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(54) Title: TOXIN ASSOCIATED WITH SUDDEN INFANT DEATH SYNDROME, AND USES THEREOF

(57) Abstract: A method diagnostic for Sudden Infant Death Syndrome (SIDS), using immunological methods of detecting the presence of a curli protein or immuno-cross reactive protein or antibody directed against a curli protein in a biological sample taken from a person. The diagnosis of SIDS has important ramification for identification and treatment of persons at risk. Additionally disclosed is a method of vaccination against organism carrying the curli toxin to provide immunity thereagainst to reduce the incidence of SIDS.

TOXIN ASSOCIATED WITH SUDDEN INFANT DEATH SYNDROME, AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to methods of detecting, screening and vaccination against Sudden Infant Death Syndrome.

BACKGROUND OF THE INVENTION

Sudden Infant Death Syndrome (SIDS) accounts for 0.81 deaths for every 1000 live births in Australia (Australian Bureau of Statistics website http://www.abs.gov.au). Although the rate of deaths from SIDS has fallen in a number of countries it still remains a major contributor to post-neonatal mortality. It is thought by some researchers that the decline in SIDS deaths reflects natural variation and this is supported by figures from some countries (eg. Sweden) which show that in recent times the rate of SIDS deaths has returned to earlier levels (Alm *et al.*, 2001).

Recent widespread publicity surrounding SIDS has highlighted the results of epidemiological studies that propose an asphyxial theory of SIDS which encompasses the prone sleep position in the causation model. Prone sleep position remains a leading factor in SIDS (Beal, 2000) and is claimed to cause respiratory obstruction. However it is not a prerequisite for SIDS and many infants succumb in supine and lateral positions. Also, rates of prone SIDS vary considerably geographically and temporally (Beal and Finch, 1991).

Despite extensive studies, the mechanism of death in SIDS still remains unknown. Proponents of a physiological basis for SIDS suggest that respiratory obstruction or sleep apnoea or other mechanistic theories may be causative. However to date a convincing explanation of how prone position contributes to death has been lacking. Asphyxia cannot provide the answer because it contradicts pathological evidence in that the number and distribution of petechiae are very different in the two conditions, and heavy and wet lungs is universal in SIDS and not in asphyxia (Beckwith, 1988; Vlades-Dapena, 1982).

There is also increasing evidence in support of an infection model of the causation of SIDS. A number of independent studies have demonstrated an increased colonisation rate by toxigenic bacteria in the gut and/or nasopharynx of babies who have died of SIDS compared with living or dead controls (Bettelheim *et al.*, 1990; Murrell *et al.*, 1993; Bettiol *et al.*, 1994; Morris, 1999; Siarakas *et al.*, 1999). Histological studies

have also shown that the intestines of SIDS babies show changes to villi and crypts compatible with bacterial enterotoxin damage (Stanton *et al.*, 1980; Variend and Sunderland, 1984).

However to date no known toxin has been detected in SIDS' babies' sera on a consistent or reproducible basis. Evidence of Staphylococcal toxins have sometimes been found in, for example, renal tissue of SIDS babies, however the quest for known toxins of the suspected bacterial candidates has not been successful. As a result it has not hereto before been shown that there is a constant or common "toxin" associated with SIDS. Consequently, there is no immunological approach to diagnosing or vaccinating against SIDS, despite such approaches potentially being both time and cost effective.

OBJECT OF THE INVENTION

The object of this invention is to provide methods for diagnosing SIDS and vaccinating against SIDS that obviates or alleviates any one of the above problems, or at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

The present invention arises out of the inventors findings of a protein that is expressed by certain strains of *E. coli* and is proposed to be a potential causative factor in SIDS. In particular, the present inventors research has shown a correlative link between a protein believed to be the fimbrial curli protein (known as CsgA) of *E. coli* and SIDS. The curli protein can be produced in both soluble and polymerised (fimbrial) forms. Rabbit antiserum raised against the soluble curli protein produced by a curli-producing SIDS *E. coli* isolate was used to detect a protein in the serum of SIDS babies that has the same MW as the curli protein (15.3 kDa).

Previous studies have shown that when the soluble curli protein was injected into mice it produced a profound drop in blood pressure and a lethal shock-like state akin to "septic shock" (Bian *et al.*, 2001). This may indicate that toxic effects associated with SIDS death may be derived at least in part from soluble curli protein.

In one form of a first aspect the invention could be said to reside in a method of diagnosing a person as SIDS by detecting the presence of curli protein in a biological sample taken from the person, the method including the steps of:

taking the biological sample from the person,

contacting the sample with an agent for specifically binding curli protein under conditions for formation of an agent:protein complex, and

assaying for the formation of the agent:protein complex to detect the presence of the curli protein.

