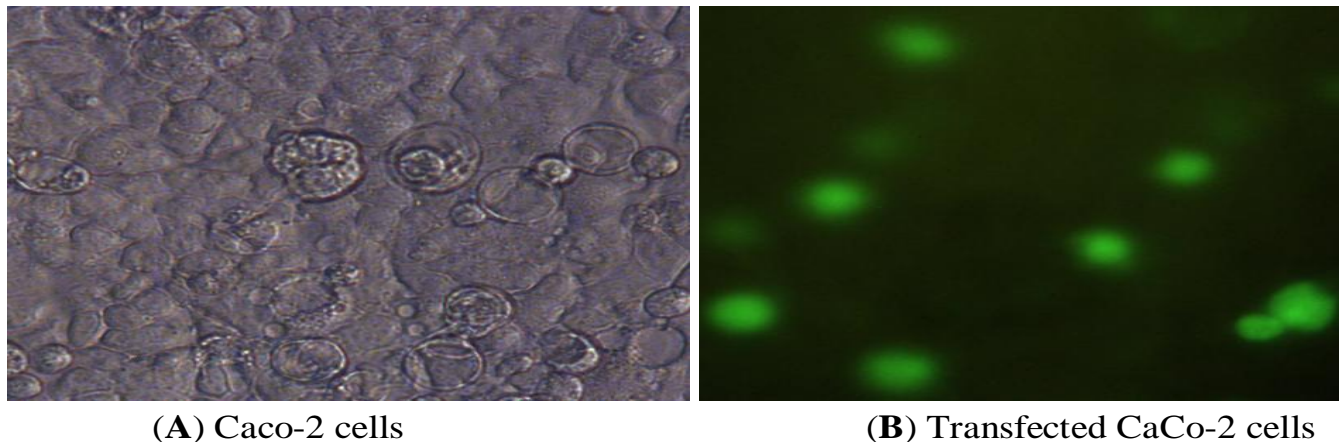
## DISCUSSION

This work describes the cloning of human DECTIN-1 isoform genes; make a GFP fusion construct, expression and sub-cellular localization of the recombinant genes in Caco-2 cell line. High fidelity RT-PCR analysis using the human monocyte cDNA revealed the identification of different transcripts as shown in Figure 1. pCR2.1 TOPO vector was used for cloning of the PCR products and the cloning products were sequenced and their sequences were analyzed by Vector NTI software programme and it revealed the presence of six different transcripts (741, 617, 547, 245, 206 and 193 bp). The full gene sequence of human Dectin-1 isoform A and the smallest isoform F were isolated and these transcripts might be used to see

the existence of size difference in relation to immune response to different antigens. Isoform C, D and E were not isolated and it needs repeated PCR testing to recover the genes from the cDNA.

The sequences were subjected to a BLAST search in the NCBI database and all of them revealed as members of the human Dectin-1 gene family. The sequences of each of the isolated genes were clustered with the sequences of the human Dectin-1 isoform transcripts using the contig express programme. The contig analysis explained the sequence similarity between the three isolated transcripts with the human Dectin-1 isoforms of A, B, F and U with a size of 741, 603, 245 and 206 bp, respectively. This result is in agreement with the work done by Willment et al. (2001) and Grunebach et al. (2002); they amplified the different human Dectin-1 isoforms from peripheral blood leukocyte cDNA. Ariizumi et al. (2000) also detected the full gene of Dectin-1 from Langerhans cells using reverse transcription PCR. Sequence alignment analysis of the sequences of isoform A and isoform B showed that there is a gap from 68 to 114aa in the sequence of isoform B, and this result explained the absence of exon 4 (encodes 46aa) which is responsible for a stalk region.

The absence of exon 4 in the sequence of isoform B which could be generated due to alternative splicing and this idea was also reported by Hernanz-Falcon et al. (2001), Willment et al. (2001) and Yokota et al. (2001). A difference in the immune response might not be detected between isoform A and B since the Carbohydrate Recognition Domain is present on both sequences, but this statement has to be proved by experimental data. Of the six isolated transcripts, only four transcripts were selected for further cloning and expression experiments since they did not have a stop codon. The sequences having a stop codon did not be used for further expression test since a full gene transcription could not take place during cloning and expression experiments. The stop codon might be occurred either due to mutation, error in cloning, purification and sequencing experiments or can be other reasons. Alignment of the amino acid sequences of the sequenced four human Dectin-1 isoform transcripts and the six isoform sequences from database were analyzed. This indicated that there is a 100% sequence similarity of the sequenced isoforms with the human Dectin-1 isoform A, B and F; thus the cloning and sequencing experiments were conducted correctly. The selected four isoform transcripts were cloned into the mammalian expression vector pEYFPn-mC2Y at the NheI/BamHI site in the place of CFP sequence. The cloned isoform gene in the plasmid DNA was again fused with the GFP gene at the 3'end of the human Dectin-1 gene, which was removed from the vector pRAB-CBPGFPmyctag, at the BamHI/SstI site in the place of YFP sequence. This is because the GFP was used as a reporter gene for the expression and localization study of the human Dectin-1 isoform genes in the human

