LeLISA: a new lectin-based immunoassay for evaluation of mucins and pancreatic cystic lesions (PCL)

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Abstract

Background

The discrimination of mucinous from serous pancreatic cysts and pseudocysts is an important clinical issue. We established an assay to detect different types of carbohydrate containing molecules like glycans and mucins in biological materials and have called it the LeLISA. The method implies a certain specificity since different lectins bind predominantly to certain di -or oligosaccharides that may appear typically on certain cell types or, as a consequence of cell transformations often called aberrant mucin expression. The presence, or absence of reactivity with some lectins may be associated with different pathological conditions and may therefore have diagnostic implications, for instance in differentiation of pancreatic cysts. We aimed at detecting mucin-calprotectin (Cp)complexes (Muc/Cp) bound to lectin (Le) coated wells using enzyme labelled anti-Cp.

Materials and methods

The LeLISA is a special type of ELISA where the catching antibody is replaced by a Le. Eight different randomly selected lectins were used for coating of microwells and subsequently incubated with pancreatic cyst fluids collected via endoscopic ultrasound fine needle aspiration (EUS-FNA) from patients with mucinous, serous cysts and pseudocysts, 10 patients in each group. The diagnosis was confirmed through histopathological examination of surgical specimens and follow-up after initial diagnosis. The binding of Muc/Cp to lectins was demonstrated by a new type of ELISA where cyst fluids were incubated in microwells coated with different types of lectins followed by enzyme (HRP) labelled monoclonal anti-Cp. The name LeLISA was introduced for this new procedure.

Results

Muc/Cpin cyst fluids bound to several of the eight lectins tested, in particular to *Galanthus nivalis, Agaricus* blazei Murill and *Phaseolus vulgaris*. This was especially noticeable for fluids from mucin-producing cysts.

Conclusions

Cyst fluids contain complexes with Cp and mucins. The LeLISA may be a new method for detection of aberrant mucin expression and possibly a way of discriminating between different types of pancreatic cysts, in particular when the *Galanthus* lectin and enzyme labelled anti-Cp monoclonals are used. The binding to lectins depends upon certain carbohydrate sequences recognized by the individual lectin.

Background

By an extended use of computer tomography (CT) and magnetic resonance imaging (MRI), pancreatic cystic lesions (PCL) are found in an increasing number of patients, creating a need for methodology in selecting patients for surgery. The current European guidelines on the management of pancreatic cystic

neoplasms were presented in 2018 (1). It is important to discriminate between mucinous (premalignant and malignant), such as intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) from benign cysts like serous cysts (SCN) and pseudocysts (2). In patients considered for surgery, endoscopic ultrasound (EUS) may improve the diagnostic accuracy. Additional fine needle aspiration (EUS-FNA) for biochemical analysis of pancreatic cyst fluid may be helpful to discriminate the mucinous with malignant potential from serous cysts and pseudocysts without. Cyst fluid analysis of carcinoembryonic antigen (CEA), cytology, glucose, and different molecular markers (KRAS, GNAS) have been performed as well as advanced EUS based interventional procedures interventional procedures and radiomics but with limited success (2). Lectins are glycoproteins with a carbohydrate-binding domain possessing reversible binding ability to specific sugar moieties in glycoproteins or glycolipids as well as the free monosaccharide and glycan structures. Evidence is now emerging that lectins are dynamic contributors to tumor cell recognition (surface markers), cell adhesion and localization, signal transduction across membranes, mitogenic stimulation, augmentation of host immune defense, cytotoxicity, and apoptosis (3). Calprotectin (Cp) consists of two subunits called S100A8 and S100A9. It is located in the cytosol of neutrophils and found throughout the human body (4, 5). In response to contact with external substances like microbes or their components, Cp is released from neutrophils, and some of it as a major component of neutrophil extracellular traps (NETs) (6), so that high concentrations are found in plasma, body fluids, secretions, and excretions.

During studies on proteins in fecal extracts, it was found that fecal Cp differs from neutrophil derived Cp in two important aspects: the fecal protein did not react with monoclonal antibodies against the S100A8 subunit, it contains complexes with the chromatin components DNA and histones, and additional negative charges cause fecal Cp to bind strongly to ion exchange materials (6). It was hypothesized that the latter may be due to complex formation between Cp and negatively charged molecules, for instance mucous proteoglycans containing sulphate or sialic acid. Cp can bind to glycans on tumor cells (8). Such complexes might bind to lectins (proteins that bind to carbohydrates) for which reason we developed a new procedure that we call LeLISA, which differs from the ELISA method by using lectins (Le) for coating of wells rather than antibody. Cp-proteoglycan complexes may bind to lectins in the microwells via the carbohydrate moiety, and the presence of Cp in the complex can be detected by enzyme conjugated monoclonal anti-Cp. Initial experiments using four different lectins showed an about 100-fold stronger LeLISA signal when fluid from a mucinous cyst was compared with fluid from a serous cyst.

