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Research article

Purification and characterization of a sialic Acid specific bacterial lectin from *Corynebacterium amycolatum* and assigning its role in Keratitis

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Abstract

A sialic acid-specific bacterial lectin, CAL from *Corynebacterium amycolatum*, isolated from the corneal smears of a keratitis patient was purified by 78-fold with a combination of anion exchange chromatography using DEAE-cellulose and affinity chromatography on fetuin coupled Sepharose 4B column with an overall recovery of 45%. The purified lectin is a monomer and showed apparent molecular mass of 19.7 kDa as elucidated by SDS-PAGE both under reducing and non reducing conditions. CAL is human blood group non specific lectin which agglutinates trypsinised and neuraminidase treated human and rabbit erythrocytes. CAL is an N-acetyl neuraminic acid (NANA) specific lectin with least MIC but it also recognizes Fetuin, Mucin, Glucose, Galactose and Lactose. CAL showed strong binding to HCECs which could be blocked by NANA and mucin. CAL showed mitogenic effect on HCECs at concentrations below 0.3 µg/mL leading to expression of proinflammatory cytokines IL-6 and IL-8. In contrast, CAL showed growth inhibitory effects on HCECs above 0.6 µg/mL concentrations at 48h with an IC_{50} of 1.2 µg/mL. Annexin- V/PI assay results shows that, at higher concentration, CAL induced apoptotic effect in HCECs with an increase in early and late apoptotic population. These findings demonstrate that CAL has a significant role in mediating host pathogen interactions eventually leading to pathogenesis and hence are of clinical significance in developing strategy in controlling the infection or bacterial keratitis.

Keywords: *Corynebacterium amycolatum Lectin; Bacterial Keratitis; Sialic Acid Specific; Proinflammatory; Apoptosis*

Introduction

Microbial keratitis, a predominant cause of corneal blindness is caused by a variety of microbes including fungi, protozoa, virus and predominantly bacteria and is regarded as one of the major health problem causing corneal morbidity worldwide [1]. Bacterial keratitis is the second common cause of corneal blindness worldwide which varies with geographical region and mostly seen in Western and developing countries. Several Gram negative bacteria like *Neisseria gonorrhea, Pseudomonas aeruginosa, Chlamydia trachomatis* and Gram positive bacteria like *Staphylococcus aureus, Streptococcus pneumonia* are implicated in causing bacterial keratitis [2]. In some cases *Corynebacterium* and *Staphylococcus* species were also being reported to cause bacterial keratitis [3]. Several components like endotoxins, lipases, collagenases, proteinases produced by these causative bacteria help in adhesion and evading the host tissues leading to pathogenesis [2].

Corneal blindness is a major public health problem worldwide and infectious keratitis is one of the predominant causes for it, which can be preventable [1,4]. Bacterial keratitis is a disease which is a resultant of an acute or chronic, transient infection of the cornea with varying predilection for different parts of the cornea. Different pathogens like bacteria, fungi, viruses and protozoa can infect the cornea, but amongst them bacteria top the list in causing microbial keratitis. Several studies have shown that bacterial keratitis can be caused by several groups of bacteria [5]. Bacterial keratitis results in corneal opacifications, which is the regarded as second common cause of blindness world-wide after cataracts [6]. Most microorganisms isolated from patients with bacterial keratitis are; coagulase-negative *Staphylococci,* followed by *Pseudomonas aeruginosa, Corynebacterium s*pp., *Staphylococcus aureus* and *Streptococcus* species [7]. Non-diphtheritic Corynebacteria are now being increasingly identified as the causative agents of neumorous infections. Among these, *Corynebacterium amycolatum* is the most commonly isolated gram positive bacilli arranged in palisades. It has been isolated from urine, pus, catheter tips, blood, prostatic secretion, cerebrospinal fluid and sputum. *Corynebacterium amycolatum* is known to be associated with septicaemia, endocarditis, meningitis, septic arthritis and other urinary tract infections [8].

The colonization of bacteria to human epithelial cells is an important phenomenon in the process of pathogenesis and is regarded as one of the most important step in host pathogen interactions [9]. In bacterial infections, adhesion of the pathogen to epithelial surfaces for colonization in the mucosal surfaces is regarded as the primary step to initiation of the infection. Bacteria are known to use many surface components in this mechanism and many molecules have been documented so far, which are known to play a significant role in adhesion or initiating process of infection leading to profound immunological response in the host. Adherence of the bacteria to host cells protects them from being swept away by the normal cleansing mechanisms operating on mucosal surfaces thereby increasing their ability to colonize, multiply and invade the host. Glycoproteins and glycolipids have been delineated to play significant role in the binding of bacteria to receptors on host epithelial cells [10]. Disease causing pathogens such as bacteria, fungi and viruses adhere to the host cells through the carbohydrates and elicit the pathogenesis where lectins secreted by these organisms play important role in the process of adhesion [10]. The specificity, affinity and concentrations of the interacting molecules will determine the degree of success of the bacterial colonization. Many surface proteins synthesized by bacteria are known to elicit many signaling mechanisms leading to inflammatory responses. For example succinate dehydrogenase (SDH), an enzyme synthesized by *Streptococcus pyogenes* also known as Group A Streptococci causes acute pharyngitis in humans by activating protein kinase C in pharangeal cells leading to the cellular communication between Streptococci and pharyngeal cells [11]. Bundle-forming pili (BFP) are well-studied adhesion molecules from *Aeromonas veronii* and *Aeromonas caviae* which are important intestinal pathogens [12]. Bacterial colonization to human host by pathogenic bacteria like *Neisseria gonorrhoeae* and *Neisseria meningitides* are mediated by virulence proteins or pili results in the development of systemic inflammatory responses [13]. LipoPolysaccharides (LPS) produced by many gram-negative bacteria such as *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa* are main virulence factors and are the primary adhesion molecules to human epithelial cells [14].

Lectins are group of proteins/glycoproteins of non immune origin which selectively and reversibly bind to sugars on glycoproteins in free form and also on the cell surfaces like RBCs [15]. Lectins are ubiquitous in nature and can be isolated from all groups of organisms such as bacteria, fungi, plants and animals [15]. Carbohydrate binding proteins from bacteria, such as lectins, adhesins or toxins have the capacity to specifically recognize complex oligosaccharides present on host tissues [16]. Due to their distinctive glycan binding specificity lectins have been implicated to play significant role in mediating host pathogen interactions [17]. Lectins have different affinities for diverse sugars including sialic acid and because of which they find immense applications. Sialic acids are regarded as unique sugars that generally occupy the terminal position of the glycan chains and get altered by external factors, such as microbial pathogens, or during peculiar physiological cellular events such as neural cell growth and embryogenesis. In the majority of these processes, sialic acid acts as a ligand for specific sialic acid-binding lectins reported from pathogenic microbes [18].