In one form of a second aspect the invention could be said to reside in a method of diagnosing SIDS by detecting the presence of antibodies to curli protein, the method including the steps of:

obtaining a sample of antibody containing fluid from a person,

contacting the sample with a protein capable of reacting specifically with the antibody, and

assaying for the formation of the protein:antibody complex to detect the presence of antibodies to the curli protein.

The invention may also be said to reside in a diagnostic kit containing one or more antibodies, or fragments thereof, specific for the curli protein, in conjunction with appropriate reagents.

In a third aspect, the invention could be said to reside in a method of vaccinating a human against SIDS, the method including the step of administering a curli protein, or an immunogenically active derivative or variant thereof, to thereby induce an immune reaction.

In a fourth aspect the invention could be said to reside in a method of vaccinating a person against SIDS, the method including the step of administering a nucleic acid sequence that encodes a curli protein so that a host cell expresses the curli protein to thereby induce an immune reaction.

In a fifth aspect, the invention may be said to reside in a composition for vaccinating a vertebrate against SIDS, the composition comprising curli protein, or immunologically active derivative thereof, and a pharmaceutically acceptable carrier.

The invention may also reside in a pharmaceutical preparation including the therapeutic agent, antibody or antibody fragment defined above in a pharmaceutically effective carrier. The formulation and preparation of any of these pharmaceutical compositions using antibodies, antibody fragments or other compounds is well known to those skilled

in the art of pharmaceutical formulation (see e.g. "Remington's Pharmaceutical Sciences", Sixteenth Edition, Mack Publishing Co, 1980).

By way of a shorthand notation the following three and one letter abbreviations for amino acid residues are used in the specification as defined in Table 1.

Where a specific amino acid residue is referred to by its position in the polypeptide of an protein, the amino acid abbreviation is used with the residue number given in superscript (i.e. Xaa^n)

TABLE 1

Amino Acid	Three-letter Abbreviation	One letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

BRIEF DESCRIPTION OF THE DRAWINGS.

Figure 1a Western immunoblot using anti-CsgA (ZB-AIII) fraction 6 to probe sera from SIDS and other deaths identifying soluble CsgA (curlin) at ~15 kDa. lane 1 SIDS serum (58), lane 2 SIDS serum (59), lane 3 SIDS serum (40), lane 4 SIDS serum (4), lane 5 molecular weight marker, lane 6 SIDS serum (57), lane 7 SIDS serum (60), lane 8 SIDS serum (50), lane 9 SIDS serum (55), lane 10 SIDS serum (62), lane 11 SIDS (51), lane 12 SIDS serum (63), lane 13 SIDS serum (52), lane 14 formic acid extracted soluble CsgA positive control (*E. coli* strain DH1), lane 15 molecular weight marker. The arrow represents 15-17 kDa

Figure 1b Western immunoblot (as performed in Figure 2a), lane 1 SIDS serum (29), lane 2 SIDS serum (30), lane 3 serum from a death resulting from Kawasaki disease (15), lane 4 serum from an asphyxial death (16), lane 5 serum from a SIDS-like death & pathological findings in a case of Pierre Robin Syndrome (19), lane 6 serum from a case of a SIDS-like death & pathological findings in a case of arginosuccinosis (22), lane 7 serum from a case of primary pulmonary hypertension (a coliform was isolated from heart blood culture obtained at autopsy) (31), lane 8 molecular weight marker, lane 9 formic acid extracted soluble CsgA positive control (*E. coli* strain DH1), lane 10 formic acid extract from plate culture of negative control (*E. coli* strain H5). The arrow represents 15-17 kDa

Figure 2 Representation of a PAGE of sera probed with anti csgA. Note the absence of the 15 to 17 kDA protein detected when probed with pre-immune rabbit serum. S=SIDS; HB= Healthy Baby; - = curlin negative control material, - = positive control material

DETAILED DESCRIPTION OF THE EXEMPLIFIED EMBODIMENTS

One form of a first aspect the invention could be said to reside in a method of diagnosing a person for SIDS by detecting the presence of curli protein in a biological sample taken from the person, the method including the steps of:

taking the biological sample from the person,

contacting the sample with an agent for specifically binding curli protein under conditions for formation of an agent:protein complex, and

assaying for the formation of the agent:protein complex to detect the presence of the curli protein.

The method may be used pathologically to assist in determining if SIDS was a cause of death. Alternatively the method may be used as a pre- or antenatal screen to detect for susceptibility to SIDS.

To the best of the inventors' knowledge to date, the protein is the curli protein, CsgA. The amino acid sequence of the soluble curli protein has been deduced (Normark *et al.*, United States Patent No. 6,030,805).

The protein may be any immunologically active derivative of the above sequence, including a smaller peptide derived therefrom providing the smaller peptide retains at least one region that is responsible for specific immunological recognition. Whether a smaller peptide provides immunogenicity can be determined routinely by following the procedures outlined herein.

It is also anticipated that there will be natural variants of the curli protein that will retain the immunogenic properties of the curli protein, and therefore the method of the present invention encompasses diagnoses based on natural variants of the curli protein.

