METHODS AND DEVICES FOR DIAGNOSIS OF APPENDICITIS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional application Serial No. 60/590,631 filed July 23, 2004.

BACKGROUND OF THE INVENTION

[0002] Appendicitis is a common acute surgical problem affecting human beings of a wide age range. There are approximately 700,000 cases annually in the United States. A large proportion of cases occur in the 10 to 30 age group. An accurate diagnosis at a sufficiently early stage is a significant factor in achieving a successful outcome.

[0003] Many people present to their physician with symptoms suggestive of appendicitis but caused by other ailments such as viral infections. Differentiating the appendicitis patients from those affected with other ailments is a daunting clinical task that physicians face daily. While medical science has an excellent understanding of appendicitis and its treatment, it is very limited in its ability to accurately recognize or diagnose the disease.

[0004] Complicating the goal of an accurate and early diagnosis is the considerable overlap of genuine appendicitis with other clinical conditions. There appears to be no individual sign, symptom, test, or procedure capable of providing a reliable indication of appendicitis. Imaging technology is inadequate in identifying and characterizing the appendix, especially in the early stages of the disease when treatment is likely to be most effective. Imaging technology is further handicapped by its expense and its dependence upon the availability of highly trained and experienced people to interpret the studies. This limitation affects thousands of people every year by inaccurately diagnosing their problem or by delaying the accurate diagnosis. In cases of appendicitis, delays in diagnosis are the single most important factor leading to worsening of the condition and more complications related to the disease. The misdiagnosis of appendicitis can lead not only to

unnecessary surgery but also to delay of proper therapy for the actual underlying condition.

[0005] A dilemma for surgeons is how to minimize the negative appendectomy rate without increasing the incidence of perforation among patients referred for suspected appendicitis. What is desperately needed to more effectively treat this very common ailment is a simple, reliable diagnostic test that is capable of recognizing the earliest stages of the disease process.

[0006] The typical pathogenesis in appendicitis begins with obstruction of the lumen, although an initial inflammation of the organ can precede and even contribute to the obstruction. The secreted mucus of the appendix fills the closed lumen, causing an increase in intralumenal pressure and distension. The increased intralumenal pressure can exceed the level of capillary perfusion pressure, resulting in perturbation of normal lymphatic and circulatory drainage. Ultimately the appendix can become ischemic. The appendix mucosa is compromised, which can allow invasion of intralumenal bacteria. In advanced cases, perforation of the appendix may also occur with spillage of pus into the peritoneal cavity.

[0007] Currently, the diagnosis of appendicitis is difficult, and the difficulty persists during various stages in the progression of the condition. The following represents a hypothetical portrayal of stages and associated clinical presentations. Artisans of ordinary skill will recognize that a considerable degree of variation will occur in a given patient population.

[0008] At the earliest stages of inflammation, a patient can present with a variety of non-specific signs and symptoms. Upon obstruction, presentation can involve periumbilical pain, mild cramping, and loss of appetite. The progress toward increased lumenal pressure and distension can be associated with presentation involving the localization of pain to the right lower quadrant of the abdomen, nausea, vomiting, diarrhea, and low grade fever. If perforation occurs, a patient can present with severe pain and high fever. At this very advanced stage, sepsis can be a serious risk with a potentially fatal outcome.

[0009] Practitioners currently use several tools to aid in appendicitis diagnosis. These tools include physical examination, laboratory tests, and other procedures.

Routine laboratory tests include complete blood count (CBC) with or without differential and urinalysis (UA). Other tests include a computed tomography (CT) scan of the abdomen and abdominal ultrasonography. Procedures can include, for example, laparoscopic examination and exploratory surgery.

[0010] Flum et al. attempted to determine whether the frequency of misdiagnosis preceding appendectomy has decreased with increased availability of certain techniques (Flum DR et al., 2001). These techniques included computed tomography (CT), ultrasonography, and laparoscopy, which have been suggested for patients presenting with equivocal signs of appendicitis. Flum et al. concluded as follows: "Contrary to expectation, the frequency of misdiagnosis leading to unnecessary appendectomy has not changed with the introduction of computed tomography, ultrasonography, and laparoscopy, nor has the frequency of perforation decreased. These data suggest that on a population level, diagnosis of appendicitis has not improved with the availability of advanced diagnostic testing." The rate of misdiagnosis of appendicitis is about 9 percent in men and about 23.2 percent in women (Neary, W., 2001).

[0011] Myeloid-related Protein Complex 8/14 (MRP-8/14) is a heterodimeric complex associated with acute inflammatory conditions (for review see Striz and Trebichavsky, 2004). The complex belongs to the S100 superfamily of proteins and is also referred to S100A8/9, L1, macrophage inhibitory related protein and calprotectin. The heterodimer consists of an 8 kilodalton (MRP-8) and 14 kilodalton (MRP-14) subunit. MRP-8 and MRP-14 are alternatively named S100A8/calgranulin and S100A9/calgranulin b, respectively. MRP-8/14 is a calcium binding protein originally discovered in macrophages. Neutrophils expressing high concentrations of MRP-8/14 are found in a variety of inflammatory conditions, including rheumatoid arthritis, inflammatory bowel disease and allograft rejections (Frosch et al., 2000; Limburg et al., 2000; Burkhardt et al., 2001).

[0012] MRP-8/14 is not always diagnostic of inflammation. For example, it does not reliably indicate the presence of inflammatory diverticuli (Gasché, C. 2005). Lymphocytes do not generally contain MRP-8/14 (Hycult Biotechnology, Monoclonal Antibody to Human S100A8/A9), and therefore MRP-8/14 is not diagnostic of inflammation characterized by the presence of lymphocytes but not neutrophils. Also,

this protein is not always associated with opportunistic infections (Froland, M.F., et al., 1994).

[0013] Haptoglobin is an acute phase protein that binds free hemoglobin following hemolysis. The haptoglobin-hemoglobin complex is removed by the liver. Haptoglobin is a heterotetramer composed of two alpha and two beta subunits. The alpha and beta units are derived from a single polypeptide chain precursor that is enzymatically cleaved to produce the subunits. The molecular weights of the subunits are approximately 9 kd-18 kd and 38 kd for alpha and beta, respectively.

[0014] In addition to being a hemoglobin scavenger, haptoglobin has a wide range of biological functions (Dobryszycka, 1997). Haptoglobin has been shown to be upregulated and modulate the immune response in certain infection and inflammatory conditions perhaps by regulating monocyte function (Arredouani et al., 2005). The alpha subunit has been demonstrated to be a potentially useful serum marker for ovarian cancer (Ye et al., 2003).

[0015] The ability to accurately diagnose appendicitis would be greatly augmented by the identification of molecules differentially associated with appendicitis.