Pseudomonas aeruginosa an opportunistic bacterium which is known for causing many acute and chronic infections in humans and colonizing vital organs namely lungs, kidney, urinary tracts and implicated in major diseases like keratitis, cystic fibrosis and necrotizing

enterocolitis [19]. LecA and LecB are respectively galactose and fucose specific lectins produced by *Pseudomonas aeruginosa* and have been shown to be involved in adhesion and initiating pathogenesis in lung infection using an *in vitro* model with A549 cells [20]. Acanthamoeba keratitis is a form of microbial keratitis caused by species of several free-living amoebae of the genus Acanthamoeba. Lectin-glycoprotein interaction is regarded as most important biological process in initiating pathogenesis in corneal epithelial cells leading to keratitis [21].

In the present study we report the purification and characterization of a sialic acid specific lectin from *Corynebacterium amycolatum* isolated from bacterial keratitis patient using anion ion exchange chromatography followed by affinity chromatography. Further interaction of CAL was studied with immortalized Human Corneal Epithelial Cells (HCECs) *in vitro* for investigating the role of lectin in mediating host pathogen interactions. The results s are of clinical significance to develop a strategy for controlling the infection leading to bacterial keratitis.

Materials and Methods

Mucin from porcine stomach, type III, fetuin (fetal calf serum), N-acetyl neuraminic acid, Cyanogen bromide, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide), Amphicillin G-100 Chemical Co, St. Louis, USA. Sepharose 4B was procured from Pharmacia Fine Chemicals, Uppsala, Sweden. Standard protein molecular weight marker was procured from Merck, India. Amicon Ultrafiltration centrifugation tubes 10 kDa were procured from Millipore, USA. Human blood samples were collected from German hospital, Dharwad (India), Dulbecco's Modified Eagle's Medium F12 (DMEM F12) and foetal bovine serum (FBS) were obtained from GibcoInvitrogen (Paisley, UK), with Costar 96-well plates obtained from Corning Incorporate (Corning NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Bovine serum albumin (BSA), Fluorescein isothiocyante(FITC), 3,3′,5,5′-Tetramethylbenzidine (TMB) were obtained from Sigma Chemical Co.(St. Louis MO, USA). Annexin V/PI apoptosis detection kit was from Biovision (Milpitas CA, USA). ELISA kits for IL-6 (#MBS261259) and IL-8 (#MBS2886709) were from My Biosources. All other reagents used were of analytical grade. Asialofetuin used for hapten inhibition studies was prepared from fetuin as described by Spiro and Bhoyroo [22].

Bacteria and media

Corynebacterium amycolatum was isolated from the eye scrapings of keratitis patient with institutional ethical clearance from L V Prasad Eye Hospital and research Institute, Hyderabad, India and culture characterization has been done in Microbiology laboratory LVPEI Hospital Hyderabad. Strains were maintained on L B agar plates containing Agar concentrate 1.7% (w/v), stored at 4 ± 1ºC until further use. A loop full of bacteria from the mother culture was inoculated on to freshly prepared L B (Luria-Bertani) broth under sterile conditions. Inoculum from 3 day old cultures from L B were used and inoculated into 500ml Erlenmeyer's flasks and incubated for 48 h at 37°C under agitation condition at 120 rpm. The strain expressed high amount of cell-associated hemagglutinating lectin titer with human erythrocytes which were grown in L B broth.

Purification and characterization of CAL

Bacterial cells were cultured and harvested at 48 h post incubation to obtain pellet by centrifugation at 10,000 rpm for 20 mints at 4ºC, washed with PBS, phosphate buffered saline (0.05 M, pH 7.2) and pressed dry using muslin cloth. Lectin was extracted in PBS by sonicating bacterial pellet at a frequency of 20 kHz and samples were maintained on ice. After sonication crude extract was collected by centrifugation (10,000 rpm for 20 mints at 4ºC) and checked for the presence of lectins/adhesins. The crude extract was subjected for ammonium sulphate precipitation (70%) and kept for 1 - 2 hrs at 4°C. The precipitates obtained was centrifuged at 10,000 rpm for 20 min, 4°C and dissolved in PBS and dialyzed against the same buffer.

The dialysate was loaded onto DEAE-cellulose anion exchange column (1.5×20 cm) equilibrated in PBS. Proteins bound to the column were eluted by using a salt gradient of 150 to 700 mM sodium chloride in PBS. The eluted factions were subjected for extensive dialysis using 50 mM PBS pH 7.2. The eluted major protein peak after concentration was loaded on to fetuin-Sepharose 4B affinity column pre-equilibrated with PBS. Unbound proteins from the coloumn were washed with PBS till absorbance of eluting fractions read zero at 280 nm. Affinity bound protein was eluted with 100mM Glycine-HCl buffer pH 2.0, containing 500 mM NaCl. Fractions with high lectin activity were pooled and dialyzed extensively against PBS, pH 7.1. Lectin-positive fractions were concentrated in Amicon Ultrafiltration centrifugation tubes (10 kDa) and stored at - 20 °C for further use. All purification steps were carried out in a cold room at 4°C. At each step of purification, lectin activity was estimated by haemagglutination assay.

Preparation of trypsin and neuraminidase treated erythrocytes

Trypsinized and neuraminidase treated human blood erythrocytes required for the determination of hemagglutination assay were prepared as described by E. Liener 1953 [23].

Hemagglutination assay

Hemagglutination activity of CAL during various stages of purification was determined by the serial two fold dilution method using trypsinized and neuraminidase treated human erythrocytes in 96 well, "U" bottom micro titer plates using a method developed by E. Liener 1953 [23]. The highest dilution of the extract causing visible hemagglutination was regarded as the titer and the minimum concentration of the protein required for agglutination (MCA) as one unit of hemagglutinating activity. The specific hemagglutination activity was expressed as unit mg^{-1}protein.

Hapten Inhibition studies

The sugar specificity of the purified lectin was determined by a hapten inhibition assay. Inhibition assays were carried out by incubating the lectin sample (with titer of 4) with serially diluted sugar/glycoprotein in a total volume of 50 uL prior to the addition of 50 uL of erythrocytes in PBS and the hemagglutination was visually observed. The minimum concentration of the sugar/glycoprotein required for complete inhibition was taken as the inhibitory titer of the hapten (MIC).

Estimation of protein

Protein concentrations in the extract at different stages of purification was determined by the modified method of Lowry., *et al*. 1951 [24] using DC protein estimation kit from BioRad using Bovine serum albumin (1mg/mL) as a standard.