Aim of the project

to investigate the possibility of using our LeLISA test for distinguishing between different types of pancreatic cysts by testing cyst fluids against many lectins.

Material and methods

From 2008 our institution established a multidisciplinary research program including all patients referred to our tertiary center with suspected pancreatic or periampullary neoplasm.

The patients in this study were included prospectively in the Thematic Pancreatic Tumor Project between 2008 and September 30th, 2013. The multimodal preoperative workup included imaging with multidetector CT using a triphasic pancreas protocol and if required for diagnosis EUS with fine needle aspiration (EUS-FNA) of cyst fluid, mainly for analysis of carcinoembryonic antigen (CEA) and amylase. The required volume of cyst fluid would be sent for CEA-analysis, and the eventually remaining cyst fluid would be stored frozen at -80C within one hour after collection.

Radiological findings including worrisome features, CEA and amylase level in the cyst fluid, and the size of the lesion were also used to evaluate the possible malignant potential. The patients were selected for surgical resection whenever there was suspicion of malignancy.

The mucinous cyst diagnosis was histologically confirmed for the 10 patients in whom surgery was performed. Surgical resection was performed in two of the patients in the group with serous cysts. The diagnosis of serous- and pseudocyst among the other eighteen patients was based on the multimodal workup. Follow-up data on these patients have confirmed the initial non-malignant diagnosis.

The diagnosis of pseudocysts was based on radiological features, clinical symptoms, high amylase (> 250 U/L) in the cyst fluid [1] and observation.

Methods

Eight different randomly selected lectins were used for coating of microwells subsequently incubated with pancreatic cyst fluids collected via endoscopic ultrasound fine needle aspiration (EUS-FNA) from patients with mucinous, serous cysts and pseudocysts, 10 patients in each of the three groups.

The following Lectins were purchased from Medicago AB, Uppsala, Sweden: *Arachis hypogaea* lectin (PNA), *Concanavalin* A lectin, *Lens culinaris* lectin, *Galanthus nivalis* lectin, *Phaseolus vulgaris P* lectin, *Triticum vulgaris* lectin (WGA), *Galanthus nivalis* lectin L, *Narcissus pseudonarcissus* lectin, *Artocarpus integrifolia* lectin (Jacalin). The extract from the fungus *Agaricus blazei* Murill was from Pharma West AS, Trondheim, Norway. Recombinant Cp was produced by Aldevron, USA and used for generation, by the ProMab (USA) Company, of eight murine monoclonal antibody that binds Cp and S100A9. Horseradish peroxidase (HRP) conjugates were prepared using reagents from Abcam plc, Cambridge, UK. The TMB substrate for the enzyme was from SurModics, USA. The Sigma Fast protease inhibitor mix, and bovine serum albumin (BSA) was from Sigma-Aldrich, USA. We used MaxiSorp 96 well microplates from Nunc, Denmark, and a BioTek reader with Gene5 software.

The LeLISA is a new procedure aiming at detection of Cp in complexes with carbohydrate containing substances, for instance mucins, glycoproteins and glycans, that can bind to Lectins (Le). Otherwise, the method is very similar to an ELISA: Wells were coated with lectins, 200 µg/ml, 50 µl per well, in tris buffered saline (TBS), pH 7.5 at + 5 C for one to six days. To block residual protein binding sites on the plastic, wells were incubated with 200 µl TBS containing 1% BSA for 20 min. Washing solutions was TBS with 0.1 per cent Tween-20 from Sigma, USA. ProClin 300 (Sigma, USA) at 0.2% was used as

antimicrobial. Before and in between incubations the wells were washed three times. Cyst fluids were diluted 1:6 in TBS with Sigma Fast antiprotease mix according to the producers. After three times washing, wells were added 50 µl diluted cyst fluid and incubated at room temperature for 60 minutes with shaking, 350 rpm. In subsequent steps, plates were incubated with 50 µl per well of a mixture of the HRP-conjugated monoclonals (MiMo) diluted 1: 8000 in TBS with one per cent BSA. The optical density (OD) at 450 nm was read after 30 min with 100 µl substrate per well and addition of 100 µl of 2 N sulfuric acid.

Statistical evaluations and generation of figures performed using GraphPad Prism program version 6.01.

Ethics

The project was approved by the Regional Committee for Medical and Health Research Ethics, South-Eastern Norway (2015/1124/REK Sør-øst C) and by the Data Protection Supervisor at Oslo University Hospital (2015/9517). The patients were originally included in the Thematic Pancreatic Tumor Project (TPTP), which was approved by the Regional Committee for Medical and Health Research Ethics, South-Eastern Norway (265–08412 C), the Norwegian Directorate of Health (08/7927) and The Norwegian Data Protection Authority (08/01409-2).