SUMMARY OF THE INVENTION

[0016] This invention provides a method for diagnosing appendicitis in a patient comprising identifying at least one classical symptom of appendicitis in said patient and identifying the presence of at least one molecule differentially associated with appendicitis in a fluid or tissue sample of said patient. It is recognized in the art that the diagnosis of appendicitis is difficult, and that it is often misdiagnosed. Thus the term "diagnosing appendicitis" as used herein does not necessarily mean diagnosing appendicitis with more than usual accuracy. However, in fact, the methods of the present invention have been shown to provide improvements in correct diagnosis, with almost no false positives and few false negatives.

[0017] The term "differentially associated" with respect to a molecule "differentially associated with appendicitis" refers: (1) to a molecule present in a patient with appendicitis and not present in a patient not having appendicitis; (2) to a molecule whose relative level (amount) is distinguishing between appendicitis and non-appendicitis; (3) to a molecule present, or present at a level, in conjunction with the presence of other symptoms of appendicitis, that is diagnostic of appendicitis; and/or (4) to a molecule present, or present at a level, in conjunction with the lack of symptoms associated with conditions other than appendicitis in which the presence of the molecule occurs, that is diagnostic of appendicitis.

[0018] The diagnostic level of such a molecule is also referred to herein as the "threshold amount" or "threshold level." The molecules differentially associated with appendicitis are preferably protein antigens.

[0019] Classical symptoms of appendicitis include: pain in the abdomen; pain that starts near the navel, then moves to the lower right quadrant of the abdomen; anorexia (loss of appetite); trouble eating accompanied by sleepiness; nausea starting after onset of pain; vomiting starting after onset of pain; vomiting accompanied by fatigue; constipation; small stools with mucus; diarrhea; inability to pass gas; low-grade fever; abdominal swelling; pain in the abdomen worsening; tenesmus (feeling of needing to move the bowels); high fever; and leukocytosis. Increased plasma viscosity is also associated with appendicitis. In one embodiment of the invention at least two or more symptoms of appendicitis are identified.

[0020] In one embodiment of this invention patients are screened to determine whether or not they have an "interfering condition," i.e., another condition in which the molecule is present in the type of sample being tested. Patients are tested for the presence of the molecule if they do not have such an interfering condition; or are tested for the presence of appendicitis-diagnostic levels of the molecule if they do have such an interfering condition. Appendix-diagnostic levels when the patient has an interfering condition are levels higher than those present in patients who have the interfering condition but do not have appendicitis. Interfering conditions include recent allograft; septicemia; meningitis; pneumonia; tuberculosis; rheumatoid arthritis; gastrointestinal cancer; inflammatory bowel disease; skin cancer, periodontitis, preeclampsia, and AIDS.

[0021] A sample can be a fluid or tissue, and can contain whole blood, plasma, serum, milk, urine, saliva and/or cells. Fecal samples may also be used. Preferably tissue and fecal samples are liquefied before testing.

[0022] In one embodiment of this invention two or more molecules differentially associated with appendicitis are tested for. Identification of additional molecules provides greater accuracy to the method.

[0023] One molecule differentially associated with appendicitis is MRP-8/14. Another is haptoglobin. Both these molecules can be tested for in diagnosing appendicitis.

[0024] MRP-8/14 levels in the range of about 1 to about 11 μg/ml are present in patients without appendicitis. Levels higher than this provide increased accuracy in diagnosing appendicitis. Levels higher than about 10, 11, 13, 15 or 20 μg/ml of MRP-8/14 can be used to diagnose appendicitis. Haptoglobin levels in the range of about 27-139 mg/dL are found in patients without appendicitis. Levels higher than this, e.g., higher than 125, 130, 135, 139 and 150 provide increased accuracy in diagnosing appendicitis.

[0025] Other molecules that can be tested for in the methods of this invention, or that can be tested for in addition to the foregoing molecules, include unique structural proteins of the gastrointestinal tract, stress-related inflammatory mediators, immunologic factors, indicators of intestinal bacterial flora, Plasminogen Activator Inhibitor-1, fatty acid binding proteins, nuclear factor kappa beta (NFκB), specific appendix antigens (HLA-DR), inflammation associated antigens; and nucleic acids coding for any of the foregoing, including nucleic acids coding for MRP-8/14 and haptoglobin. Methods for testing for the presence of nucleic acids are known to the art.

[0026] The methods of this invention involving obtaining a first sample from a patient suspected of having appendicitis can also comprise identifying at least one molecule differentially associated with appendicitis by a process including obtaining a second fluid or tissue sample from a second patient, wherein the second patient has appendicitis; obtaining a third fluid or tissue sample from a third patient wherein the third patient has a non-appendicitis condition characterized by at least one

symptom of appendicitis; and analyzing the second and third samples so as to detect a molecule differentially associated with the appendicitis in the second patient, and then identifying the presence of that molecule, or presence of an increased level of that molecule, in the first sample, thereby diagnosing appendicitis. Candidate molecules for this process of identifying molecules differentially associated with appendicitis include unique structural proteins of the gastrointestinal tract, stress-related inflammatory mediators, immunologic factors, indicators of intestinal bacterial flora, Plasminogen Activator Inhibitor-1, fatty acid binding proteins, nuclear factor kappa beta (NFκB), specific appendix antigens (HLA-DR), inflammation associated antigens, and nucleic acids coding for any of the foregoing.

[0027] This invention also provides a method for identifying a molecule differentially associated with appendicitis, the method comprising obtaining a sample from each of a plurality of patients who are undergoing surgery for suspected appendicitis; determining during surgery whether each said patient has appendicitis or not; and analyzing said samples for the presence of a molecule differentially associated with appendicitis. The samples can be blood samples or samples of appendix tissue. This method can also include determining the amount of each molecule found to be differentially associated with appendicitis in the sample. In one embodiment of the invention, following identification of the molecule in tissue, it is also identified in plasma. This requires that samples of blood be taken from patients suspected of having appendicitis. The amount of the molecule differentially associated with appendicitis in patients who have appendicitis compared with those who do not is also determined.

[0028] The methods for diagnosing appendicitis of this invention can include using test devices, e.g., cartridge test devices and dipstick test devices, and/or other means for determining the presence or absence of a molecule differentially associated with appendicitis, e.g., performing western blots, northern blots, ELISA tests, protein function tests, PCR and other assays known to the art. In testing molecules differentially associated with appendicitis that are present in patients without appendicitis, but upregulated in patients with appendicitis, assays that test for the relative amount of the molecule present in patient fluids or tissues as well as the mere presence of the molecule are required. Cartridge immunoassays can be

designed to provide information on relative amounts of such molecules as described herein. Other assays known to the art including ELISAs and hospital assay devices such as the Synchron LX system of Beckman Coulter can be used to provide the amount of such molecules present in the patient, which can then be compared with amounts present in patients without appendicitis to determine whether or not the patient has appendicitis.

[0029] The methods for diagnosing appendicitis can include performing an immunological assay using a monoclonal or polyclonal antibody to the molecule differentially associated with appendicitis. Such antibodies are known to the art or can be generated by means known to the art without undue experimentation.