The agent for specifically binding curli protein may be any ligand having a high specificity for binding to the curli protein, including other peptides, proteins, organic molecules and inorganic molecules. In one preferred form the agent is an antibody, or fragment thereof, to the curli protein. Various procedures known in the art may be used for the production of such antibodies and fragments.

The specificity of the agent or antibody is to be understood in the context of requiring a positive result that is distinguishable from a negative result in an immulogically meaningful way.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, 1983). In a preferred method, the antibody

producing cells may be fused with a myeloma cell to produce a pool of hybridoma cells which can then be screened for cells that produce the monoclonal antibody.

Monoclonal antibody fragments may also be used in the above method. Thus, the protein containing biological sample may be contacted with a fragment of a monoclonal antibody specific for the curli protein. It is to be understood that where reference is made to a fragment of a monoclonal antibody the term includes, but is not limited to, Fab, Fv and peptide fragments of the monoclonal antibody, and it may also include such fragments when made as part of a different, larger peptide or protein, which may be the product of a recombinant vector.

Alternatively, additional antibodies capable of binding to the curli protein may be produced in a two step procedure through the use of anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and that, therefore, it is possible to obtain an antibody which binds to a second antibody. Thus, curli protein specific antibodies can be used to immunise an animal and the splenocytes of the animal are used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to block the curli protein specific antibody can be blocked by the protein. Such antibodies comprise anti-idiotypic antibodies to the curli protein specific antibody.

The step of assaying for formation of the antibody:protein complex may include the step of separating the bound antibody:protein complex from unbound antibody. Thus, the raised antibodies, or fragments thereof may be labelled as discussed below. Any antibody:protein complex formed by contacting the sample with the antibody may be separated from unbound antibody using suitable techniques such as immunoprecipitation or techniques for separation based on size. For example, a mixture obtained after contacting the sample with antibody may be filtered through a suitable membrane so that antibody:protein complex is retained on the membrane and unbound antibody passes through the membrane. The labelled antibody:protein complex can then be quantitatively assayed using standard techniques for the label used.

Alternatively the antibodies may be used in an ELISA based assay to bind the curli protein in the sample, and a labelled antibody specific for another immuno-recognition site on the curli protein can be used to assay for bound protein. Thus, the raised antibodies or fragments thereof may be attached or conjugated to a solid support. After contacting the raised antibodies or fragments thereof under conditions suitable for

formation of an antibody:protein complex any component of the biological sample that is not bound to the antibodies or antibody fragments on the support may be washed or otherwise separated from the bound complex.

The assay for the formation of antibody:protein complex preferably involves adding a labelled antibody specific for the curli protein and assaying for the presence of the labelled antibody as discussed below. These assays employ a wide variety of labels including radionuclides, enzymes, fluorescers, chemiluminescers, particles, ligands, enzyme substrates, enzyme cofactors, enzyme inhibitors, light emitter-quencher combinations and the like.

The labelled antibodies may be labelled with a radioisotope, which can then be determined by such means as the use of a gamma counter or a scintillation counter.

Another way in which the antibodies specific for antibodies can be detectably labelled is by linking to an enzyme. This enzyme, in turn, when later exposed to its substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or visual means. Examples of enzymes which can be used to detectably label the antibody include malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase. Avidin-biotin binding may be used to facilitate the enzyme labelling.

It is also possible to label the antibodies specific for antibodies with a fluorescent compound. When the fluorescently labelled antibody is exposed to light of an appropriate wavelength, its presence can be detected due to the fluorescence of the dye. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibodies specific for antibodies can also be detectably labelled using fluorescent emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody molecule using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibodies specific for antibodies can also be detectably labelled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labelling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibodies specific for antibodies. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent antibody is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labelling are luciferin, luciferase and aequorin.

Another technique which may also result in greater sensitivity when used in conjunction with the present invention consists of coupling the antibodies specific for antibodies to low molecular weight haptens. The haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin (reacting with avidin) or dinitrophenyl, pyridoxal and fluorescamine (reacting with specific anti-hapten antibodies) in this manner.

In addition, the sensitivity of the assay may be increased by use of amplification strategies including substrate cycling and enzyme channelling as described by Lindbladh *et al.* (1993).

Other known methods may also be used to assay for levels of curli protein in the biological sample. For example protein expression in tissues can be studied with classical immunohistological methods. In these, specific recognition is provided by the primary antibody but the secondary detection systems can utilise fluorescent, enzyme, or other conjugated secondary antibodies. As a result an immunological staining of tissue section for pathological examination is obtained. Tissues can also be extracted, for example with urea and neutral detergent, for the liberation of the protein for Western-blot or dot/slot assay (Jalkanen *et al.*, 1987).