SDS PAGE

SDS-PAGE of CAL and crude extracts was carried out using 15% gel as described by Laemmli 1970 [25]. Molecular mass of the purified CAL was determined by comparison of its electrophoretic mobility with those of standard molecular weight marker proteins both under reducing and non-reducing conditions. The gel was stained with both Coomassie brilliant blue and by silver staining.

Effect of temperature and pH on CAL

In order to determine the thermo stability of CAL, CAL (200 µg/mL) in PBS pH - 7.2 was incubated at different temperatures (0°C to 90°C) for 20 min in temperature controlled water bath. The stability of the CAL at various pH was determined by evaluating the hemagglutination activity of the purified lectin incubated in various buffers of different pH between 2.0 to 11.0. CAL (200µg/mL) was incubated with an equal volume of buffers (100 mM) of pH 2.0 (Glycine-HCl), pH 4.3 (Acetate buffer), pH 7.0-8.0 (Tris-HCl buffer) and pH 9.0 - 11.0

(Carbonate buffer) for 24h at 4°C. After incubation, the lectin samples were neutralized to pH 7.0 by adding 0.1N NaOH or 0.1N HCl before testing hemagglutination activity.

FITC conjugation of lectin

Conjugation of CAL to FITC was performed as described by Goldman, 1968 [26]. Briefly, CAL (5 µg/mL) was incubated with FITC at 25 µg/mL of protein in carbonate buffer (0.05M, pH 9.5) with gentle stirring overnight at 4°C. Excess, unbound FITC was washed off by extensive dialysis against phosphate-buffered saline with pH 7.2, and FITC-conjugated CAL was kept at 4°C until further use.

Cell culture

Immortalized human corneal epithelial cells (HCECs) were procured from ATCC and were grown and maintained in DMEM/F-12 medium (Invitrogen) containing 10% FCS, at 37 ºC with 5% CO_2 and used for the study.

Binding of CAL to immortalized HCECs by flow cytometry

HCECs (0.2 x 10^6) were treated with 3% BSA in PBS for 1 h at 4ºC for blocking the non-specific binding sites. HCECs were washed and treated with lectin, CAL and CAL-hapten mix prepared earlier and allowed to bind for 1 h at 4ºC. Lectin-hapten mix required for inhibition studies was prepared by incubating purified lectin (5 µg/mL) with competing happen NANA (200 mM) and mucin (100 µg/mL) in PBS for 1 h at 37ºC. After incubation, HCECs were washed twice with PBS and further processed for flow cytometry using Beckman Coulter FC500. Data were obtained for 10000 events using untreated cells as control and analyzed using CXP Software.

Effect of CAL on HCECs viability by MTT assay

To study the effect of CAL on HCECs viability, cells were seeded in 96-well plates (5 × 10^4 cells/mL) and grown in complete DMEM F12 medium for 48h prior to lectin treatment. After 48h medium was replaced with serum-free DMEM F12 and then treated with CAL at different concentrations (0.03 - 5.0 µg/mL) and maintained in humidified atmosphere (37°C, 5% CO_2) for 24h and 48h. At each time point, 50 µL of MTT (5 mg/mL) was added to each well followed by lysis with 100µL dimethyl sulfoxide (DMSO). To notice the effects of competing glycan or hapten, CAL (1.5 µg/mL) was pre-incubated for 1h with NANA (200 mM), before addition to cells and processed for MTT assay at 24h and 48h as mentioned above. Cell viability was quantified by measuring absorbance at 570nm using a spectrophotometer. Percentage of viable cells was calculated, by comparing to lectin untreated controls considered as 100%.

ELISA for quantification of interleukins secreted in HCECs by CAL at lower concentration

In order to monitor the expression of IL-6 and IL-8 in immortalized HCECs following CAL treatment for 24 and 48 h, the spent media from lectin-treated cells were collected and further used for the quantification of ILs. IL-6 and IL-8 secreted in the spent media were estimated by following the manufacturer's instructions using MyBioSources ELISA kit using different dilutions of the spent media. Spent media from the untreated cells were used as negative control. Data was represented as picogram of IL-6 and IL-8 per 100 µl of spent media.

Assessment of CAL-mediated cellular apoptosis in immortalized HCECs at higher concentration

To ascertain the noticed growth inhibitory effect of CAL in HCECs at higher concentrations is because of apoptosis, Annexin V/PI assay was carried out and FITC-annexin V staining was used for monitoring phosphatidylserine externalization which is a characteristic feature of cellular apoptosis. HCECs (5 × 10^5 cells/well) in 35 mm petri dish treated with CAL (1.5 µg/mL) for 24 and 48h were harvested by gentle trypsinization and resuspended in binding buffer. In short, cells were incubated with 5 µL FITC-AnnexinV and 5 µL PI for 15 min

at 37°C in the dark, before analysis by flowcytometry. The percentage of cells positive for Annexin V, PI alone and both Annexin V and PI were calculated by dot plot analysis using CXP analysis software version 1.2, (Beckman Coulter).

Statistical analysis

Results were expressed as mean ± SD. Statistical comparisons were achieved using the Student's t-test in order to calculate statistical significance. Microsoft Excel was used to perform statistical analysis.

Results

Purification of CAL

Purification of CAL, from 70% ammonium sulphate precipitate of the crude extract performed by anion exchange chromatography on DEAE-cellulose resulted in 23.8 fold purification (Figure 1). Affinity chromatography of DEAE eluted major peak on fetuin coupled Sepharose-4B column resulted in purification of CAL by 48.5 fold (Figure 2 and Table 1) indicating the efficiency of the protocol developed. The fold purification and the% recovery of the lectin purified from 10 g of bacterial pellet and are summarized in table 1.