[0030] This invention also provides an immunoassay test device for detecting the presence of a molecule differentially associated with appendicitis in a sample. The device comprises a first monoclonal or polyclonal antibody specific to the molecule, a support for the first monoclonal or polyclonal antibody, means for contacting the first monoclonal or polyclonal antibody with the sample, and an indicator capable of detecting binding of the first monoclonal or polyclonal antibody with the molecule.

[0031] Detecting binding of the antibody with the molecule can include binding the antibody/molecule complex to a second, labeled antibody which binds to the molecule or to the antibody of the complex.

[0032] Test devices can be in the form of cartridges, dipsticks, or other conformations known to the art. The test device can also be part of a kit which can contain instructions for use, instructions for comparison of test results with results of the same test done on non-appendicitis patient, additional reagents, such as cells or fluids from non-appendicitis patients, and other reagents known to the art. These types of assay devices are known to the art and described, e.g., in U.S. Patent Publication No. 2003/0224452.

[0033] The methods for diagnosing appendicitis can include comparing the level of the molecule in the sample with a background level of the same molecule in persons not having appendicitis. This comparison can be made by any means known to the art. It can include comparing sample results with results from a second sample taken from a person known not to have appendicitis, or comparing sample

results with a photograph or other representation of results from a person not having appendicitis. Test devices having means for masking non-appendicitis levels, e.g. a support having the same color or tone as indicators showing non-appendicitis levels, or a filter having the same color or tone as a non-appendicitis level, so that only higher, appendicitis-indicating levels of the molecule are detectable, e.g., by eye, can also be used. The methods of this invention can include use of control fluids having background levels of the molecule typical of non-appendicitis samples, as well as colored supports and/or light filters as discussed above.

[0034] When the sample is blood, the method can also include processing the blood by a means known to the art, such as filtration or centrifugation, for separating plasma or serum which is to be assayed.

[0035] Antibody supports are known to the art. In an embodiment of this invention, antibody supports are absorbent pads to which the antibodies are removably or fixedly attached. In the devices of this invention, any indicator means known to the art to detect antibody binding with the molecule can be used. The indicator means can include second, labeled, monoclonal or polyclonal antibodies which bind to the selected protein, which preferably bind to a substantially different epitope on the selected protein from that to which the first monoclonal or polyclonal antibodies bind, such that binding of the first monoclonal or polyclonal antibody will not block binding of the second antibody, or vice versa. The indicator means can also include a test window through which labeled antibodies can be viewed. Any label known to the art can be used for labeling the second antibody. In an embodiment of this invention, the label is colloidal gold. The second antibody can be monoclonal or polyclonal. In an embodiment of this invention, the first antibody is a polyclonal or a monoclonal antibody made using a specific polypeptide sequence of the molecule differentially associated with appendicitis, and the second antibody is a different monoclonal or polyclonal antibody which binds to a different site of the molecule or binds to the first antibody. Antibodies for MRP-8 and MRP-14 are commercially available through Cell Sciences, Canton, MA. Monoclonal antibodies to haptoglobin useful in the methods of this invention are also known to the art, e.g., as described in U.S. Patent No. 5,552,295.

[0036] In one embodiment of this invention, the sample to be assayed is a liquid, and the immunoassay test device is a lateral flow device comprising inlet means for flowing a liquid sample into contact with the antibodies. The test device can also include a flow control means for assuring that the test is properly operating. Such flow control means can include control antigens bound to a support that capture detection antibodies as a means of confirming proper flow of sample fluid through the test device. Alternatively, the flow control means can include capture antibodies in the control region which capture the detection antibodies, again indicating that proper flow is taking place within the device.

[0037] Methods for detecting the presence of a molecule differentially associated with appendicitis using the foregoing devices are also provided, the methods comprising: providing an immunoassay test device of this invention; contacting a first antibody with a sample; and reading an indicator which is capable of detecting binding of the first antibody. Preferably, binding indicates appendicitis in the patient being tested. Methods of using these devices can be performed in the doctor's office, emergency room, or surgery, rather than requiring sending the patient or the sample to a separate laboratory.

[0038] The devices of this invention are useful for testing the above-mentioned samples. When cells are tested, e.g., when the molecule differentially associated with appendicitis is suspected to be in blood or tissue cells rather than serum, the method and/or device can include a cell-lysing step or means using detergent, puncture or other physical or chemical process known to the art.

BRIEF DESCRIPTION OF THE FIGURE

[0039] Figure 1: Two-dimensional electrophoresis image of proteins from (A) normal and (B) diseased appendix tissue. Proteins were separated by isoelectric focusing on the x axis and by molecular weight on the y axis. The molecular weight in kilodaltons is shown on the left. The arrow indicates the upregulated protein, AP-93.

[0040] Figure 2: MRP-14 western blot analysis of normal (N) and diseased (A) tissue. The numbers are sample ID numbers. Molecular weights are shown in kilodaltons.

[0041] Figure 3: MRP-8 western blot analysis of normal (N) and diseased (A) tissue. The numbers are sample ID numbers. Molecular weights are shown in kilodaltons.

[0042] Figure 4: Relative levels of MRP-8/14 in normal and appendicitis serum as determined by ELISA. The levels are given as a fraction of the mean for the patients not having appendicitis, said fraction also being referred to herein as a "fold increase." Dark bars represent samples from patients having appendicitis. White bars represent samples from patients not having appendicitis.

[0043] Figure 5: Two-dimensional electrophoresis image of proteins in depleted serum samples from (A) normal and (B) appendicitis patients. Proteins were separated by isoelectric focusing on the x-axis and by molecular weight on the y-axis. The molecular weight in kilodaltons is shown in the right. The tailed arrow indicates the upregulated protein, AP-77 (haptoglobin alpha subunit). The untailed arrow indicates a control protein that is equally abundant in diseased vs. normal.

[0044] Figure 6: Two-dimensional electrophoresis image of proteins from (A) normal and (B) diseased (perforated) appendix tissue. Proteins were separated by isoelectric focusing on the x axis and by molecular weight on the y axis. The molecular weight in kilodaltons is shown on the left. The arrow indicates the upregulated protein, AP-91 (haptoglobin alpha subunit).

[0045] Figure 7: Haptoglobin distribution. Haptoglobin western blot analysis of normal (N) and diseased (A) tissue. The numbers are sample ID numbers. Molecular weights are shown in kilodaltons. The alpha and beta subunits are >20 kd and 38 kd, respectively.