Results

As shown in Fig. 1, signals for the four different lectins were much stronger in the mucinous cyst fluids compared to serous cyst fluids. When mucous cyst fluids were tested against eight lectins, two fluids gave strong reactions against all lectins, while the others generally gave weaker signals (Fig. 2). Remarkably, all cyst fluids gave optical density (OD) greater than 0.2 against the *Galanthus* lectin which binds mannose residues. Similarly, all ODs were greater than 0.2 against the *Agaricus* extract which typically binds Galactoseβ1-3N-Acetylgalactosamine, but here the signals were weaker than those with *Galanthus* lectin. OD of blanks, i.e., incubation with TBS with BSA followed by HRP-conjugate, were between 0.04 and 0.08 in all runs for all three types of cyst fluids.

Much weaker signals were seen when serous cyst fluids were tested (Fig. 3), and ODs above 0.2 were found only with two cyst fluids, and mostly the values were below 0.1. The reaction with *Galanthus* lectin were generally somewhat higher for all cyst fluids.

Fluids from pseudocysts gave more varied patterns (Fig. 4). Only five fluids gave signals above OD 0.2, but again stronger signals were seen with *Galanthus* coat.

When comparing the three types of cyst fluid signals against *Galanthus* lectin coats no overlap between the serous and mucinous groups was observed, while there is a clear overlap between the pseudocyst and mucinous types (Fig. 5).

Discussion

In this project we wanted to see if pancreatic cyst fluids might contain mucin/calprotectin complexes that can bind lectin dependent upon the type of lectin and type of cyst. Reactions with different lectins may reflect the presence of different mucins in such fluids which may contribute to the discrimination between different types of cysts. Our finding of lectin binding of mucins in pancreas cysts by the LeLISA has not previously been reported. The lectin Fap2 on the *Fusobacterium nucleatum* can bind Gal-GalNAc, a polysaccharide overexpressed in colorectal cancer cells leading to a severe, metastatic cancer (9). A similar lectin dependent specificity may occur in pancreatic neoplasia which may be explored using our LeLISA. The strong reactivity with all lectins in two of the mucinous and pseudo mucinous cysts may suggest that these fluids contain different individual or complex mucins with carbohydrate sequences corresponding to binding sites on the various lectins. It is also possible that some fluids may contain Cp that can bind directly to lectins.

The LeLISA may become a useful method for demonstration of mucin modifications caused by pathology. There was no overlap between the serous and mucinous LeLISA results in contrast to the pseudocyst and mucinous cyst data. Clearly, further studies on many more patient samples and lectins are needed before the LeLISA may be used clinically. Ideally, patients should have their diagnosis confirmed by biopsy/surgery, but that is not usually the case for serous cysts and pseudocysts. In this patient cohort the diagnosis was initially based on imaging, cyst fluid analysis and discussion in multidisciplinary team including symptoms and past medical history like pancreatitis and pseudocysts. Histologic confirmation of the diagnosis of pancreatic cystic neoplasms is only possible with surgical resection of the lesion and surgery is only performed when there is suspicion of premalignancy or malignancy. The mucinous cyst diagnosis was histologically confirmed for all the 10 patients by surgery. Among patients with serous cysts lesions, the diagnosis was confirmed by surgical resection in two of them due to suspicion of possible malignancy. The rest underwent long-time follow-up to exclude malignancy.

The LeLISA opens a new aspect of the Cp biology, namely its capability of binding to substances in stool extracts (data not shown) and pancreatic cyst fluids that secondarily can bind to lectins. It has been shown that transformed/malignant cells have glycans in their cell membrane, and that these can bind Cp (10). The specificity we have seen may be related to changes of mucins in cells as a consequence of pathology.

Because microorganisms cause infection by use of lectins for attachment to membrane proteoglycans, we hypothesize that Cp may prevent infection by binding to proteoglycans. Such binding may also contribute to blocking of deleterious effects of lectins in food, for instance from mushrooms. Early experiments (data not shown) showed that Cp can bind to elderberry extracts that are used for abdominal complains, and the *Agaricus* mushroom extract called Andosan has been used for treatment of IBD with some success (12, 13). The latter may also protect against intestinal tumor development (14).