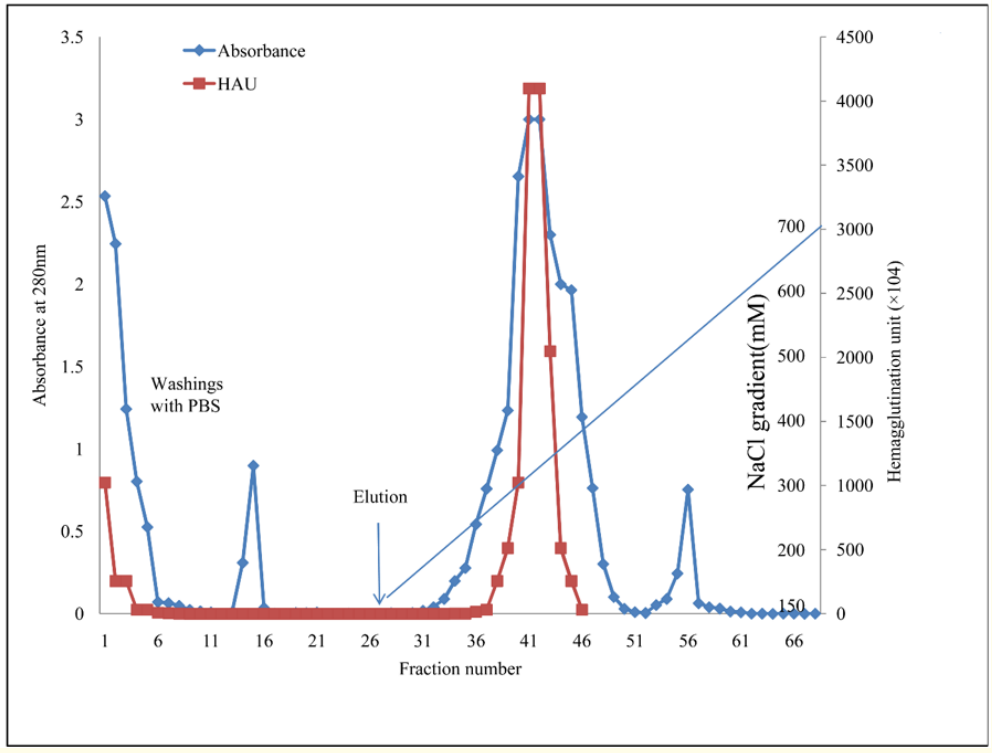

Figure 1: *Partial purification of Corynebacterium amycolatum lectin (CAL) by Ion exchange chromatography: Partial purification of CAL by Ion exchange chromatography using DEAE-Cellulose column (1.5×20 cm) equilibrated in PBS. Bound lectin was eluted with a salt gradient of 150 mM to 700 mM of NaCl in PBS, pH 7.2. Fractions of 3.0 ml were collected at flow rate of 30 ml/h.*

Sample	Volume (ml)	Total protein (mg)	MCA[a] (µg)	Specific activity[b] (units)	Total activity[c] (units)	Fold purification	%Recovery of activity
Crude	80	384	0.468	2.1×10^3	2.0×10^5	-	100
Ammonium sulphate ppt	12	60	0.39	2.4×10^3	1.42×10^4	1.14	61
IEC	20	23	0.0158	5.0×10^4	1.4×10^4	23.8	54
Affinity purified	0.9	0.9	0.00195	5.1×10^5	1.2×10^4	48.5	50

Table 1: *Summary of purification of Corynebacterium amycolatum lectin.*

Wet pellet weight: ~10g.

a - Minimum concentration of protein required to agglutinate human 'B' erythrocytes.

b - Specific activity: hemagglutinating activity/ mg protein.

c -Total activity: hemagglutinating activity of lectin in total protein.

Hemagglutination and hapten inhibition assay

CAL showed hemagglutination activity with trypsinized and as well as with neuraminidase treated human A, B, AB, O and rabbit erythrocytes. There were no significant differences in terms of heamagglutination with trypsinized and as well as with neuraminidase treated human erythrocytes. Hapten inhibition studies showed that the hemagglutinating activity of CAL was inhibited strongly by NANA compared to fetuin and mucin but L-fucose, galactose, lactose, glucose, N-acetyl galactosamine and N-acetyl glucosamine also showed inhibition but with higher MICs. CAL has complex sugar specificity and inhibited by glycoproteins like fetuin and mucin (Table 2). NANA, fetuin and mucin showed highest inhibitory effect with MIC - 1.56 µg/50 µl for NANA and 3.125 µg/50 µl for fetuin and mucin compared to galactose (MIC - 11.5 µg/50 µl), N-acetylglucosamine, N-acetylgalactosamine (MIC - 6.25 µg/50 µl), lactose, glucose (MIC - 11.5µg/50 µl) and L-fucose (MIC - 25 µg/50 µl).The carbohydrate binding specificity of *Corynebacterium amycolatum* lectin (CAL) was determined by hemagglutination assay using simple sugars, sugar derivatives (200 mM) and glycoproteins (1mg/ml).

Hapten Tested	Minimum Concentration Required for Inhibition in µg (MIC)
Simple sugars	
D-Galactose	12.5
L-Fucose	25
D-Fucose	Nil
Lactose	12.5
Glucose	12.5
Mannose	Nil
Sugar derivatives	
N-Acetyl Neuraminic acid	1.562
N-Acetyl D-Galactosamine	6.25
N-Acetyl D-Glucosamine	6.25
Glycoproteins	
Fetuin	3.125
Asialofetuin	Nil
Mucin	3.125
Asialomucin	Nil

Table 2: *Hapten inhibition studies of Corynebacterium amycolatum lectin.*

3.3 Estimation of molecular mass of CAL by SDS-PAGE

CAL eluted from fetuin Sepharose-4B column was found to be homogenous as revealed by a single band on SDS-PAGE under both reduced and non-reduced conditions with a subunit molecular mass of 19.7 kDa (Figure 2; b, c). CAL is a monomer with Mr of 19.7 kDa as revealed by treatment of lectin at reducing conditions (Figure 2, b; lane 6, c). Silver staining of CAL further confirms the homogeneity of the lectin (Figure 2, c).

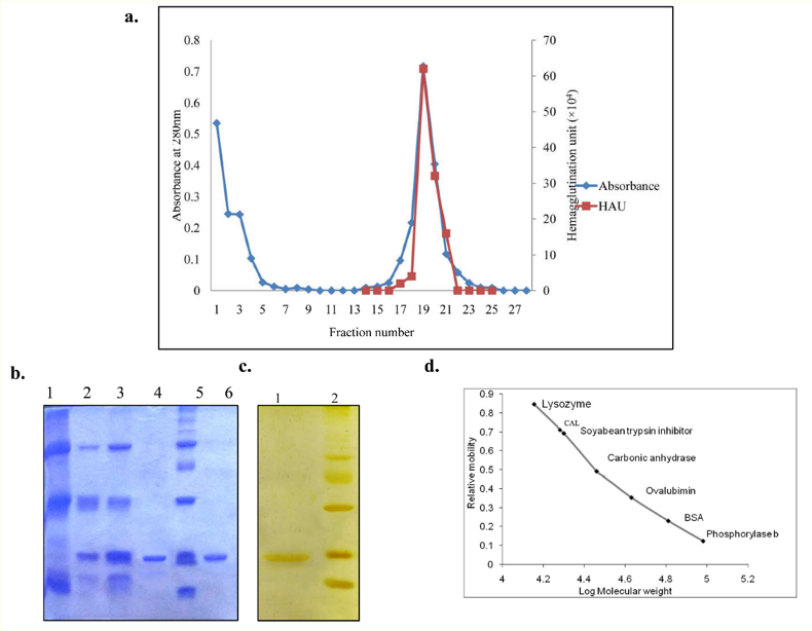

Figure 2: *Purification of Corynebacterium amycolatum lectin (CAL) by affinity chromatography.*

a. Purification of CAL by affinity chromatography using Fetuin-Sepharose-4B column (1.5×10 cm) equilibrated in PBS. Ion exchange eluted major peak (Peak II) was subjected to affinity chromatography on Fetuin-Sepharose-4B column. Column was washed with PBS till absorbance was read zero. Bound lectin was eluted with 100 mM Glycine-HCl buffer, pH 2.0 containing 500 mM NaCl. Fractions of 3.0 ml were collected at flow rate of 15 ml/h.