In one form of a second aspect the invention could be said to reside in a method of diagnosing SIDS by detecting the presence of antibodies to curli protein, the method including the steps of:

obtaining a sample of antibody containing fluid from a person,

contacting the sample with a protein capable of reacting specifically with the antibody, and

assaying for the formation of the protein:antibody complex to detect the presence of antibodies to the curli protein.

The method may be used pathologically to assist in determining if SIDS was a cause of death, or as a screen to test for immunity to SIDS associated toxin and hence SIDS.

The antibody containing fluid may be any biological fluid that contains antibodies including, but not limited to, serum, plasma, whole blood, cerebro spinal fluid, amniotic fluid, and synovial fluid.

In contacting the biological sample with the curli protein, the protein may be attached or conjugated to a carrier molecule or attached or conjugated to a solid support. A solid support in the present invention means any solid material to which the curli protein can be complexed or attached. Examples of such solid supports include, but are not limited to, microtitre plates, petri dishes, bottles, slides, and other such containers made of plastic, glass, polyvinyl, polystyrene, and other solid materials which allow detection of labelled antibodies. Other suitable carriers for binding the curli protein exist or will be able to ascertained by routine experimentation.

After contacting the curli protein under conditions suitable for formation of a protein:antibody complex, any component of the biological sample that is not bound to the protein on the solid support may be washed or otherwise removed from the bound complex.

In a preferred form of the invention the curli protein, or derivative thereof, is covalently or non-covalently bound to the surface of a microtitre well. A serum sample suspected of containing antibody may then be added, unbound sample washed away and the level of antibodies bound in the protein:antibody complex assayed.

The assay for the formation of protein:antibody complex preferably involves adding a compound that enables detection of the antibodies which are specifically bound to the

protein. These assays employ a wide variety of labels and provide for a varying range of sensitivity and susceptibility to interference. Labels include radionuclides, enzymes, fluorescers, chemiluminescers, particles, ligands, enzyme substrates, enzyme cofactors, enzyme inhibitors, light emitter-quencher combinations and the like, as described in the first aspect of the invention. The immunoassays may be homogenous or heterogenous, where the distinction relates to the use of a separation step for separating uncomplexed label from complexed label.

In a preferred form of the invention the assay is an ELISA and labelled anti-antibodies that are specific for the antibodies may be added to bind the antibodies, thereby allowing detection and quantification of the antibodies.

It will be appreciated that the antibodies being assayed may be members of any of the five major classes of antibodies and therefore the diagnostic method encompasses IgA, IgD, IgE, IgG and IgM antibodies and the labelled anti-antibodies for use in an ELISA may be IgA, IgD, IgE, IgG, and IgM anti-antibodies, respectively.

Alternatively, antibodies may be detected by binding with anti-antibodies specific for the antibodies, and then a second labelled antibody specific for the anti-antibody may be used to detect the anti-antibodies. Thus for instance in the case of a human diagnostic test, the antibodies may be detected with labelled rabbit anti-human IgG and then labelled anti-rabbit IgG may be added to bind and detect the anti-human IgG. This latter form of assay is believed to give better specificity in that it tends to give less false positives.

Alternatively, a protein:antibody complex may be used to detect the presence of antibody in the sample. Preferably the antibody that forms part of the complex is labelled with a suitable label.

The invention may also be said to reside in a diagnostic kit containing one or more antibodies, or fragments thereof, specific for the curli protein, in conjunction with appropriate reagents.

In a third aspect, the invention could be said to reside in a method of vaccinating a human against SIDS, the method including the step of administering a curli protein, or an immunogenically active derivative or variant thereof, to thereby induce an immune reaction.

This might be especially useful in vaccinating a pregnant or prospectively pregnant female to potentially provide protection for the progeny.

Several different approaches that may be used to reduce the toxicity of the curli protein to allow safe use as a vaccine include manufacturing a toxoided product. This is attained by chemically altering the protein molecule by reaction with formaldehyde while retaining immunogenicity. This is a tried and true method used to inactivate viruses (e.g. Salk poliovirus vaccine) or bacterial toxin (e.g tetanus and diphtheria toxins). Other methods of reducing toxicity include creating antigenic polypeptide subunits of the protein and if necessary coupling the polypeptide to a suitable carrier protein.

The curli protein may form part of a larger protein by cross linking to a suitable carrier. The carrier may be a carrier protein including but not limited to bovine serum albumin, tetanus toxoid, cholera toxin and deactivated diptheria toxin. For example, the protein may be chemically coupled to the ß-subunit of the heat labile (LT) enteroteoxigenic *E. coli* (ETEC) toxin which has previously been used as a carrier protein for other bacterial epitopes.