The pattern of reactivity against different lectins probably reflects their carbohydrate specificity. For instance, PNA binds galactose while *Galanthus* binds mannose. The patterns shown in Figs. 2 and 4 suggest that mucinous and pseudocysts cysts may contain different mucoproteins, and that some mucins contain several carbohydrate sequences that may bind to many different lectins. Mucins binding to *Galanthus* may be a type becoming more dominant in malignant and premalignant cysts. For the studies reported here a mixture of anti-Cp was used; monoclonals were selected so that all the subfractions of fecal calprotectin would be detected (7). It is possible that some monoclonals can perform better in this context. As shown in Fig. 2, all mucinous cysts had LeLISA values above the arbitrary cut off 0.2 when tested against the lectin *Agaricus blazei* Murill (Agar)

The binding of S100A8 to GNA (data not shown) opens a link to a better understanding of many different types of disease since the former may cause severe Covid-19 disease (15), may promote epithelial-mesenchymal transition and metastasis in colorectal cancer (16), may protect alveolar cells against injury and emphysema development (17), may cause poor prognosis in mammary cancer (18) while appearance of S100A8 on stromal cells may be beneficial in colorectal cancer (19). Theoretically, treatment with lectins binding S100A8 might influence such phenomena.

Weaknesses of the current study: Limited experience with the novel LeLISA procedure. Relatively few patient samples tested.

Abbreviations

BSA: bovine serum albumin; CEA: carcinoembryonic antigen; Cp: calprotectin; EUS-FNA: endoscopic ultrasound fine needle aspiration; HRP: horseradish peroxidase; CT: computer tomography; Le: lectin; Muc-Cp: mucin-Cp complexes; MRI: magnetic resonance imaging; PCN: pancreatic cystic neoplasm; CA 19.9: serum cancer antigen; NETs: neutrophil extracellular traps; MiMo: mixed monoclonal antibodies; DNA: deoxyribonucleic acid; TMB: tetramethylbenzidine; TBS: tris-buffered saline; OD: optical density

Declarations

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The authors reported that there is no funding associated with the work described in this article.

Disclosure

The authors have no conflict of interest to disclose.

Availability of data sets:

The datasets and the antibody (MiMo) on which this study is based are available from the corresponding author.

The study was approved by the Regional ethical committee (6.2008.1844/1442, biobank 2428) and the institutional Data Protection Officer of Research (2015/9517) at Oslo university hospital. All patients signed written, informed consent.

Ethics approval and informed consent

The study was approved by the Regional ethical committee (6.2008.1844/1442, biobank 2428) and the institutional Data Protection Officer of Research (2015/9517) at Oslo university hospital. All patients signed written, informed consent.

Consent for publication was included in the approval.

Data and materials are available from the corresponding author.

Competing interests Authors declare no compete ng interests

Authors contributions: Magne: Idea, planning, analysis, manuscript. Isabel: material sampling, planning, analysis, and manuscript. Geir: analysis, manuscript. Anne: surgeon, follow-up, and manuscript. Truls: Endoscopic ultrasound, material sampling, planning, manuscript