b. SDS PAGE of CAL in 15% gel: A. Gel stained with Coomassie brilliant blue, lane 1- Crude extract, lane 2- Ammonium sulphate Ppt, lane 3- IEC eluted peak II fraction, lane 4- Affinity purified CAL(non-reducing), lane 5- Standard protein molecular weight markers, lane 6- CAL (reducing).

c. Silver staining of the gel: Lane 1- CAL under non-reducing conditions lane 2- Standard protein molecular weight marker.

d. Calibration curve for the determination of molecular mass of CAL by SDS PAGE: X-axis relative mobility and Y-axis log molecular weight of protein markers; phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor

Effect of pH and temperature on CAL activity

Purified CAL showed optimum lectin activity at neutral pH 7.2. Lectin activity was found to be stable over a wide range of from pH 4.0 to 10.0 with complete loss of activity at pH 11.0. CAL is thermo stable up to 0 - 70°C. Lectin activity was completely lost at higher temperatures after 90ºC.

CAL strongly binds to HCECs

Interaction of CAL with HCECs was determined by staining cells with FITC-conjugated lectin followed by flowcytometric analysis. A total of 98.23% HCECs showed positive staining for CAL with mean fluorescence intensity (MFI) of 518, compared to unstained cells with MFI of 0.539 (Figure 3 A1). Carbohydrate-mediated interaction of CAL to HCECs was confirmed by preincubating CAL with competing sugars/glycoprotein, which resulted in significant reduction in cell-surface binding of lectin to HCECs. For HCECs stained with FITC-CAL, MFI decreased to 15.7 and 28.6 in presence of NANA and mucin respectively (Figure 3 A2).

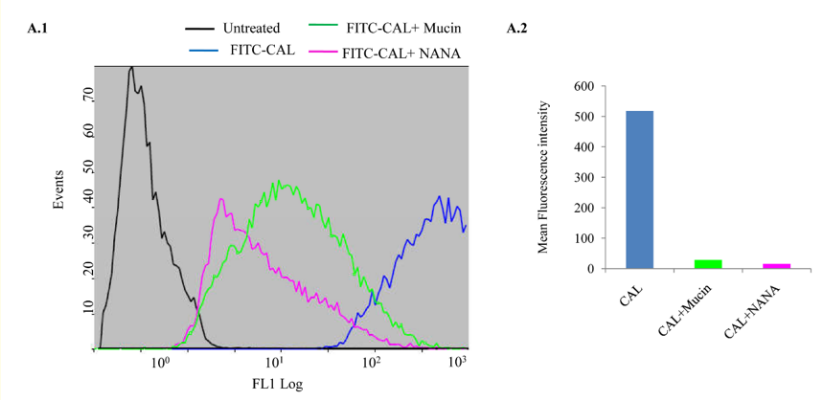

Figure 3: *Binding of Corynebacterium amycolatum lectin to HCECs.*

Binding of CAL by incubating FITC-conjugated CAL with HCECs in absence (blue line) or presence of competing sugar (NANA; pink) or glycoconjugate (mucin; green line), and cells untreated with lectin (black line). Binding analyzed by flow cytometry; the overlays are representative data with X-axis and Y-axis expressed as mean fluorescence intensity (MFI) and cell count, respectively. Panel A2. represents inhibition of binding (decrease in MFI) in presence or absence of competing glycans, NANA (pink bar) or mucin (green bar) compared to FITC-conjugated lectin alone (blue bar).

(20.1 kDa), lysozyme (14.3 kDa).

Effect of CAL on HCECs growth (proliferative/ anti proliferative)

At lower concentrations below 0.6µg/mL, CAL increased the viability of HCECs in a dose- and time-dependent manner. At concentrations of 0.3µg/mL viability was increased by 127.3 ± 0.8% (n = 9, $p < 0.005$), at 0.15 µg/mL by 144.6 ± 0.1% (n = 9, $p < 0.005$) and at 0.07µg/mL by 156.6 ± 0.8% (n = 9, $p < 0.005$), 0.03 µg/mL by 166 ± 0.3% (n = 9, $p < 0.05$). Maximum proliferation of HCECs was seen at 0.03 µg/mL of CAL which resulted in an increase of HCECs population to 166% at 48h post lectin treatment. Similarly, at higher concentrations i.e. more than 0.6 µg/mL of CAL significantly inhibited HCECs growth in a dose- and time-dependent manner (Figure 4). Maximum reduction in cell viability was seen at 5 µg/mL lectin over 48h, with CAL inhibiting cellular growth of HCECs at concentrations of 1.25 µg/mL by 54.9 ± 1.2% (n = 9, $p < 0.001$), 1.5 µg/mL by 64.7 ± 1.8% (n = 9, $p < 0.001$) and at 5 µg/mL by 77.0 ± 0.9% (n = 9, $p < 0.001$). Carbohydrate mediated effect of CAL on HCECs was evident and effectively blocked by 98% in presence of competing sugar mucin. IC_{50} value of CAL for HCECs was found to be 1.15 µg/mL at 48h. These results show that effect of CAL on HCECs growth is mediated by binding to cell surface glycans specific to CAL.