DETAILED DESCRIPTION

[0046] The vermiform appendix is recognized as a separate organ from the large and small intestines. It extends as a finger-like pouch from the base of the ascending colon, which is also called the cecum. The appendix, like the large intestine, is hollow and composed of the same three tissue layers. These three layers are a mucosa, muscularis and a serosa. The appendiceal lumen communicates with the lumen of the cecum via a round opening (os) through which the appendix adds its

secretions to the fecal stream. These secretions are excess mucus produced from the appendiceal mucosa. In addition to containing mucus, the appendix also contains numerous bacteria common to the right colon. Obstruction of the appendiceal lumen is the dominant factor causing acute appendicitis. While fecaliths are the usual cause of appendiceal obstruction, hypertrophied lymphoid tissue, inspissated barium from previous x-ray studies, vegetable and fruit seeds, and intestinal worms like ascarids can also block the appendiceal lumen.

[0047] Following luminal obstruction an escalating cycle of events ensues. The proximal obstruction of the appendix produces a closed-loop obstruction that blocks the normal flow of appendiceal mucus into the cecum. The continuing normal secretion of the appendiceal mucus very rapidly fills the luminal capacity of the appendix (approximately 0.1 cc). Once the luminal capacity of the appendix is reached additional mucus production from the obstructed appendix rapidly elevates the intraluminal pressure within the organ. This elevated intraluminal pressure is exerted outward against the appendiceal wall and causes the appendix to distend. Distention stimulates nerve endings of the visceral afferent pain fibers, producing vague, dull, diffuse pain in the midabdomen or lower epigastrium. Peristalsis is also stimulated by the rather sudden distention, so that some cramping may be superimposed on the visceral pain early in the course of appendicitis.

[0048] Distention of the appendix continues, not only from continued mucosal secretion, but also from rapid multiplication of the resident bacteria of the appendix. As pressure in the organ increases, venous pressure within the appendiceal wall is exceeded. This rising intraluminal pressure then occludes capillaries and venules, but arteriolar inflow continues, resulting in engorgement and vascular congestion. Distention of this magnitude usually causes reflex nausea and vomiting, and the diffuse visceral pain becomes more severe. The inflammatory process soon involves the serosa of the appendix and in turn parietal peritoneum in the region, producing the characteristic shift in pain to the right lower quadrant (RLQ). The disease process is fairly advanced when pain is localized to the RLQ.

[0049] The mucosa of the gastrointestinal tract, including the appendix, is very susceptible to impaired blood supply. Thus mucosal integrity is compromised early in the process, allowing bacterial invasion of the deeper tissue layers. This bacterial

invasion leads to appendiceal destruction and systemic liberation of various bacterial toxins. Fever, tachycardia, and leukocytosis develop as a consequence of this systemic release of dead tissue products and bacterial toxins. As progressive appendiceal distention rises, encroaching on the arteriolar pressure, ellipsoidal infarcts develop in the antimesenteric border of the appendiceal serosa. As distention, bacterial invasion, compromise of vascular supply and infarction progress, perforation occurs through one of the infarcted areas on the antimesenteric border. This perforation then releases the bacteria and its toxins into the abdominal cavity.

[0050] Appendicitis has been called the "great imitator," as its symptoms are frequently confused with those of other conditions. This confusion stems from the nonspecific nature of the pain early in its course and the variability in how appendicitis progresses. Pain in the right lower quadrant of the abdomen is the hallmark of appendicitis but this is not typically what the patient first perceives. When the appendiceal lumen first obstructs, the patient will have few if any symptoms because the appendiceal lumen has not yet had the chance to fill with mucus. The time required to fill the appendiceal lumen is proportional to the lumen volume available behind the obstruction. This is variable and unpredictable, as that volume is dependent upon the individual's appendix size and precisely where the fecalith or other obstruction is located along that length. Should the fecalith or other obstruction be close to the tip of the appendix the available volume is relatively small and the time to symptoms or perforation short. In contrast, the opposite will be true should the fecalith or other obstruction be near the base of the appendix and provide for the largest possible appendiceal volume.

[0051] Once the appendix begins to distend, the appendicitis patient will begin to experience a nonspecific discomfort usually in the mid portion of the abdomen. This discomfort can be easily confused with common ailments such as indigestion, constipation or a viral illness. Continued appendiceal distention is also accompanied by some nausea and frequently vomiting. Rarely is the vomiting severe or unrelenting, which reinforces the confusion with common ailments.

[0052] Later in the progression of appendicitis, inflammation will have progressed to the outermost layer of the appendix. This outmost layer is called the serosa and it touches the inner lining of the abdominal cavity called the peritoneum. This contact

irritates the peritoneum, producing peritonitis that is perceived by the appendicitis patient as focal pain wherever the appendix is touching the peritoneum. This too can vary between different individuals. The appendix is most usually located in the right lower quadrant under an area known as McBurney's point. McBurney's point is a position on the abdomen that is approximately two-thirds of the distance from the anterior superior iliac spine in a straight line toward the umbilicus. The appendix can, however, reside in other locations in which case the peritonitis produced by the appendix will be in an atypical location. This again is a common factor producing an erroneous diagnosis and delays surgical treatment in cases of appendicitis.

[0053] Regardless of its location, if appendicitis is allowed to progress the organ will eventually perforate. This contaminates the abdominal cavity around the perforated appendix with bacteria producing a severe infection. This infection will usually lead to a localized intra-abdominal abscess or phlegmon and can produce generalized sepsis.

[0054] To identify molecules differentially associated with appendicitis, a proteomic approach was used. A protein complex, MRP-8/14, that is present in appendix tissue in patients with acute appendicitis was identified. The highly correlative nature of this complex with appendicitis led us to examine MRP-8/14 serum levels in patients with apparent appendicitis. MRP-8/14 is significantly elevated ($p<0.02$) in patients with appendicitis as compared to levels in patients with apparent appendicitis yet having no appendiceal inflammation. The source of MRP-8/14 in the serum is the inflamed appendix tissue. This is consistent with the known functions of MRP-8/14.

[0055] The role of MRP-8/14 in inflammation is not fully understood but it does seem to play a vital role in retaining leukocytes in microcapillaries. Extracellular MRP-8/14 interacts with endothelial cells by binding to heparin sulfate and specifically carboxylated glycans (Robinson et al., 2002). The intracellular signal pathways and effector mechanisms induced by binding of MRP-8/14 to endothelial cells are not well defined. However, interaction of MRP-8/14 with phagocytes increases binding activity of the integrin receptor CD11b-CD18. This is one of the major adhesion pathways of leukocytes to vascular endothelium (Ryckman et al., 2003). It is believed that the MRP-8/14 utilizes the receptor for advanced glycation

end products (RAGE). A relative of MRP-8/14, S100A12, is a specific ligand of RAGE expressed by endothelial cells and their interaction activates NF-kappa B binding in these cells (Hsieh et al., 2004). The NF-kappa B binding subsequently induces expression of many proinflammatory molecules, such as various cytokines or adhesion molecules. Thus, release and extracellular functions of S100 proteins represent a positive feedback mechanism by which phagocytes promote further recruitment of leukocytes to sites of inflammation. Taken together, these proteins appear to play a role in a fundamental inflammatory response in certain inflammatory conditions, and are excellent markers of appendix tissue inflammation.