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Figures

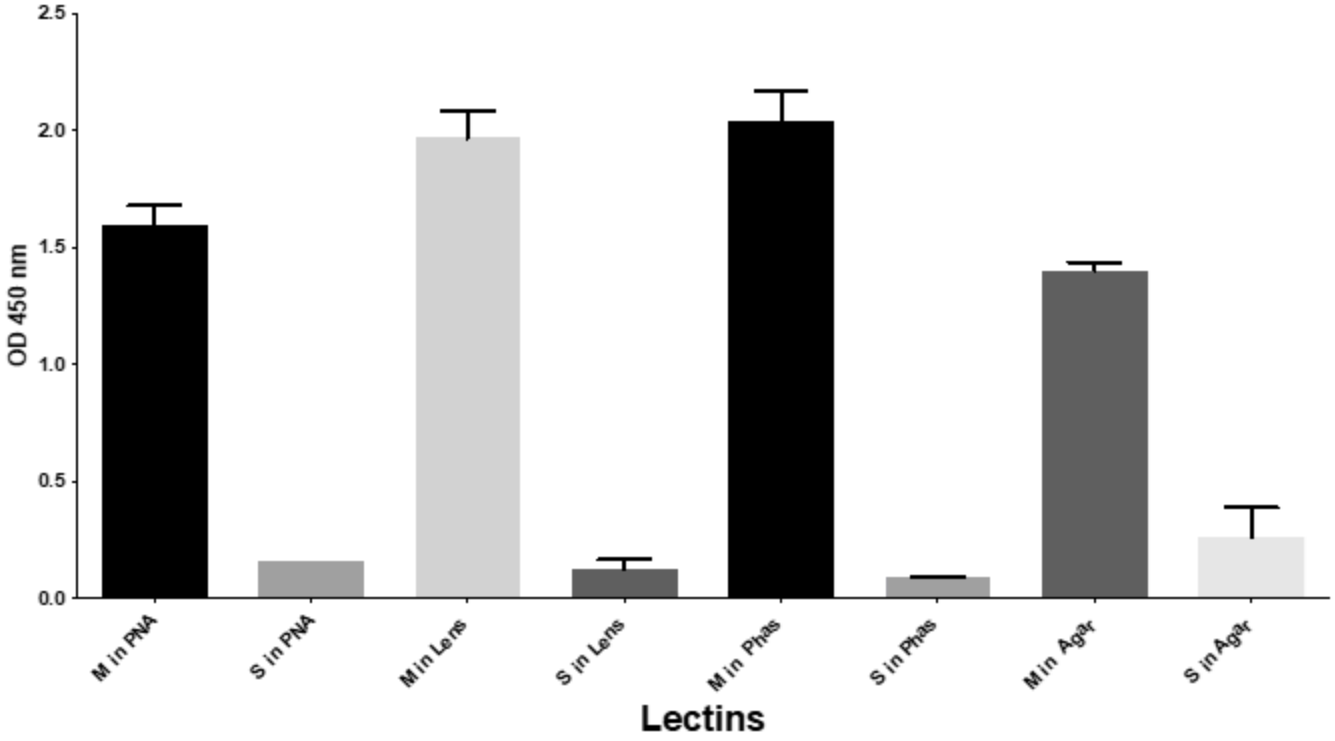

Figure 1

LeLISA testing of cyst fluid from one mucinous (M) and one serous (S) pancreatic cystic lesion against the lectins Peanut agglutin (PNA), *Lens culinaris* (LCH), *Phaseolus vulgaris* (PHA) and *Agaricus blazei* Murill (Agar). Coating with 50 ml at 200 µg/ml in TBS, for two hours at RT. Fifty µl of M and S diluted 1:6 in TBS added Sigma Fast (SF) protease inhibitor mix, was incubated in wells for one hour at RT followed by HRP-conjugated anti-Cp for one hour, and finally substrate for 20 min.

The SigmaFast (SF) protease inhibitor mix consists of SF + 10 mM EDTA. The figure gives means and one SD from testing in duplicate.