Alternatively the carrier may be an organic molecule, or a solid phase carrier. In one preferred form the carrier is a Ni-NTA (nickel-nitriloacetic acid) agarose to which the protein is bound through a $(Histidine)_6$-tag at either the N or the C terminus. In another form the protein may be displayed on bacteriophage particles. Thus nucleic acid vectors are used wherein an oligonucleotide encoding the protein is fused to a portion of a gene encoding the transmembrane portion of an integral protein. Upon expression of the fusion protein it is embedded in the outer cell membrane with the curli protein portion of the fusion protein facing outward. Thus the protein is linked to a solid support which is the bacterial cell itself.

Preferably the protein is administered in pharmaceutical dosage form as a composition or formulation comprising an immunogenically effective amount of the protein. The amount of protein administered will vary depending on the pharmacokinetic parameters, severity of the disease treated or immunogenic response desired. Doses may be set by a physician considering relevant factors including the age, weight and condition of the person including, in the case of immunogenic dosage forms, whether the person has been previously exposed to the microorganism responsible for the disease to be vaccinated against as well as the release characteristics of the protein.

The person may be vaccinated directly according to the method of the invention, or alternatively a progeny may be passively vaccinated by maternal vaccination.

In one specific form the protein has the sequence set out in United States Patent number 6030805 to Nomark *et al.*

Alternatively the protein may be homologous to the above sequence. In this context, a protein is considered homologous when it is immuno cross-reactive with antibodies specific for the protein. It will be recognised by those skilled in the art that some amino acid sequences within the protein can be varied without significant effect on the structure or function of the protein. Thus, for instance, it is anticipated that conservative amino acid substitutions still retain immuno cross reactivity and as such a neutral amino acid may be conservatively substituted with another neutral natural or non-natural amino acid, an acidic amino acid may be conservatively substituted with a natural or non-natural acidic amino acid, a hydrophilic amino acid may be substituted with another hydrophilic amino acid, and so on, provided that the immunological function of the protein is not altered by the substitution.

Typically seen as conservative substitutions are the replacement of one for another among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitutions between the amide residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among the aromatic residues Phe and Tyr. Preferably the homologous protein shares 50% homology with the above sequence, more preferably shares 70% homology, and most preferably shares 90% homology.

In this form of the invention it might be convenient that the curli protein is expressed on a bacterial cell surface. Whole live, or killed bacteria may be delivered in an appropriate form per orally. Alternatively semi purified curli protein may be administered. It will be understood that the administration of live or killed bacteria or partially fractionated bacteria will be in a manner known in the art and is likely to be delivered in an appropriate excipient and may or may not require delivery in a protective capsule or matrix that resists degradation in the stomach of the individual to the be treated. The bacterial for delivery should be a non-harmful bacterium.

In a fourth aspect the invention could be said to reside in a method of vaccinating a person against SIDS, the method including the step of administering a nucleic acid sequence that encodes a curli protein so that a host cell expresses the curli protein to thereby induce an immune reaction.

The techniques for cloning and expressing nucleic acid sequences that encode the amino acid sequences corresponding to the curli protein, for example synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems and the like are well known in the art and standard reference materials may be consulted for specific conditions and procedures (Sambrook *et al.*, eds., Molecular Cloning, A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, 1989).

The nucleic acid sequence may be any sequence disclosed in United States Patent No. 6,030,805 to Nomark *et al.*, which is incorporated herein specifically for the purpose of providing nucleic acid sequences.

The nucleic acid sequence may encode a chimeric protein in which the curli protein is tagged into the protein, wherein the chimeric protein is expressed by a suitable host.

In one specific example, a mutagenised gene encoding the chimeric protein and expressing the protein may be inserted into a suitable DNA vaccine vector such as pcDNA3 which is a mammalian expression vector often used in DNA vaccines.

The method may also include the step of assaying for the presence of antibodies to the curli protein to ensure that there is an immune reaction to the expressed protein. The assay for antibodies may be any of the assays discussed with respect to the first and second aspects of this invention.

In a fifth aspect, the invention may be said to reside in a composition for vaccinating a vertebrate against SIDS, the composition comprising curli protein, or immunologically active derivative thereof, and a pharmaceutically acceptable carrier.

The composition may be added to the pharmaceutically acceptable carrier as will be apparent to those skilled in the art and as set out in "Remington's Pharmaceutical Sciences", Sixteenth Edition, Mack Publishing Co, 1980, and include water and other polar substances, including lower molecular weight alkanes, polyalkanols such as ethylene glycol, polyethylene glycol and propylene glycol as well as non-polar carriers.

The method of administering the vaccine may vary and could include intravenous, buccal, oral, transdermal and nasal as well as intradermal, intramuscular or subcutaneous administration.

Other compounds capable of binding the curli protein may be isolated by screening for binding thereto. For example, a scramble of randomly synthesised compounds could be passed through a solid matrix to which the curli protein is bound. Following washing the strongly binding compounds remain and can be eluted and characterised using standard techniques. The screening may also be a competitive binding screen used to identify compounds that bind the curli protein in preference to a monoclonal antibody specific for the curli protein.