[0056] Neutrophils are white blood cells that are the first to migrate from the circulation into sites of inflammation. Within neutrophils, constituting approximately 40% of total cytosolic proteins is the MRP-8/14 complex. This protein is specifically expressed only in cells of macrophage lineage, making blood monocytes and acutely activated macrophages other potential white blood cell sources of these proteins. MRP-8/14 is not usually expressed in lymphocytes nor resident macrophages or those macrophages involved in chronic inflammation. These two proteins are also known to be independently expressed by mucosal epithelium in specific states of acute inflammation.

[0057] In the case of appendicitis, the luminal obstruction and the resultant distention of the appendiceal wall triggers an inflammatory response. The circulating neutrophils are then recruited into the area, as are activated macrophages. While the expression of this protein complex is related to the activity of the macrophages in inflammation, the exact relationship between MRP-8/14 and cellular activity is not fully known. What is known is that the intracellular distribution of MRP-8/14 varies with the activation state of macrophages. Normal macrophages contain the complexes in the cytosol, but once stimulated, MRP-8/14 translocates from the cytosol to the cell membrane (specifically with the proteins of the cytoskeleton). This would imply that MRP-8/14 may be related to cell movement, phagocytosis or inflammatory signal transduction. The roles of cellular movement and signal transduction may also explain why MRP-8/14 is produced directly from vascular epithelium such as that lining the blood vessels within the appendix.

[0058] Regardless of its role in certain inflammatory conditions, MRP-8/14's abundance within cells of acute inflammation makes it an excellent detector and monitor of acute appendicitis. The first step in the inflammatory process is the recruitment of neutrophils and macrophages to a specific site. In our study, the specific site is the appendix, where those MRP-8/14-containing cells will engage the offending stimulus. This engagement will usually result in MRP-8/14 cell death and the liberation of MRP-8/14 from either the cytosol or cell membrane into the patient's circulation. At the same time, the mucosal linings of the appendix will start to produce and release MRP-8/14 to facilitate macrophage migration or inflammatory amplification. This process will then escalate as increasing amounts of MRP-8/14 cells are recruited by the appendicitis to ultimately release more MRP-8/14 into the circulation. Other examples of inflammatory states causing increases of extracellular MRP-8/14 and the tendency of these increases of MRP-8/14 to correlate with extent of inflammation are known. Specifically, chronic bronchitis, cystic fibrosis and rheumatoid arthritis are all associated with elevated serum levels of MRP-8/14 and the severity of these diseases is generally proportional to the serum levels of MRP-8/14 detected.

[0059] The physiological role of MRP-8/14 makes it an ideal clinical marker for acute appendicitis. As patients with appendicitis are generally young and healthy, they generally produce a vigorous inflammatory response. This vigorous response is believed to liberate MRP-8/14 in the earliest stages of the disease, which then escalates as appendicitis progresses. Additionally, the diseases known to be associated with elevated levels of MRP-8/14 are not common in this younger age group and usually do not produce symptomology similar to appendicitis. Finally, as MRP-8/14 is not located in nor associated with lymphocyte proliferation, this marker is not believed to be elevated in viral infections. This is an especially powerful advantage for diagnosing appendicitis, as viral infections are one of the most common imitators of appendicitis.

[0060] Haptoglobin was also identified in this invention as a useful marker for appendicitis. A differential proteomic screen of depleted serum identified haptoglobin as a marker for appendicitis. A second differential screen of appendix tissue confirmed that haptoglobin is upregulated in the appendix tissue of patients with

appendicitis. This finding was confirmed by western blotting of tissue protein. In particular the alpha subunit isoforms were present only in diseased tissue. Since haptoglobin is a plasma protein, it is highly valuable as a biomarker for appendicitis.

EXAMPLES

[0061] Example 1. MRP-8/14.

[0062] The objective of this study was to identify a tissue-specific marker that could contribute to the decision matrix for diagnosing early acute appendicitis. A proteomic screen was used to identify a protein in the appendix specifically upregulated in acute appendicitis. MRP-8/14 was identified as present both in the diseased appendix and in serum of acute appendicitis patients.

[0063] MATERIALS AND METHODS

[0064] Specimen and Serum Collection. All patients enrolled in this study were treated according to accepted standards of care as defined by their treating physicians. Prior to being approached for inclusion in our study, all patients were evaluated by a surgeon and diagnosed by that surgeon as having appendicitis. The treating surgeon's plans for these appendicitis patients included an immediate appendectomy. The specifics of all treatments such as use of antibiotics, operative technique (either open or laparoscopic) were determined by the individual surgeon.

[0065] Exclusion Criteria: Any patients with pre-existing chronic inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease, psoriasis, or neutropenia. Pregnancy was also considered an exclusion criterion.

[0066] An investigator counseled all patients about the study and informed consent was obtained. At the time of informed consent, the subject was assigned an identification number and non-personal demographic and clinical information was obtained (age, sex, race, duration of symptoms, white blood count (WBC), results of imaging studies, etc). At the time of surgery, following induction of general anesthesia, a whole blood sample (5-10 cc volume) was obtained via peripheral venopuncture. This blood specimen was then placed on ice. As soon as possible, a small sample (approximately 1 gram) of inflamed appendix was taken from the

pathologic specimen and also placed on ice. The iced blood specimens were then centrifuged for 20 minutes at 3000 rpm and the separated serum isolated. This isolated serum and the piece of appendix tissue were then stored separately, frozen at -80° C.

[0067] Appendicitis Tissue Processing. Appendix tissue from appendectomy patients was harvested and stored at -80°C until processed. Individual tissue samples were ground into powder using a sterile mortar and pestle under liquid nitrogen. Protein was extracted from tissue powder by incubating at 37°C in Extraction Buffer (0.025M Tris-base, 200 mM Sodium Chloride, 5 mM EDTA, 0.1% Sodium Azide, pH 7.5). Samples were centrifuged for 10 minutes at 14K rpm. Supernatants were stored at -80°C until analysis.

[0068] 2D Gel Analysis of Extracted Tissue Samples. 2D gel analysis was performed on depleted serum samples and extracted tissue samples. Isoelectric focusing (IEF) and SDS-PAGE were performed according to the Zoom (Invitrogen) protocol for 2D Gel analysis. Equal quantities of protein were analyzed on each gel.

[0069] Comparisons between negative serum gel and positive serum gel were made to determine which proteins were present in positive samples and absent in negative samples. Candidate gel spots were identified and submitted to MALDI-TOF protein identification analysis (Linden Biosciences).