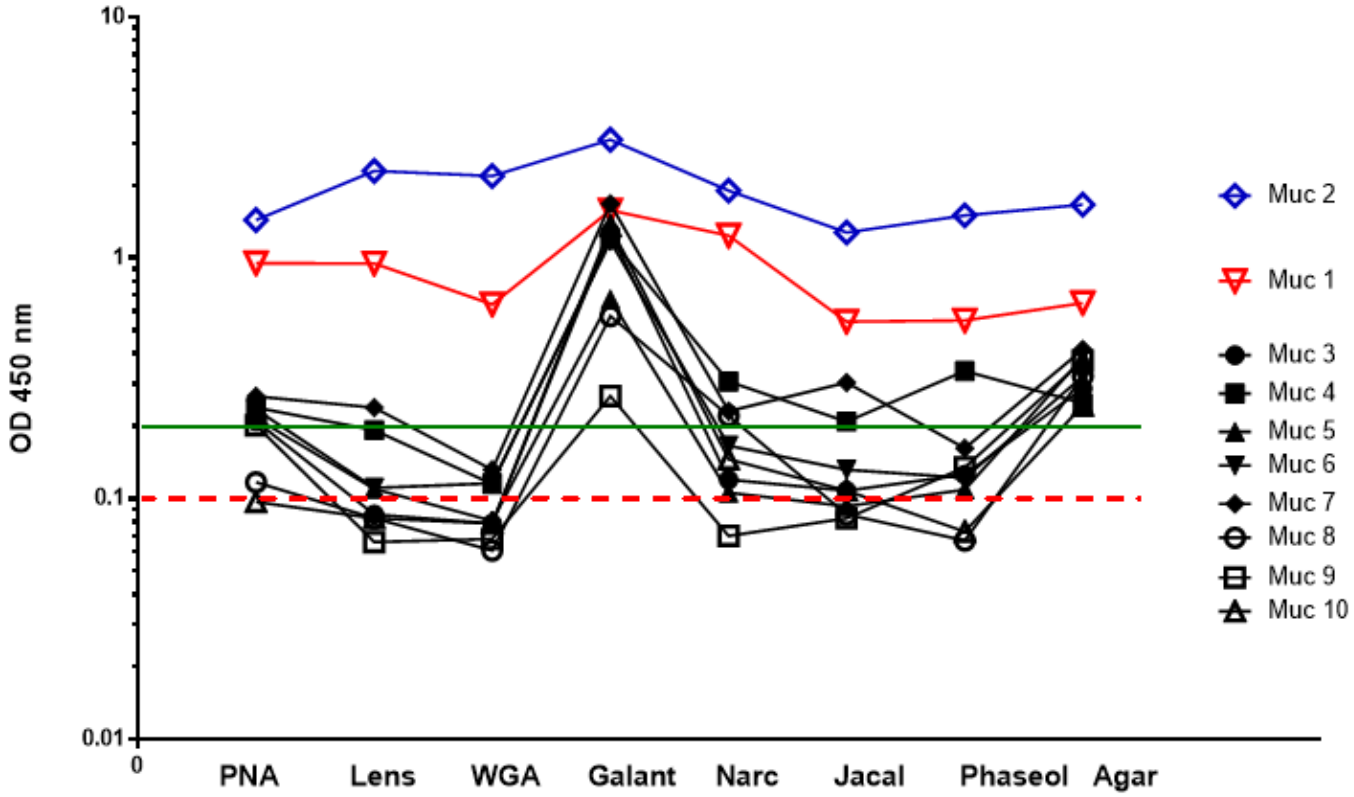

Figure 2

LeLISA testing of cyst fluids from 10 pancreatic mucinous cysts (Muc) against eight different lectins. Wells were coated with lectins, 200 µg/ml in TBS, 50 µl per well, for 18 hours. Cyst fluids were diluted 1: 6 with TBS with Sigma Fast protease inhibitor mix; incubated at RT for 60 mins followed by 60 mins with HRP conjugated monoclonal anti-Cp followed by 20 mins with substrate.

PNA: Peanut Agglutinin; Lens: *Lens culinaris* agglutinin; WGA: *Triticum vulgaris* lectin; Galant: *Galanthus nivalis* lectin L; Narc: *Narcissus pseudonarcissus* lectin; Jacal: *Artocarpus integrifolia* lectin; Phaseol: *Phaseolus vulgaris* lectin; Agar: *Agaricus blazei* Murill.