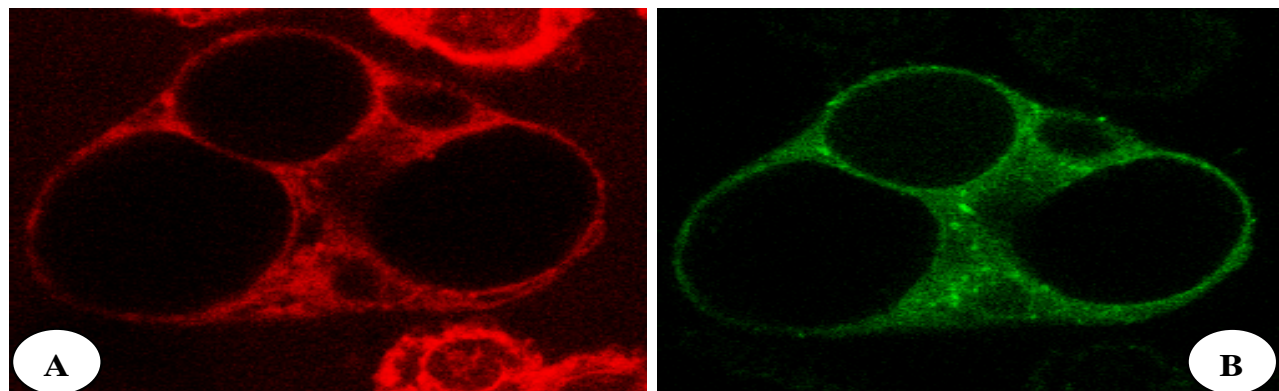


**Figure 3.** A picture taken using fluorescent microscope (40x) for the non-transfected cells (A) and transfected Caco-2 cells (green fluorescence signals) of the different isoform-GFP fusion constructs (B) after 24 h of nucleofection incubated at 37°C/5%CO<sub>2</sub>.

intestinal epithelial cell line (Caco-2). The plasmid DNA which contains the cloned fusion genes of Dectin-1 and GFP was digested with NheI/BamHI/SstI restriction enzymes and it was confirmed that both the cloned fusion construct of the different size human Dectin-1 isoforms and GFP genes in the expression vector was successful, that is there was a clear separated bands of the digestion product of the three digested fragments of the vector, hDectin-1 isoform and GFP genes as shown in Figure 2. Thus the digestion test using the three enzymes proved that the human Dectin-1 isoform gene was cloned between the 3' end of the CMV promoter and 5' end of the GFP gene. This type of ligation construct is used to detect the localization of the recombinant genes in the mammalian cells since Dectin-1 is a type II membrane protein; and also helps to study the level of protein-protein interaction between the recombinant genes with other proteins since the CRD domain is located outside the cell membrane. Nucleofection experiment was conducted for transiently transfection of the different constructs of hDectin-1 isoforms and GFP gene fusions in Caco-2 cells. Nucleofection technique was selected since it gives high transfection efficiency as indicated in the previous study conducted by Distler et al. (2005) and Jacobsen et al. (2006). The plasmid DNA which contains the different cloned genes of isoform and GFP genes was purified with the Endofree plasmid purification kit as it was recommended by the nucleofection kit manufacturer so as to remove or minimize the amount of endotoxins that was produced during purification of the plasmid from the *E. coli* cells and thus the purified plasmid does not produce a toxicity problem to the mammalian cells after transfection. Thus one positive control (pmaxGFP) and five different plasmid GFP constructs (pEYFP-GFP fusion, pEYFP-isoform B-GFP, pEYFP-isoform F-GFP and pEYFP-isoform U-GFP) were used for transient transfection experiment. The Caco-2 cells that were transiently transfected with the different plasmid DNA's

were examined by fluorescent microscope for the presence of green fluorescence signal after 24 h of nucleofection and the result proved that all the tested samples were positive with most of the cells appeared green fluorescence as shown in Figure 3.

This result is in line with the research done by Rizzuto et al. (1995) that they transfected the GFP fused gene into the HeLa cells and examination of the cells under the fluorescent microscope after 36 h of cells incubation revealed green fluorescence signals. At 48 h of post nucleofection, the transfected cells were again analyzed to determine the percentage of transfection efficiency and the level of viable cells by the fluorescence-activated cell sorting system. As shown in Figure 4, that the transfection efficiency of 40.2, 44.3, 51.0, 58.0 and 47.5% were recorded for the positive control, pEYFP-GFP, pEYFP-isoform B-GFP, pEYFP-isoform F-GFP and pEYFP-isoform U-GFP, respectively. This finding is in agreement with the finding of (Retrieved on May 10, 2007) that they reported around 58% transfection efficiency in Caco-2 cell examined after 24 h of nucleofection. 2.8% GFP was recorded in the negative control Caco-2 cells and this might be due to contamination from other transfected samples during processing. Similarly the level of viable cells were also analyzed using flow cytometry and for the negative control, positive control, pEYFP-GFP, pEYFP-isoform B-GFP, pEYFP-isoform F-GFP and pEYFP-isoform U-GFP tested samples 90.1, 70.7, 73.6, 82, 80.0 and 79.6% were recorded, respectively. This finding is also in line with the values recorded by (Retrieved on May 10, 2007) that cell viability varies between 70 to 76% examined at 24 h post nucleofection. The cell viability data also explained that the constructed plasmids did not have a toxicity effect to the transfected cells since the lowest cell viability was 70%. Thus, the data recorded in this experiment proved that the transfection experiment was done well even though it is the result of a single