[0070] Western Blot Analysis of Extracted Appendix Tissue Samples. Samples (10 µg) were subjected to standard Laemmli SDS-PAGE and proteins were transferred to nitrocellulose membrane for western blot analysis using standard techniques with chemiluminescent detection. Magic Mark Western Standard (Invitrogen) was used to determine molecular weight. MRP-8 (Calgranulin A C-19, Santa Cruz, SC-8112) was used in a 1:100 dilution in 0.5x Uniblock (AspenBio, Inc) for primary antibody. The secondary antibody was Peroxidase anti-goat IgG (H+L), affinity purified (Vector, PI-9500) in a 1:2000 dilution in Uniblock. MR-14 (Calgranulin B C-19, Santa Cruz, SC-8114) was used in a 1:100 dilution in 0.5x Uniblock for primary antibody. The secondary antibody was Peroxidase anti-goat IgG (H+L), affinity purified (Vector, PI-9500) in a 1:2000 dilution in Uniblock.

[0071] Serum MRP-8/14 Determinations. Serum levels of MRP-8/14 were determined by ELISA using a commercially available ELISA (Buhlmann S100-Cellion S100 A8/A9) according to the manufacturer's protocol.

[0072] RESULTS

[0073] Identification of Proteins Present in Appendix Tissue from Appendicitis Patients. A differential proteomic analysis was performed on depleted serum samples with the goal of identifying proteins elevated in patients with acute appendicitis. The analysis involved comparing samples from normal patients versus patients with perforated appendices. Blood samples were obtained immediately prior to surgery. A normal patient in this study is one that presented with abdominal pain, underwent surgery, and was found to have a normal appendix. Normal and diseased appendix tissue was collected during surgery.

[0074] The proteomic approach was to compare a pool of 4 normal samples with a pool of 4 appendicitis samples using two-dimensional electrophoresis. Figure 1 shows the 2D profile of proteins analyzed. Comparison between the gels was performed and the most obvious difference is indicated in Figure 1B as AP- 93. Based on the gel in Figure 1, the molecular weight of AP-93 is approximately 14 kilodaltons. The corresponding gel slice was analyzed by MALDI-TOF and a positive identification was made. The identification was based upon spectra of two tryptic peptides, NIETIINTFHQYSVK [SEQ ID NO:1] and LGHPDTLNQGEFKELVR [SEQ ID NO:2]. The peptides correspond to the underlined residues in the following amino acid sequence of MRP-14 (GenBank Accession Number P06702):

[0075] MTCKMSQLER<u>NIETIINTFHQYSVKLGHPDTLNQGEFKELVR</u>KDLQNFLK KENKNEKVIEHIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEGDEGPGHHH KPGLGEGTP [SEQ ID NO:3].

[0076] The MALDI-TOF identification of AP-93 as MRP-14 was confirmed by the matching molecular weights. Based on this data, MRP-14 protein was more highly abundant in the diseased sample pool than in the normal sample pool.

[0077] Presence of MRP-14 and MRP-8 in Diseased Appendix Tissue. In order to confirm the presence of MRP-14 in diseased tissue, an anti-MRP-14 antibody was

used in western blotting of tissue extracts from individual normal and diseased appendices. Figure 2 shows the western blot data from 9 normal and 11 appendicitis samples. A 14 kilodalton band is present in every appendicitis sample. There is no detectable signal in the normal samples. This data confirms the proteomic screen data and shows that the protein is an indicator of diseased appendix tissue.

[0078] Since it is known that MRP-8 exists as a dimer with MRP-14, tissue specimens were also examined for the presence of MRP-8. Figure 3 shows the western blot data using an anti-MRP-8 antibody on the normal and diseased tissue samples. As expected, MRP-8 is present in all of the diseased appendix samples and not detectible in the normal appendix tissue. These western blot data show that the MRP-8 and MRP-14 proteins are markedly more abundant in appendicitis than in normal appendix tissue.

[0079] Elevated Serum Levels of MRP-8/14 Patients with Acute Appendicitis. The high correlation between appendicitis and the presence of MRP-8/14 in the appendix led us to examine the MRP-8/14 levels in serum of those patients and other patients subsequently added to the study. The sera were collected before surgery, banked and analyzed after the disease status was known. MRP-8/14 levels were measured using a sandwich ELISA specific for the complex.

[0080] Table 1 lists serum MRP-8/14 levels for 39 patients as determined by an ELISA manufactured by Hycult (Netherlands) and available commercially through Cell Sciences, Canton, MA. The amounts are given as fractions compared to an average level for patients in the study without appendicitis. Note that all patients with appendicitis show a fold-increase of MRP-8/14 over average normal levels. The procedure was conducted according to instructions accompanying the ELISA product. The sample numbers do not correspond to the sample numbers shown in Figures 2 and 3 as the samples were renumbered.

TABLE 1

Sample Number	Clinical Diagnosis	Pathology	Grading	Fraction of Normal
1	Advanced Appendicitis	Mild Acute Appendicitis	2	2.80428
2	Normal Appy	**Normal**	1	0.960805
3	Advanced Appendicitis	Transmural Appendicitis	3	5.554904
4	Perforated Appy	Perforated Appy-Necrosis	4	6.53913
5	Early Appy	Mild Acute Appendicitis	2	4.562059
6	Early Appy	Mild-Acute Appendicitis	1	2.881124
7	Horrible perforated	Perforated Appy-Necrosis	4	7.906886
8	Normal Appy	Mild Acute Appendicitis	2	3.971489
9	Early Appy	Transmural Appendicitis	3	3.83328
10	Advanced Appendicitis	Transmural Appendicitis	3	3.566665
11	Appendicitis	Mild Acute Appendicitis	2	3.205335
12	Appendicitis	Transmural Appendicitis	3	5.51224
13	Advanced Appendicitis	Transmural Appendicitis	3	2.92671
14	Advanced Appendicitis	Transmural Appendicitis w Necrosis	4	3.866306
15	PERFORATED	Transmural Appendicitis	3	4.54657
16	Advanced Appendicitis	Perforated Appy	4	7.01877
17	Advanced Appendicitis	Transmural Appendicitis	3	4.25998
18	Appendicitis	Transmural Appendicitis	3	6.90312
19	Normal Appy	**Normal**	1	0.838679
20	Normal Appy	**Normal**	1	0.590095
21	Early Appy	Appendicitis with Peri appy changers	3	1.682291
22	Normal Appy	**Normal**	1	1.128849
23	Advanced appendicitis	Transmural Appendicitis	4	2.338583
24	Normal Appy	**Normal**	1	2.478035
25	Hot appy			2.807046
26	Perforated	Perforated	4	4.954136
27	Normal	**Normal**	1	0.918438
28	Hot	Hot	2	4.387589
29	Early	Transmural Appendicitis	3	4.015013
30	Hot	Transmural Appendicitis	3	2.460902
31	Normal	**Normal**	1	0.594943
32	Hot	Transmural Appendicitis	3	4.211086
33	Perforated	Transmural Appendicitis	4	3.835219
34	Normal	**Normal**	1	1.968859
35	Perforated	Transmural Appendicitis	4	4.126198
36	Advanced	Transmural Appendicitis	3	2.423726
37	Hot Appy	Transmural Appendicitis	3	4.178647
38	Early	Transmural Appendicitis	3	9.584398
39	Normal	Transmural Appendicitis	2	2.835339

[0081] We have identified a protein complex that is present in the appendix and serum of appendicitis patients. Based on the western blot data, the presence of

MRP-8/14 in appendix tissue is highly correlative with disease. Furthermore, levels of MRP-8/14 in serum are predictive of appendicitis. We presume that this increase is due to increased production of these proteins from systemic neutrophil infiltration of the appendix and possibly direct mucosal production of the proteins by the appendix itself. This study demonstrates that MRP-8/14 is a useful clinical marker for acute appendicitis. After our discovery that MRP-8/14 was a molecule differentially associated with appendicitis, our work was confirmed by the finding of Power, C. et al., 2004 and 2005, who reported detection of this molecule in feces of patients having acute appendicitis.