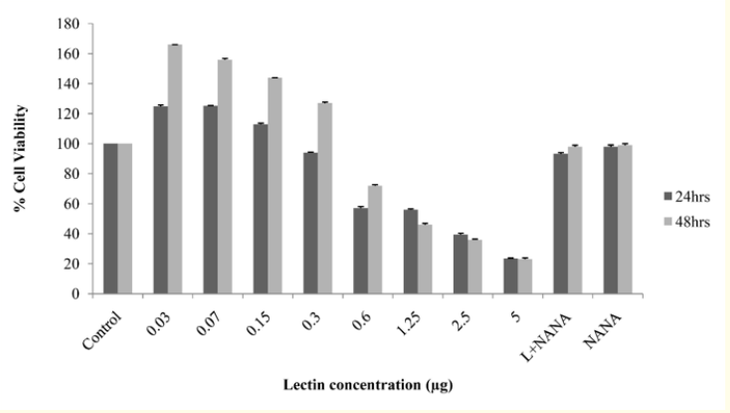

Figure 4: *CAL stimulates cell proliferation at lower concentrations and inhibits HCECs growth at higher concentrations: Immortalized HCECs seeded in 96-well plates (5× 104cells/mL) were grown in complete DMEM F12 medium for 48h prior to lectin treatment. Medium was replaced with serum-free DMEM F12 and treated with CAL at different concentrations (0.03–5µg/mL) for 24h and 48h and processed for MTT assay. Data is expressed as % cell viability compared to untreated controls (100%). NANA at 200 mM/mL, effectively blocked CAL mediated inhibition of growth (2.5µg/mL). Data expressed as mean ± SD of three independent experiments.*

CAL elicits pro-inflammatory response in HCECs by inducing interleukin secretion

In order to assess the pro-inflammatory effects induced in HCECs upon CAL treatment, the spent media was collected at different time intervals and tested for the expression of inflammatory interleukins after lectin treatment. IL-6 (Figure 5. A1) and IL-8 (Figure 5 A2) secretion in HCECs spent media were quantified and data are depicted as pg of IL released per 100 µl of spent media. When treated with 0.07 µg/mL of CAL, HCECs induced maximum secretion of IL6 viz. 992 ± 14.3 pg ($n = 6$, $p < 0.001$). When treated with 0.15 µg/mL and 0.3 µg/mL of CAL, IL6 levels decreased to 664 ± 7.23 pg and 460 ± 16.07 pg, respectively ($n = 6$, $p < 0.05$). At the same concentrations, IL8 was secreted in HCECs when treated with maximal concentrations of CAL i.e 0.07 µg/mL viz 998 ± 18.06 pg ($n = 6$, $p < 0.01$) whereas when treated with 0.15 µg/mL and 0.3µg/mL of CAL, IL8 levels decreased to 694 ± 16.12 pg and 470 ± 11.23 pg, respectively ($n = 6$, $p < 0.05$). These results demonstrated that CAL indeed elicits proinflammatory effect in HCECs eventually leading to pathogenesis.

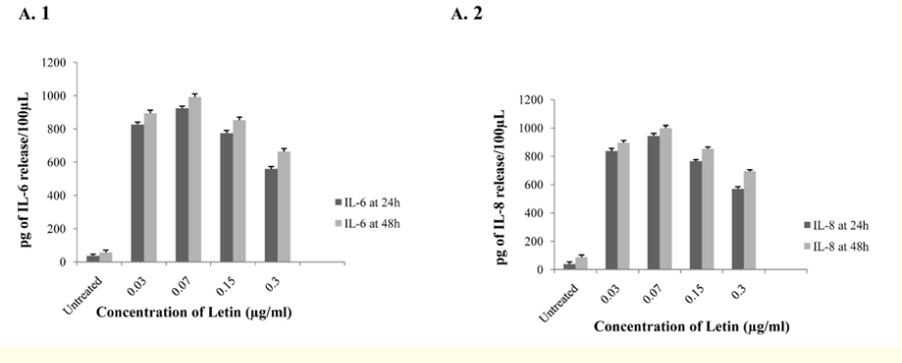

Figure 5: *CAL induces secretion of pro-inflammatory Cytokines IL-6 and -8 in HCECs.*
HCECs treated with different concentrations (0.3- 0.03 µg/mL) of CAL for 24 h, 48h, and spent media was used to quantify the release of IL6 (A) and IL8 (B) by CAL using ELISA kits following the manufacturer's instructions. Data are presented as pg of IL6 or IL8 release per 100 ul of spent media. Data represent mean ± SD of triplicate determinations from two different assessments. (P<0.05 was considered significant).

CAL induces apoptosis in HCECs at higher concentrations

To determine whether the observed growth inhibitory effect of CAL on HCECs at higher doses was due to the induction of apoptosis, cells were analyzed for phosphatidylserine externalization by staining cells with Annexin-V/PI. HCECs treated with 2.5 µg/mL of CAL showed 56.1 and 61.5% of the cell population in the early phase of apoptosis at 24h and 48h respectively, compared to untreated control cells with 0.1 and 0.6% at 24h and 48h respectively; Similarly, CAL also increased numbers of cells in the late apoptotic phase 19.5% and 23.2% (Figure 6). Significant increase in early and late apoptotic population supports induction of apoptosis in both HCECs by CAL.

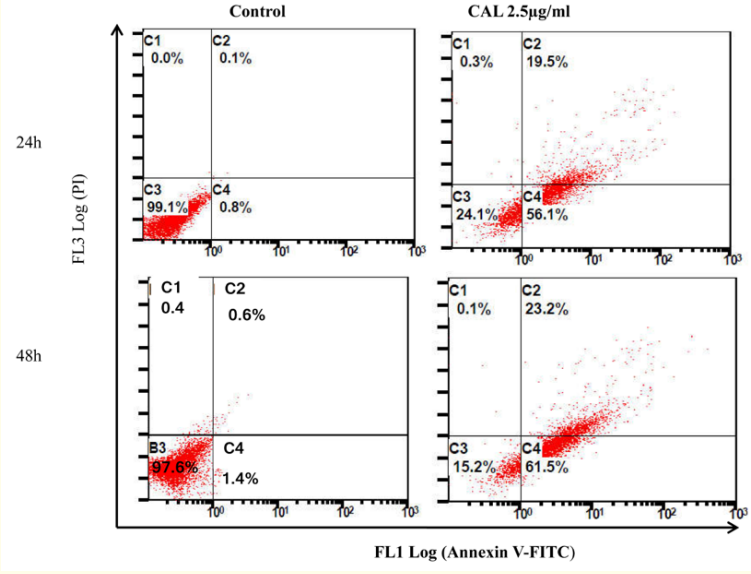

Figure 6: *CAL induces apoptosis in HCECs at higher concentrations.*

HCECs treated with CAL (2.5ug/mL) were analyzed by Annexin V/propidium iodide (PI) staining to monitor CAL induced apoptosis by flow cytometry. HCECs (5 × 105 cells/well) in 35 mm petri dish were treated with CAL (2.5µg/mL) for 24 and 48h and harvested by gentle trypsinization and processed for Annexin V/PI staining following manufactures instructions (Biovision). X-axis depicts Annexin V positive cells and Y-axis depicts PI-positive cells. C1, C2, C3 and C4 quadrants represent the necrotic, late apoptotic, normal and early apoptotic cell populations respectively, with numbers indicating the percentage number of viable, early apoptotic, late apoptotic, and necrotic cells that are present in the respective quadrants.