**Figure 4.** Laser scanning confocal microscopic imaging using 60X oil objective of Caco-2 cells that were examined after 24 h of nucleofection with the chimeric gene construct of human Dectin-1 and GFP fusion. **A.** The cells were stained with the molecular probe ( $\beta$ -Bodipy®, red color) and it is shown that the cell membrane appeared red color; a second picture was taken. **B.** The GFP is localized on the cell membranes as it appears green.

**Table 1.** Primers used for the isolation of the gene.

Isoform	Primer	
	Forward	Reverse
A, B and E		5'- <b>GGATCCC</b> ATTGAAACTTCTTCTCACAAATAC-3' = Dec1R
C and D	5'- <b>GGCTAGC</b> ATGGAATAT	5'- <b>GGGATCC</b> TAAATGACTGACACGTGAATCCATAC-3' = Dec2R
F	CATCCTGATTTAG-3' =DecF	5'- <b>GGATCC</b> TCCTTTGAATTCCACAGCTTTGAAACC-3' = Dec3R
	Internal Forward	Internal Reverse
A	5'- <u>CACACAATCATCTTTAGAA</u> <u>GACAGTGTGACTCCTAC</u> -3'	5'- <u>ACTGTCTTCTAAAGATGATTGTGTGGGTTGACTGTGG</u> -3'

- **GCTAGC** - NheI    **GGATCC** – BamHI

- The underlined is the overlap sequence

- **GCTAGC** - NheI    **GGATCC** – BamHI, The underlined is the overlap sequence.

experiment. Thus, optimization of the transfection procedure and time of Fluorescence-activated cell sorting (FACS) analysis, and conducting all the above tested experiments in one laboratory might increase the transfection efficiency. Sub-cellular localization of the GFP fusion construct was examined using the laser scanning confocal microscope and it revealed that the GFP, which is a reporter gene, was localized on the cell membrane as shown in Figure 4. This result explained that the fusion construct of GFP gene with the Dectin-1 isoform gene was properly cloned since human Dectin-1 is a type II transmembrane protein and the GFP gene was fused on the 3'end of the Dectin-1 gene which is outside the cell membrane. This statement proved our hypothesis that the hDectin-1 gene is a type II membrane protein and the GFP ligation experiment at the 3'end of the Dectin-1 gene. Localization of the recombinant gene using GFP fusion technique was similar with the work

done by Rizzuto et al. (1995) that they localized the recombinant GFP gene in Hela cell. Thus based on the findings from the present study it can be concluded that the isolation of human Dectin-1 transcripts from the human monocyte cDNA, cloning of human Dectin-1 isoforms into the mammalian expression vector, make a GFP fusion construct, expression and localization of the chimeric genes in the human intestinal epithelial cells were well conducted. Based on the results of the cloning, expression and sub-cellular localization experiments conducted the following points were recommended: a study should be done to see immune stimulating properties of the cloned gene, determine the level of protein-protein interaction between the recombinant isoform-GFP protein with any other tested protein of plant or animal or pathogen origin, and assess the existence of therapeutic or preventive properties of the cloned genes for any selected pathogens that can cause



a disease or problem to human.

## Abbreviations

**CRD**, Carbohydrate recognition domain; **cDNA**, complementary deoxyribonucleic acid; **RT-PCR**, reverse transcription-polymerase chain reaction; **GFP**, green fluorescent protein; **BLAST**, basic local alignment search tool; **CMVp**, cytomegalovirus promoter; **PRRs**, pattern recognition receptors; **PAMPs**, pathogen associated molecular patterns; **CTLs**, C-type lectins; **DCs**, dendritic cells; **APC**, antigen-presenting cells; **ITAM**, immunoreceptor tyrosine-based activation motif; **CTLD**, C-type lectin-like domain; **NK**, natural killer; **NKC**, natural killer complex; **TNF**, tumor necrosis factor; **PCR**, polymerase chain reaction; **IPTG**, isopropyl-beta-D-thiogalactopyranoside; **LB**, luria-bertani; **CFP**, cyan fluorescence protein; **YFP**, yellow fluorescent protein; **EDTA**, ethylenediaminetetraacetic acid; **FACS**, fluorescence-activated cell sorting; **NCBI**, national center for biotechnology information; **EtBr**, ethidium bromide.

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