The nature of the compounds obtained by screening is not limited and may include, but is not limited to, peptides, oligonucleotides, amino acids, nucleic acids or sugars. The methods used for the binding assay can be any one of the many common techniques known to those skilled in the art. Such methods may include affinity selection chromatography, ultrafiltration assays, the scintillation proximity assay, interfacial optical techniques, the quartz crystal microbalance, the jet ring cell, interferometric assays using porous silicon to immobilise the receptor. Reference to such techniques can be found in Woodbury *et al.*, 1999. By way of example, a scramble of randomly synthesised oligonucleotides could be passed through a solid matrix to which the curli protein is bound. Following washing the strongly binding oligonucleotides remain and can be eluted under different conditions (salt, pH etc). The sequence can be determined by PCR and tested for inhibition of curli protein.

It is to be understood that where reference is made to a fragment of a monoclonal antibody the term includes, but is not limited to, Fab, Fv and peptide fragments of the monoclonal antibody, and it may also include such fragments when made as part of a different larger peptide or protein, which may be the product of a recombinant vector. Thus the variable region of the respective monoclonal antibody may be cloned and be made part of a hybrid protein with properties appropriate for the therapeutic purposes of the respective agent. Thus for example the monoclonal antibody may be "humanised" by recombining nucleic acid encoding the variable region of the monoclonal antibody with nucleic acid encoding non-variable regions of human origin in an appropriate expression vector.

The invention may also reside in a pharmaceutical preparation including the therapeutic agent, antibody or antibody fragment defined above in a pharmaceutically effective carrier. The formulation and preparation of any of these pharmaceutical compositions using antibodies, antibody fragments or other compounds is well known to those skilled in the art of pharmaceutical formulation (see e.g. "Remington's Pharmaceutical Sciences", Sixteenth Edition, Mack Publishing Co, 1980).

EXAMPLES

While a toxigenic cause of SIDS has been considered and investigated by several groups worldwide, and a variety of candidate bacteria including *Clostridium* spp., *Staphylococcus aureus* and *Escherichia coli* as well as chemical toxins have been considered, there has never before been shown a consistent or common "toxin" associated with SIDS. This specification discloses evidence for a protein expressed by *E. coli* that could, on the basis of its physiological effect, cause SIDS. Because of the strong evidence linking *E. coli* and SIDS and the fact that no specific known *E. coli* toxin has been consistently found in SIDS strains, a study was made to look for unusual virulence/pathogenicity factors that could provide *E. coli* with the necessary lethal qualities. The gene encoding the curli structural subunit (crl) is present and transcribed in most natural isolates of *E. coli* but only certain strains are able to assemble the protein subunit into curli. (Olsen *et al.*, 1989). Because curli play a major role in determining urinary tract pathogenicity of *E. coli* strains and that curliated strains have a profound effect on blood pressure and induce significant hypotension when curliated bacteria are injected into mice via the intraperitoneal route (Bian *et al.* 2001), a role for curli-producing strains in the causation of SIDS was explored by investigation of curli production of a random selection of *E. coli* strains collected from SIDS babies, dead controls and healthy babies.

IDENTIFICATION OF A SIDS RELATED TOXIN

A random selection of *E. coli* strains, maintained on Columbia agar slopes, which had been collected from the small and large intestines of 92 SIDS babies, 17 dead control babies and 91 healthy baby stools were examined for curlin production using cultural methods. Two types of Congo-red agar i.e. Congo-red-magnesium oxalate agar (as described by Riley & Toma , 1989) and YESCA agar (as described by Hammar *et al.* 1996) plates were incubated at 26°C for 48 hours and examined for the typical appearance of colonies taking up the Congo-red dye representing curli-positivity.

All 92 of the SIDS *E. coli* isolates produced curli protein when grown for 48h on Congo Red Agar at 26°C. Significantly fewer control isolates from non-SIDS deaths (13 of 17 individual isolates) (76.5%; Chi square 17.79; p=0.00002) and significantly fewer strains from healthy babies (74 of 91 individual isolates) (80.4%; Chi square 16.3; p=0.00005) produced curli compared with SIDS strains.

TESTING OF SIDS SERA USING A CURLI-SPECIFIC ANTISERUM

The strong association between curli production and *E. coli* strains from SIDS cases indicated that sera from SIDS cases might contain soluble curlin antigen (CsgA). Therefore sera from SIDS cases and age-matched controls were examined immunochemically for the presence of CsgA.

Heart blood samples from SIDS and babies who died of other causes were obtained following approval for this work from the Women's & Children's Hospital Research Ethics Committee. The work complied fully with the South Australian legal requirements relating to the retention of tissues obtained at autopsy.