Discussion

A sialic acid specific lectin from pathogenic bacterium *Corynebacterium amycolatum* isolated from corneal smears of a keratitis patient was purified by combination of ion exchange and affinity chromatography using DEAE-cellulose and fetuin coupled Sepharose 4B affinity columns. The purified lectin, CAL has a molecular mass of 19.7 kDa as revealed by SDS–PAGE both under reducing and non reducing conditions. CAL is a human blood group non specific lectin and also agglutinates rabbit erythrocytes. CAL is heat stable up to 70°C with in pH range of 4.0 to 10.0. In order to understand the role of lectin, CAL in mediating host pathogen interactions leading to bacterial keratitis, its interaction was studied with immortalized HCECs *in vitro* by using different assays. CAL showed proliferation/mitogenic response at lower concentrations and inhibitory/apoptotic effect at higher concentrations. Further, CAL also induced the secretion of proinflamma-

tory cytokines/chemokines IL-6, 8 at lower concentration in a time dependent manner. On the contrary, at higher concentration, CAL induced apoptosis in HCECs as identified by Annexin V/PI assay.

Several bacteria have been reported to use sialic acid-containing glycans as ligands for lectins or adhesins although the identity of the specific adhesin remains uncertain in many cases [27]. Sialic acid specific lectins/adhesins have been reported from pathogenic bacteria like *Escherichia coli* and *Vibrio cholerae* which play important role in bacterial colonization and causing virulence in host cells [28]. *Psuedomonas aeruginosa* produces two types of lectin proteins namely, Lec A (galactose-specific) and Lec B (fucose-specific), which are involved in *Psuedomonas aeruginosa* virulence and biofilm formation respectively [29,30]. Non-fimbrial surface proteins or adhesins specific to NANA has been reported from pathogenic bacterium *Corynebacterium diphtheria,* however information on its involvement in pathogenicity is not known [31]. Wheat Germ Agglutinin is one of the extensively studied sialic acid specific lectin from *Triticum vulgaris* which is purified by conventional methods, like ion exchange column chromatography [32]. Purification of CAL was also performed by using ammonium sulfate precipitation, ion exchange and affinity chromatography which is found to be an effective protocol. Another example is N-acetyl neuraminic specific lectin from the bacterium *Bacillus subtilis* B-7025 purified by ammonium sulfate precipitation, gel filtration chromatography on Sepharose CL-6B [33]. The sugar specificity, temperature and pH stability and molecular properties of CAL are comparable with other bacterial lectins reported such as *Escherichia coli* and *Vibrio cholerae* [28,31].

Pattern Recognition receptors (PRRs) present on the human corneal epithelial surface are known to play a crucial role in corneal defense mechanisms against microbial infections causing microbial keratitis (MK) [34]. Amongst PRRs, Toll-like (TLRs) and Nod-like receptors are major class of receptors which play important and foremost inflammatory responses against bacterial and fungal infections of cornea [35]. Corneal destruction is a characteristic feature of microbial keratitis which is mainly mediated by profound inflammatory responses by host immune system against pathogenic components resulting in blindness [34]. S*taphylococcus aureus* LP (saLP) is known to induce the expression of pro-inflammatory cytokines and chemokines like IL-6, 8 and ICAM-1 [36]. An Endosymbiotic *Wolbachia* bacterium which is implicated in bacterial keratitis through lipoproteins is known to induce the expression of IL-6 and IL-8 in cultured HCECs [37].

Pseudomonas aeruginosa induces signaling cascade which activates the transcription of CXCL1 and other CXC chemokine that in turn leads to the secretion of IL-1a and IL-1b subsequently leading to the activation of host neutrophils against the bacterial insult and killing the pathogen resulting in the tissue destruction [38].

Herpes stromal keratitis is also an important vision threatening condition which is caused by herpes simplex virus (HSV)-1 infection which is known for activating innate and adaptive immune responses of host cells leading to the activation of inflammatory cytokines and chemokines [39]. Expression of inflammatory cytokines like IL-6, 8 is a characteristic feature observed in many bacterial infections of the cornea leading to keratitis especially in *Pseudomonas aeuroginsa* infection [40]. The expression of pro inflammatory cytokines IL-6, 8 by pathogenic fungal lectins from *Cephalosporium curvulum* and *Aspergillus niger* in immortalized HCECs was recently reported while implicating the role of lectins in pathogenesis [41,42].

In contrast, when inflammatory response is profound/high against invading micro biomes it results in the destruction of host epithelial tissues mediated by different signaling mechanisms like apoptosis/necrosis [43]. At higher concentrations CAL induced the apoptosis in immortalized HCECs in a time dependent manner. As the infection persists/prolongs due to high bacterial load or replication which in turn increases lectins concentration and this high profound host immune response leads to the destruction of host epithelial cells. Hence at higher concentrations of CAL, probably when the bacterial load or concentration is high, it results in destruction or breaching the epithelial barrier resulting in bacterial keratitis.

Hence based on the present findings we implicate the involvement of CAL in pathogenesis or causing bacterial keratitis. The study is of clinical significance as it can be basis for developing therapeutic strategies in future in controlling infection by *cornebacterium amycolatum*.

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Conflict of Interest Statement

There is no conflict of interest declared by the authors.

Bibliography

1. Leck AK., *et al*. "Aetiology of suppurative corneal ulcers in Ghana and south India, and epidemiology of fungal keratitis". *British Journal of Ophthalmology* 86.11 (2002): 1211-1215.
2. Keay Lisa., *et al*. "Microbial keratitis: predisposing factors and morbidity". *Ophthalmology* 113.1 (2006): 109-116.
3. Funke Guido., *et al*. "Corynebacterium macginleyi has to date been isolated exclusively from conjunctival swabs". *Journal of Clinical Microbiology* 36.12 (1998): 3670-3673.
4. Gopinathan Usha., *et al*. "The epidemiological features and laboratory results of fungal keratitis: a 10-year review at a referral eye care center in South India". *Cornea* 21.6 (2002): 555-559.
5. Green Matthew., *et al*. "Risk factors and causative organisms in microbial keratitis". *Cornea* 27.1 (2008): 22-27.
6. Al-Mujaini Abdullah., *et al*. "Bacterial keratitis: perspective on epidemiology, clinico-pathogenesis, diagnosis and treatment". *Sultan Qaboos University Medical Journal* 9.2 (2009): 184.
7. Ly Cameron N., *et al*. "Bacteria commonly isolated from keratitis specimens retain antibiotic susceptibility to fluoroquinolones and gentamicin plus cephalothin". *Clinical and Experimental Ophthalmology* 34.1 (2006): 44-50.
8. Belmares Jaime., *et al*. "Corynebacteriumendocarditis species-specific risk factors and outcomes". *BMC Infectious Diseases* 7.1 (2007): 1-8.
9. Jones Garth W. "The attachment of bacteria to the surfaces of animal cells". *Microbial interactions. Springer, Boston, MA* (1977): 139-176.
10. Espinosa-Cantellano Martha and Adolfo Martinez-Palomo. "Pathogenesis of intestinal amebiasis: from molecules to disease". *Clinical Microbiology Reviews* 13.2 (2000): 318-331.
11. Pancholi Vijaykumar and Vincent A Fischetti. "Regulation of the phosphorylation of human pharyngeal cell proteins by group A streptococcal surface dehydrogenase: signal transduction between streptococci and pharyngeal cells". *The Journal of Experimental Medicine* 186.10 (1997): 1633-1643.
12. Kirov Sylvia M., *et al*. "Functional characterization of type IV pili expressed on diarrhea-associated isolates of Aeromonas species". *Infection and Immunity* 67.10 (1999): 5447-5454.