[0082] Example 2. Haptoglobin.

[0083] Using a proteomic screen of serum and appendix tissue, we determined that haptoglobin is upregulated in patients with acute appendicitis. The alpha subunit of haptoglobin is an especially useful marker in screening for the disease.

[0084] MATERIALS AND METHODS

[0085] Specimen and serum collection, appendicitis tissue processing, 2D gel analysis of extracted tissue samples, and western blot analysis of extracted appendix tissue samples were as described above in Example 1, except that for the western blot, affinity-purified anti-human haptoglobin (Rockland, 600-401-272) was used at a 1:5000 dilution in 0.5x uniblock for the primary antibody; and the secondary antibody was peroxidase anti-rabbit IgG (h+l), affinity purified (vector, pi-1000) in a 1:5000 dilution in uniblock.

[0086] RESULTS

[0087] Identification of proteins present in appendix tissue from appendicitis patients. A differential proteomic analysis was performed on depleted serum samples with the goal of identifying proteins elevated in patients with acute appendicitis. The analysis involved comparing samples from normal patients versus patients with perforated appendices. Blood samples were obtained immediately prior to surgery. A normal patient in this study is one that presented with abdominal pain, underwent surgery, and was found to have a normal appendix. Normal and diseased appendix tissue was collected during surgery.

[0088] The proteomic approach was to compare a pool of 4 normal samples with a pool of 4 appendicitis samples using two-dimensional electrophoresis. Figure 5 shows the 2D profile of proteins analyzed from serum depleted of IgG and albumin. Comparison between the gels was performed and the most obvious difference is indicated in Figure 5B as AP- 77. The protein in gel spot AP-77 was digested with trypsin and analyzed by MALDI-TOF. The resulting two peptides have the following sequences: TEGDGVYTLNNEKQWINK [SEQ ID NO:4] and AVGDKLPECEADDGCPKPPEIAHGYVEHSVR [SEQ ID NO:5]. The sequences were aligned with the alpha subunit of haptoglobin. The sequence of haptoglobin precursor (GenBank Accession Number NP005134) is shown below with the tryptic fragments underlined.

[0089] MSALGAVIALLLWGQLFAVDSGNDVTDIADDGCPKPPEIAHGYVEHSVR YQCKNYYKLRTEGDGVYTLNDKKQWINKAVGDKLPECEADDGCPKPPEIAHGYVE HSVRYQCKNYYKLRTEGDGVYTLNNEKQWINKAVGDKLPECEAVCGKPKNPANPV QRILGGHLDAKGSFPWQAKMVSHHNLTTGATLINEQWLLTTAKNLFLNHSENATAK DIAPTLTLYVGKKQLVEIEKVVLHPNYSQVDIGLIKLKQKVSVNERVMPICLPSKDYA EVGRVGYVSGWGRNANFKFTDHLKYVMLPVADQDQCIRHYEGSTVPEKKTPKSPV GVQPILNEHTFCAGMSKYQEDTCYGDAGSAFAVHDLEEDTWYATGILSFDKSCAV AEYGVYVKVTSIQDWVQKTIAEN [SEQ ID NO:6].

[0090] Figure 6 shows the two-dimensional electrophoresis profile comparison between diseased and normal appendix tissue proteins. Two spots, AP-91 and AP-93, were analyzed by MALDI-TOF and positive identifications were determined. AP-91 protein was determined to be identical to AP-77, haptoglobin-alpha.

[0091] Elevated haptoglobin in diseased appendix tissue. In order to confirm the presence of haptoglobin in diseased tissue, an anti-haptoglobin antibody was used in western blotting of tissue extracts from individual normal and diseased appendices. Figure 7 shows the western blot data from 6 normal and 6 appendicitis samples. Nearly every sample contained some level of the 38 kd beta subunit, however, there appeared to be an elevated level in cases of appendicitis. A >20 kilodalton band is present in every appendicitis sample and absent from all of the normal tissue samples. This data confirms the proteomic screen data and shows that the protein is

an indicator of diseased appendix tissue. The alpha subunit has higher specificity than the beta subunit.

[0092] Example 3. Method of identifying molecules using fluid samples.

[0093] In variations of this example, fluid samples can include whole blood, serum, or plasma. The samples are whole blood collected from human patients immediately prior to an appendectomy. The specimens are placed on ice and transported to the lab. The blood is then processed by centrifugation at 3000 rpm for 15 minutes. Plasma is then separated by pouring into another container

[0094] Upon performing an appendectomy, a patient is classified as having appendicitis (AP) or non-appendicitis (NAP). The classification is based on clinical evaluation, pathology, or both as known in the art. For cases of appendicitis, the clinical condition is also characterized as either perforated or non-perforated.

[0095] The samples from AP patients are optionally pooled and divided into aliquots. Optionally, a pooled aliquot is treated so as to remove selected components such as antibodies and serum albumin. Similarly, the samples from NAP patients are optionally pooled and divided into aliquots with optional treatment to remove the same selected components. Preferably the AP samples and NAP samples are processed in a similar manner.

[0096] Next, the pooled aliquots of AP and NAP samples are each subjected to two-dimensional gel electrophoresis as known in the art. The results of each sample type are compared with respect to the presence, absence, and relative expression levels of proteins. Preferably, one detects a signal corresponding to a protein derived from an AP sample that is either absent or expressed at relatively lower levels in a NAP sample. Further characterization is performed for such an AP protein.

[0097] The further characterization can include partial amino acid sequencing, mass spectrometry, and other analytical techniques as known in the art. A full length clone of the gene corresponding to the partial amino acid sequence can be isolated and expressed as a recombinant protein. The recombinant protein can be used as an antigen for detection. Alternatively, a partial or complete recombinant protein can be used to induce or otherwise generate a specific antibody reagent, polyclonal or

monoclonal. The antibody reagent is used in the detection of antigen in a patient so as to aid in appendicitis diagnosis. A combination of antigenic molecules can be employed in appendicitis diagnosis.

[0098] Example 4. Method of identifying molecules using tissue samples.