Dot-immunoblots of SIDS and control sera were prepared by spotting 0.5µL volumes of serum diluted in native sample buffer (NSB) onto nitocellulose membranes (0.45µ pore size) (Invitrogen). After fixing in transfer buffer containing 10% methanol and then blocking with 5% skim milk in tris buffered saline (0.05% tween) (TBST), the antigens were incubated overnight at room temperature with gentle rocking with a 1:2000 dilution (in TBST) of affinity-purified rabbit anti-CsgA (ZB-AIII) kindly provided Dr Zhoa Bian, Karolinska Institute, Sweden. Pre-immune rabbit antiserum at the same dilution was used as negative control.

After washing, the membranes were treated with HRP-conjugated goat anti-rabbit immunoglobulins (1:2,500) (Dako A/S, Denmark, code P0448) for 2 hours and washed in TBS and colour developed using One Step™ TMB-Blotting (No. 34018) Pierce, Rockford, IL 61105. The titre of CsgA activity in each serum specimen was determined following 2-fold dilutions in NSB.

Western Blots were performed using 4% and 4-20% tris-glycine precast 10-well 1.5mm gels (Invitrogen) to perform native polyacrylamide gel electrophoresis (PAGE) of SIDS sera and E. coli positive soluble curlin control antigen, prepared by solubilising a loopful of a 48 h CFA agar[14,15] (Hammar *et al.*, 1996; Evans *et al.*, 1997) growth of a known curli-positive E. coli K12 strain (DH1) in 100µL 90% formic acid. We

microfuged the solution at 13,000 rpm for 10 minutes and added 10μL of supernatant to 30μL of native sample buffer (NSB). We added 20μL volumes of SIDS sera to 20μL of NSB and subjected them to the same centrifugation; we loaded 25μL volumes of supernatant onto gel slots as well as a 20μL molecular weight marker (Multimark ™ Novex, San Diego, California). We ran gels at 125V, 20mA for 1 h 15 min and transferred onto Hybond-N+ nylon transfer membrane, 0.45μ pore size (Amersham Pharmacia Biotech, Little Chalfont, Bucks, England) at 25V, 120mA for 1h. We blocked membranes with 5% skim-milk/TBST and incubated overnight at room temperature in a 1 in 5000 dilution of anti-CsgA (ZB-AIII) and then treated as described above for the dot-immunoblots.

All 68 SIDS sera tested in a dot-immunoblot assay using fraction 6 of affinity-purified rabbit-anti-CsgA at 1 in 2000 gave positive reactions indicating the presence of CsgA curli protein in these sera and 45 gave titres up to the last dilution of 1:64. Further titration yielded two with titres of 1: 32,768. Six babies' sera had coliforms grown from heart blood cultures obtained at autopsy. The isolates and respective titres were *E. coli* O66:H1 1:4, *E. coli* O16:H48 1:512 and *E. coli* O4:H1 1:4096, coliform (unspecified) 1:64, coliform (unspecified) 1:512 and coliform (unspecified) 1:32,768. . Forty-one sera, sterile at autopsy, yielded the following respective titres: four (1:4), three (1:8), three (1:16), four (1:32), sixteen (1:64), four (1:128), three (1:256), and four (1:512). Typical contaminants (coagulase negative staphylococci, *Micrococcus* spp. diphtheroids, etc.) were found in seventeen heart blood cultures and their respective number and titre were as follows: one (1:4), one (1:8), four (1:16), one (1:32), two (1:64), four (1:512), two (1:4096) and two (1:32,786). Heart blood cultures were not performed in four babies. Age-matched control sera from 61 healthy babies were all negative for curli protein. Fourteen sera obtained from non-SIDS deaths (n = 21) tested negative including five with sterile heart blood cultures. Seven of the 21 sera from non-SIDS deaths gave positive results. Of the positive babies, one had a coliform, and one had a Gram positive coccus isolated from heart blood obtained at autopsy. Three cases of the seven (above-mentioned) non-SIDS deaths had sterile heart blood and the remaining two had no blood culture performed. There was no relationship between post-mortem interval and titre of curlin. No sera gave positive reactions after membranes had been incubated with pre-immune rabbit serum. Anti-CsgA treated transfer blots of SIDS sera showed 15 kDa bands clearly identifying CsgA in these sera, confirming the identity of the dot-immunoblot reactions. The sera, which upon sterility testing, grew low numbers of typical contaminants and environmental organisms gave negative reactions in colony blot assays.

VACCINE DEVELOPMENT

Curli protein is known to be immunogenic through the raising of antibody in rabbits. Given its evident immunogenicity, vaccine development would entail the three standard phases involving 1) immunogenicity studies and challenge/protection studies in an animal model; 2) definition of safety and side-effects in animals, and 3) human trials for safety, tolerability, immunogenicity and protective efficacy.