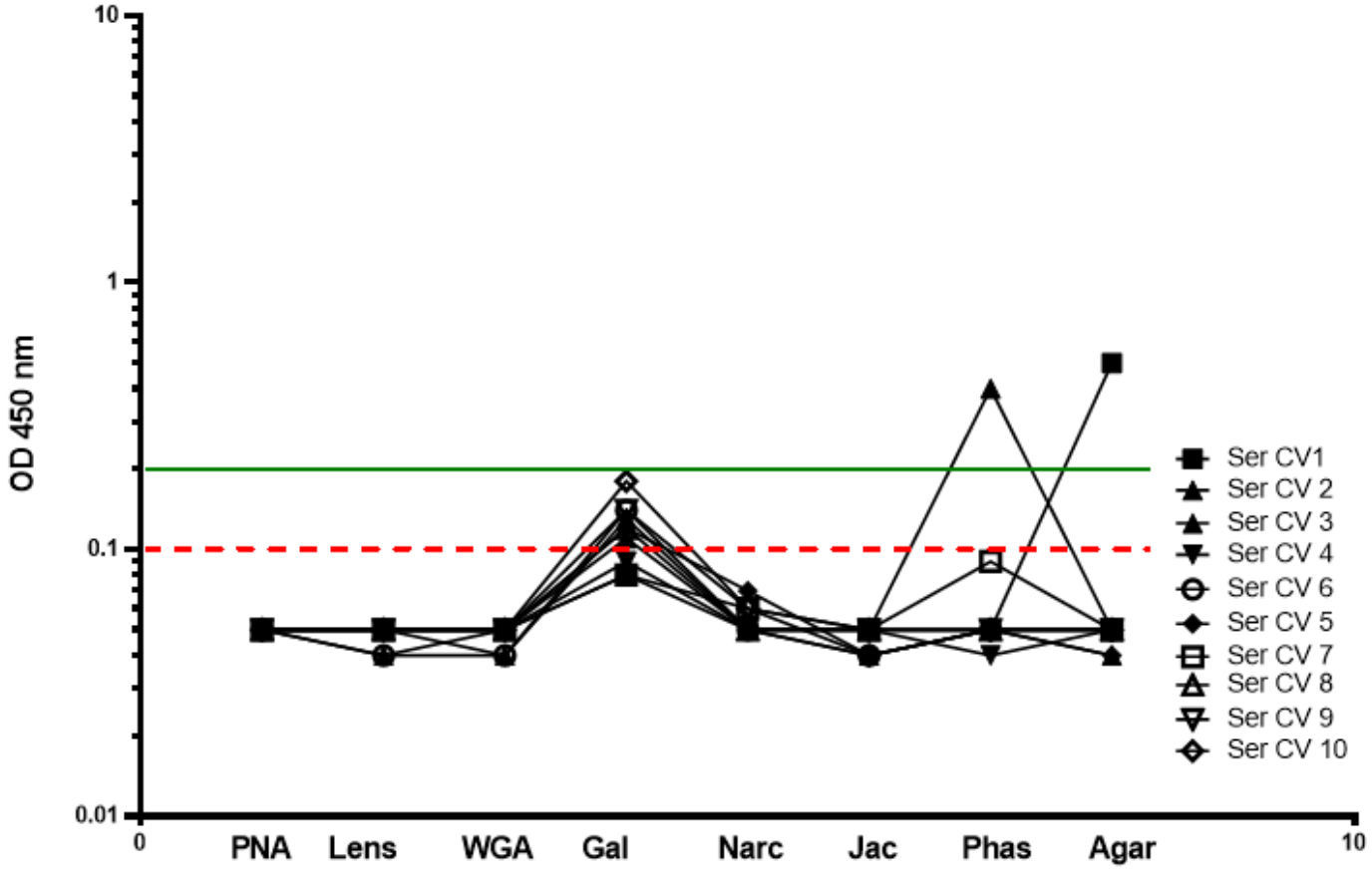

Figure 3

Binding of HRP-anti-Cp when 10 pancreatic serous cyst fluids (Ser CV) were incubated in lectin coated wells. Eight different lectins, 200 µg/ml in TBS, were used, 50 µl per well, for 18 hours. Cyst fluids were diluted 1: 6 with TBS with Sigma Fast protease inhibitor mix, incubated at RT for 60 mins followed by 60 mins with HRP conjugated monoclonal anti-Cp and for 20 mins with substrate.

PNA: Peanut Agglutinin; Lens: *Lens culinaris* agglutinin; WGA: *Triticum vulgaris* lectin; Galant: *Galanthus nivalis* lectin L; Narc: *Narcissus pseudonarcissus* lectin; Jacal: *Artocarpus integrifolia* lectin; Phaseol: *Phaseolus vulgaris* lectin; Agar: *Agaricus blazei* Murill.