[0099] Tissue samples are collected from appendicitis (AP) and non-appendicitis (NAP) patients. Preferably the tissue is the appendix. The AP or NAP tissues samples are optionally pooled so as to generate an AP tissue pool or an NAP tissue pool. The AP and NAP tissue samples are each used as a source for isolation of total RNA and/or mRNA. Upon isolation, the AP-RNA and NAP-RNA are maintained separately and used for preparation of cDNA.

[00100] A subtraction library is created using techniques available in the art. A cDNA library is optionally amplified. The cDNA library is treated so as to remove undesirable constituents such as highly redundant species and species expressed both in diseased and normal samples. Examples of the techniques include those described by Bonaldo et al. (1996) and Deichmann M et al. (2001).

[00101] Upon generation of the subtraction library, one analyzes, isolates, and sequences selected clones corresponding to sequences differentially expressed in the disease condition. Using molecular biology techniques, one selects candidates for recombinant expression of a partial or complete protein. Such a protein is then used as an antigen for detection. Alternatively, a partial or complete recombinant protein can be used to induce or otherwise generate a specific antibody reagent, polyclonal or monoclonal. The antibody reagent is used in the detection of antigen in a patient so as to aid in appendicitis diagnosis. It is envisioned that a combination of antigenic molecules can be employed in appendicitis diagnosis

[00102] Example 5. Method of appendicitis diagnosis by evaluation of plasma sample viscosity.

[00103] Whole blood is drawn from a suspected appendicitis patient immediately prior to appendectomy. The specimens are placed on ice and transported to the clinical lab. The blood is processed by centrifugation at 3000 rpm for 15 minutes followed by separation of plasma from the sample by pouring into another container.

[00104] During the step of pouring, the samples are evaluated with respect to viscosity. Increased viscosity is indicative of appendicitis. Approximately 80% of samples corresponding to appendicitis cases demonstrate increased viscosity, whereas approximately none to less than 5% of samples corresponding to non-appendicitis cases demonstrate increased viscosity. It is noted that the degree of increased viscosity can correlate with the severity of appendicitis.

[00105] Viscosity measurements can be conducted by visual observation or by using techniques known in the art. For example, a Coulter Harkness capillary viscometer can be used (Harkness J., 1963) or other techniques (Haidekker MA, et al., 2002).

[00106] The presence of increased viscosity in plasma may be used in combination with other diagnostic techniques, for example with one or more of the following: physical examination, complete blood count (CBC) with or without differential, urinalysis (UA), computed tomography (CT), abdominal ultrasonography, and laparoscopy.

[00107] All references throughout this application, for example publications, patents, and patent documents, are incorporated by reference herein in their entireties, as though individually incorporated by reference, to the extent each reference is at not inconsistent with the disclosure in this application.

[00108] Where the terms "comprise", "comprises", "comprised", or "comprising" are used herein, they are to be interpreted as specifying the presence of the stated features, integers, steps, or components referred to, but not to preclude the presence or addition of one or more other feature, integer, step, component, or group thereof.

[00109] The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention. It will be apparent to one of ordinary skill in the art that compositions, methods, devices, device elements, materials, procedures, techniques, and embodiments, and variations respectively thereof, other than those specifically described herein can be applied to the practice of the invention as

broadly disclosed herein without resort to undue experimentation. All art-known functional equivalents are intended to be encompassed by this invention. Whenever a range is disclosed, all subranges and individual values are intended to be encompassed. This invention is not to be limited by the embodiments disclosed, including any shown in the drawings or exemplified in the specification, which are given by way of example and not of limitation.

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CLAIMS

1. The use of an immunoassay capable of detecting the presence of MRP8/14 for diagnosing appendicitis in a patient suspected of having appendicitis, wherein appendicitis is diagnosed in the patient when the presence of MRP8/14 at a level higher than 10 µg/ml in a fluid sample of blood, serum or plasma obtained from the patient is detected.

2. The use of the immunoassay according to claim 1 in combination with the identification of at least one classical symptom of appendicitis in said patient, said symptom being selected from the group consisting of: pain in the abdomen; pain that starts near the navel, then moves to the lower right quadrant of the abdomen; anorexia (loss of appetite); trouble eating accompanied by sleepiness; nausea starting after onset of pain; vomiting starting after onset of pain; vomiting accompanied by fatigue; constipation; small stools with mucus; diarrhoea; inability to pass gas; low-grade fever; abdominal swelling; pain in abdomen worsening; tenesmus; high fever; leukocytosis; and increased plasma viscosity.

3. The use of the immunoassay according to claim 2 in combination with the identification of two or more said symptoms of appendicitis.

4. The use of the immunoassay according to claim 1, wherein is the sample is a blood sample.

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5. The use of the immunoassay according to claim 1, wherein said sample is a plasma sample.

6. The use of the immunoassay according to claim 1, wherein said sample is a serum sample.

7. The use of the immunoassay according to any preceding claim, wherein the patient is not known to have an interfering condition associated with the presence of said molecule in a sample of the type in which said molecule is tested for.

8. The use of the immunoassay according to claim 7, wherein said interfering condition is selected from the group consisting of recent allograft; septicaemia; meningitis; pneumonia; tuberculosis; rheumatoid arthritis; gastrointestinal cancer; inflammatory bowel disease; skin cancer, periodontitis, pre-eclampsia, and AIDS.

9. The use of the immunoassay according to any preceding claim, wherein at least one additional molecule differentially associated with appendicitis is tested for.

10. The use of the immunoassay according to claim 9 to test for the presence of MRP8/14 using antibody 27e10.

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11. The use of the immunoassay according to any preceding claim, wherein a level of MRP-8/14 higher than about 15 µg/ml of MRP-8/14 is diagnostic of appendicitis.

12. The use of the immunoassay according to any preceding claim, wherein a level of MRP-8/14 higher than about 20 µg/ml of MRP-8/14 is diagnostic of appendicitis.

13. The use of the immunoassay according to any preceding claim, comprising testing for the presence of haptoglobin when testing for the presence of MRP8/14.

14. The use of the immunoassay according to claim 13, wherein a level of haptoglobin higher than 139 mg/dL is tested for.

15. The use of the immunoassay according to claim 13, wherein a level of haptoglobin higher than 150 mg/dL is tested for.

16. The use of the immunoassay according to claim 9, wherein said at least one additional molecule differentially associated with appendicitis is identified selected from the group consisting of: unique structural proteins of the gastrointestinal tract, stress-related inflammatory mediators, immunologic factors, indicators of intestinal bacterial flora, Plasminogen Activator Inhibitor-1, fatty acid binding proteins, nuclear

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factor kappa beta (NFκB), specific appendix antigens (HLA-DR), inflammation associated antigens, and nucleic acids coding for any of the foregoing.

17. The use of the immunoassay according to claim 16, wherein said identification of said molecule is performed by detecting a nucleic acid sequence coding for said molecule in said sample.

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