13. Kerr JR. "Cell adhesion molecules in the pathogenesis of and host defence against microbial infection". *Molecular Pathology* 52.4 (1999): 220.

14. Munford Robert S and Alan W Varley. "Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria". *PLoS Pathogens* 2.6 (2006): e67.

15. Lis Halina and Nathan Sharon. "Lectins: carbohydrate-specific proteins that mediate cellular recognition". *Chemical Reviews* 98.2 (1998): 637-674.

16. Schauer Roland. "Sialic acids: fascinating sugars in higher animals and man". *Zoology* 107.1 (2004): 49-64.

17. Wang Wen-Hung., *et al*. "The role of galectins in virus infection-A systemic literature review". *Journal of Microbiology, Immunology and Infection* 53.6 (2020): 925-935.

18. Varki Ajit. "Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins". *Nature* 446.7139 (2007): 1023-1029.

19. Baltch Aldona L and Raymond P Smith. "Pseudomonas aeruginosa: infections and treatment". *Pseudomonas Aeruginosa: Infections and Treatment* 12 (1994).

20. Chemani Chanez., *et al*. "Role of LecA and LecB lectins in Pseudomonas aeruginosa-induced lung injury and effect of carbohydrate ligands". *Infection and Immunity* 77.5 (2009): 2065-2075.

21. Clarke Daniel W and Jerry Y Niederkorn. "The pathophysiology of Acanthamoeba keratitis". *Trends in Parasitology* 22.4 (2006): 175-180.

22. Spiro Robert G and Vishnu D Bhoyroo. "Structure of the O-glycosidically linked carbohydrate units of fetuin". *Journal of Biological Chemistry* 249.18 (1974): 5704-5717.

23. Liener Irvin E and Eldon G Hill. "The effect of heat treatment of the nutritive value and hemagglutinating activity of soybean oil meal: one figure". *The Journal of Nutrition* 49.4 (1953): 609-620.

24. Classics Lowry O., *et al*. "Protein measurement with the Folin phenol reagent". *Journal of Biological Chemistry* 193.1 (1951): 265-275.

25. LAEMMLI Ulrich Karl. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". *Nature* 227.5259 (1970): 680-685.

26. Goldman Morris. "Fluorescent antibody methods". New York: Academic press (1968).

27. Angata Takashi and Ajit Varki. "Chemical diversity in the sialic acids and related α-keto acids: an evolutionary perspective". *Chemical Reviews* 102.2 (2002): 439-470.

28. Morschhäuser JOACHIM., *et al*. "Functional analysis of the sialic acid-binding adhesin SfaS of pathogenic Escherichia coli by site-specific mutagenesis". *Infection and Immunity* 58.7 (1990): 2133-2138.

29. Gilboa-Garber Nechama., *et al.* "Identification and characterization of Pseudomonas aeruginosa PA-IIL lectin gene and protein compared to PA-IL". *FEMS Immunology and Medical Microbiology* 29.1 (2000): 53-57.

30. Cioci Gianluca., *et al*. "Structural basis of calcium and galactose recognition by the lectin PA-IL of Pseudomonas aeruginosa". *FEBS Letters* 555.2 (2003): 297-301.

31. Colombo Andréa Vieira., *et al*. "Corynebacterium diphtheriae surface proteins as adhesins to human erythrocytes". *FEMS Microbiology Letters* 197.2 (2001): 235-239.

32. Nagata Yoshiho and Max M Burger. "Wheat germ agglutinin: molecular characteristics and specificity for sugar binding". *Journal of Biological Chemistry* 249.10 (1974): 3116-3122.

33. Lakhtin VM., *et al*. "Purification and some properties of extracellular sialic acid-specific lectin from Bacillus subtilis 316 M". *Prikladnaya Biokhimiya i Mikrobiologiya* 29 (1993): 389-397.

34. Taube MA., *et al*. "Pattern recognition receptors in microbial keratitis". *Eye* 29.11 (2015): 1399-1415.

35. Leal Jr Sixto M., *et al*. "Distinct roles for Dectin-1 and TLR4 in the pathogenesis of Aspergillus fumigatus keratitis". *PLoS Pathogens* 6.7 (2010): e1000976.

36. Li Qiong., *et al*. "Staphylococcus aureus lipoproteins trigger human corneal epithelial innate response through toll-like receptor-2". *Microbial Pathogenesis* 44.5 (2008): 426-434.

37. Liesegang Thomas J. "The role of endosymbiotic Wolbachia bacteria in the pathogenesis of river blindness". *American Journal of Ophthalmology* 134.2 (2002): 309.

38. Sun Yan., *et al*. "TLR4 and TLR5 on corneal macrophages regulate Pseudomonas aeruginosa keratitis by signaling through MyD88-dependent and-independent pathways". *The Journal of Immunology* 185.7 (2010): 4272-4283.

39. Wang Li., *et al*. "Pathogenesis of herpes stromal keratitis: immune inflammatory response mediated by inflammatory regulators". *Frontiers in Immunology* 11 (2020): 766.

40. Zhang Dekai., *et al*. "A toll-like receptor that prevents infection by uropathogenic bacteria". *Science* 303.5663 (2004): 1522-1526.

41. Ballal Suhas., *et al*. "Mitogenic lectins from Cephalosporium curvulum (CSL) and Aspergillus oryzae (AOL) mediate host–pathogen interactions leading to mycotic keratitis". *Molecular and Cellular Biochemistry* 434.1 (2017): 209-219.

42. Jagadeesh Narasimhappagari., *et al*. "Aspergillus niger lectin elicits MyD88 dependent proliferation and apoptosis at lower and higher doses in immortalized human corneal epithelial cells leading to pathogenesis". *International Journal of Biological Macromolecules* 165 (2020): 2089-2095.

43. Kaur Amandeep., *et al*. "Toll-like receptor-associated keratitis and strategies for its management". *3 Biotech* 5.5 (2015): 611-619.

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