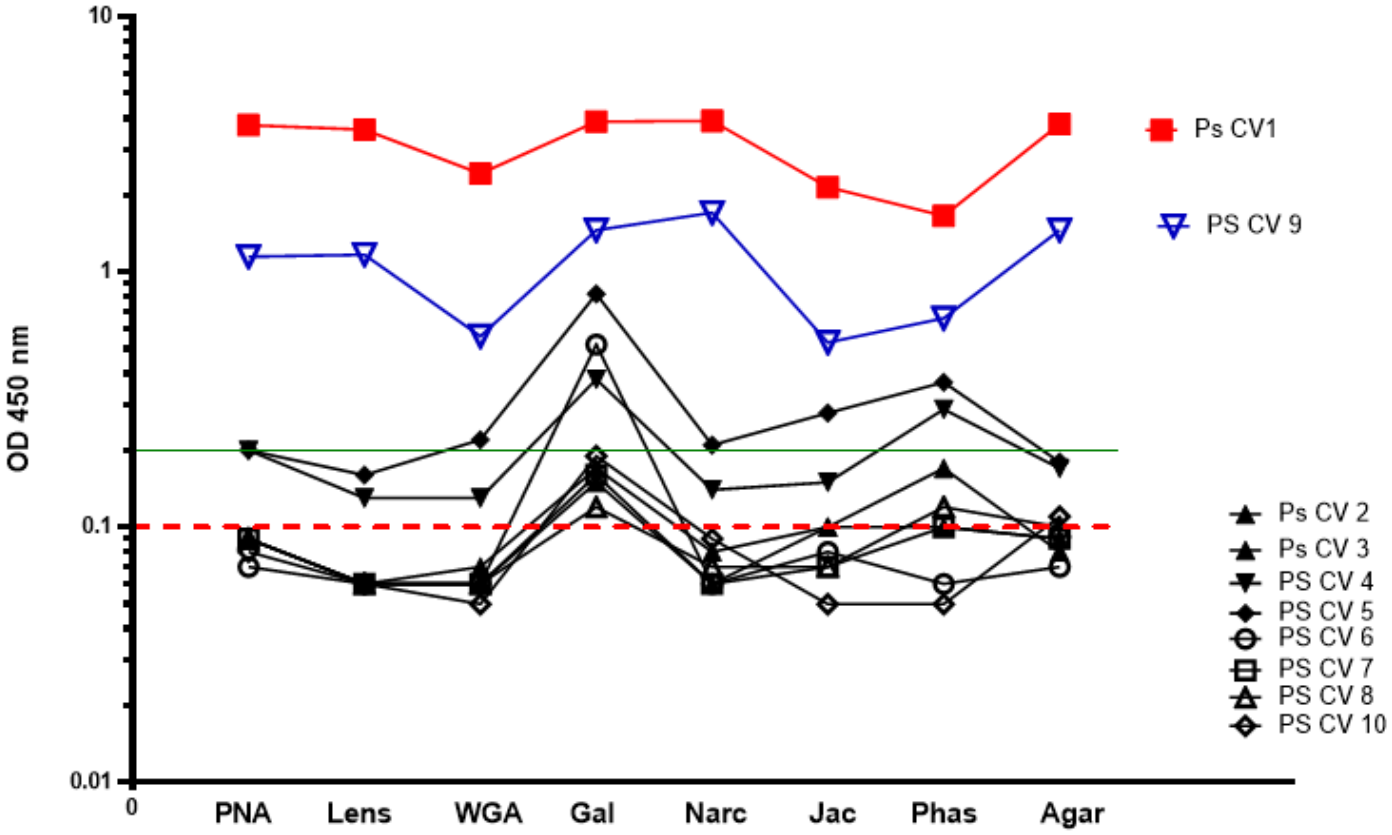

Figure 4

Binding of HRP-anti-Cp when 10 pancreatic pseudocyst fluids (Ps CV) were incubated in lectin coated wells, eight different lectins, 200 µg/ml in TBS, 50 µl per well, for 18 hours. Cyst fluids were diluted 1: 6 in TBS with Sigma Fast protease inhibitor mix and incubated at RT for 60 mins followed by 60 mins with HRP conjugated monoclonal anti-Cp followed by 20 mins with substrate.

PNA: Peanut Agglutinin; Lens: *Lens culinaris* agglutinin; WGA: *Triticum vulgaris* lectin; Galant: *Galanthus nivalis* lectin L; Narc: *Narcissus pseudonarcissus* lectin; Jacal: *Artocarpus integrifolia* lectin; Phaseol: *Phaseolus vulgaris* lectin; Agar: *Agaricus blazei* Murill.

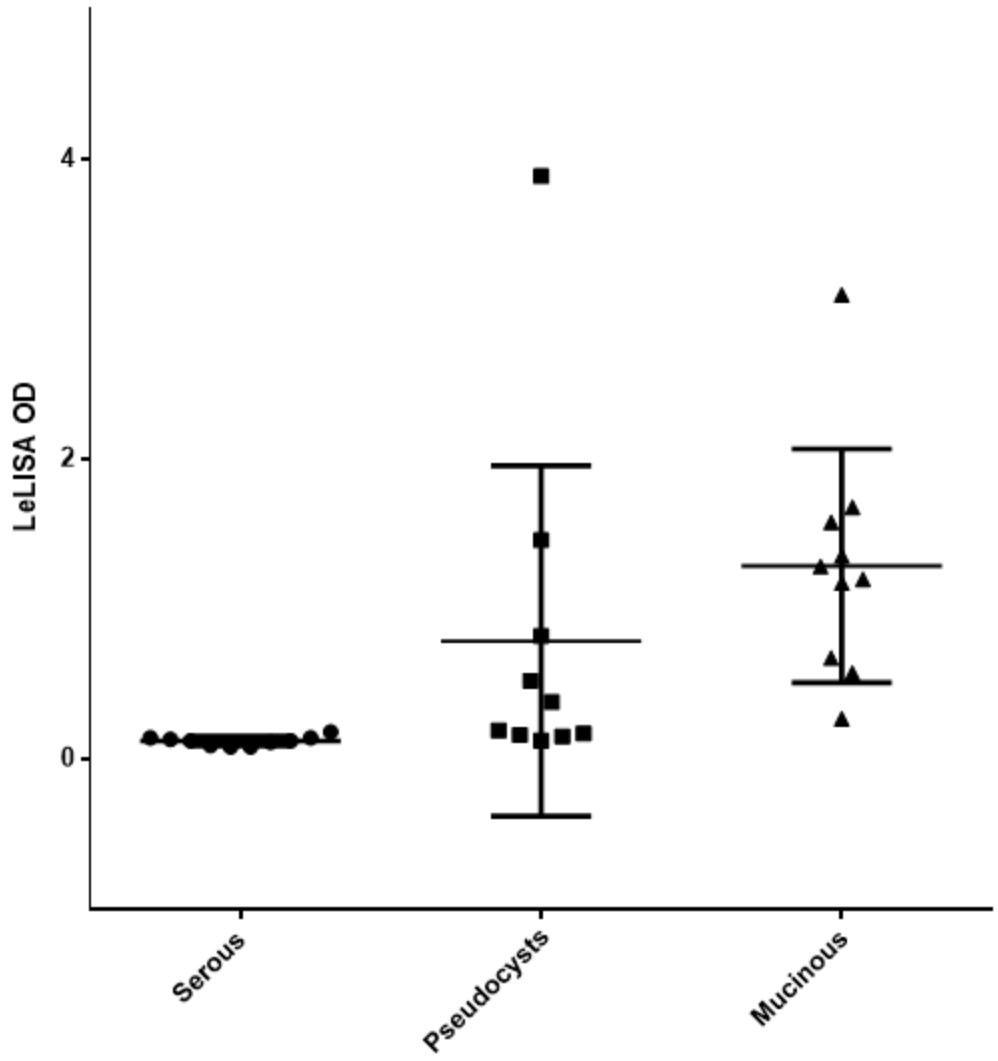

Figure 5

LeLISA with *Galanticus*lectin and HRP-conjugated anti-Cp. Optical density (OD) values for 10 cystic fluids of each type: serous, pseudocysts and mucinous cysts; means and 95 % confidence intervals.