Several possible presentations of the vaccine (as outlined elsewhere) could be used (e.g. an injectable toxoid vaccine, a live attenuated oral bacterial vaccine, an injectable DNA vaccine). The target population is babies at risk for SIDS (mainly babies 2-4 months old, in lower socioeconomic group, born premature with low birth weight/gestational age, etc.). Several approaches to achieve immunity in this target population includes immunisation of potential mothers, pregnant women, or new born babies. Mothers would convey transplacental antibody in babies of full term gestational age. Premature babies (<34 weeks gestation) may have lower levels of maternally acquired antibody and may therefore have less passive immunity than full term infants. Immunisation of all babies at birth would provide the best opportunity for protection. Premature babies would develop best protective antibody if immunisation was delayed until they achieved full-term implied gestational age. In Australia there are some 200,000 births annually. Add this to annual births in developed countries (in which SIDS accounts for most post-neonatal deaths), the potential number of vaccinees and thence preventable deaths is very large.

Table. Summary of findings for sera from SIDS, other deaths and healthy babies

Diagnosis	n	mean age *(SD)*	M:F sex ratio	No. with sterile blood culture	No. coliform bacteria in blood culture	No. blood culture not done	Curlin +ve in dot immunoblot	Reciprocal mean GMT *(SD)*
SIDS	68	3.02 *(4.6)*	1.95	41	7	5	68	1273 *(5587)*
other death	21	8.75 *(2.8)*	1.1	10	2	5	7	3.9 *(8.1)*
healthy baby	42	3.5 *(2.8)*	0.67	nd	nd	nd	0	nt

nd (not done); nt (not tested); SD (standard deviation); GMT (geometric mean titre).

DISCUSSION

Examination of the risk factors for SIDS derived from multiple epidemiological studies points to infection or vulnerability (increased susceptibility) to infection being important. Much evidence links the risk factors for SIDS (derived from multiple epidemiological studies) to infection or vulnerability (increased susceptibility) to infection. One recent finding is that 70% of SIDS babies carry a particular allele of the IL-10 gene, IL-10-592*A allele (Summers *et al.*, 2000), which results in a low-producer haplotype. IL-10 is important in immunity against bacterial toxins and a low-producer state could predispose the baby to these toxins. Pre-natal risk factors for SIDS include intrauterine growth retardation/lower birth weight, multiple births (twin/triplet), shorter gestational age, maternal smoking (independent risk factor), and urinary infections (mainly *E. coli*) in pregnancy. Neonatal and postneonatal risk factors for SIDS strongly suggest infection; these include illness (mainly respiratory viral [cough] or gastrointestinal [diarrhoea]), listlessness/droopiness, poor health in last week, hospital admissions, GP & outpatient attendances in the weeks prior to death, sweatier than usual (probable fever), decreased feeding (frequency & demand for food) in last days, peak age 2-4 months (corresponding with declining passive maternal immunity), never breast-fed (but several studies have failed to show bottle feeding to be an independent risk factor), previously used mattress, poor socioeconomic conditions, ethnicity (genetic predisposition/susceptibility), and passive smoking. Several maternal and environmental factors also reflect potential infective connotations. Moreover, poor socioeconomic conditions, low educational attainment and overcrowding are contributors to most communicable diseases by virtue of the fact that hand contamination with these agents is more likely to occur than in those with higher educational levels and better uncrowded conditions. The above risk factors are easily explainable on the basis of infection *per se* or increased susceptibility to infection (contaminated environment, loss of maternal antibody, immaturity of immune responses). Viral infections potentiate bacterial toxins as do nicotine and compounds in cigarette smoke. Furthermore, bacterial toxins synergistically potentiate each other in terms of lethality. Winter seasonality for SIDS also supports a contributory role of viruses. Pathological evidence of acute viral infection is evident in lymph nodes from cases of SIDS.

To date, a convincing explanation of how prone position contributes to death has been lacking. Cases of SIDS captured on 24 hour computerised memory-monitors (tracing pulse, temperature, respiratory rate and blood pressure) show asphyxia was an impossibility. Furthermore, supine sudden infant deaths have not been adequately

addressed. A mechanism of death accounting for all sleep positions is thus required. An explanation to cover all sleep positions can be explained on the basis of possible differences in rates of delivery to the systemic circulation of gut-derived lethal toxin. Absorbed toxin may reach the circulation via the portal system which takes it to the liver. Fatty change (for which toxaemia is a cause) is a frequent finding in the livers of SIDS babies. A second, but most important route of toxin absorption would be via the lymphatic system and the thoracic duct. Toxin would be delivered via the duct to the Innominate vein and thence to the right side of the heart. The first organ exposed to toxin would be the lungs followed by the heart and the thymus. These are the organs in which classical pathological findings in SIDS are seen (petechiae, and wet/heavy lungs). Bacterial toxins/products can perturb basement membranes of small blood vessels leading to the small haemorrhages (petechiae) seen in SIDS. The pattern of distribution of petechiae is distinctive and could be explained by the fact that these are the first organs exposed (as explained above) or that these organs are replete with toxin receptors. This hypothesis explains why more deaths occur in the prone position in which a lethal amount of toxin is delivered; and in other positions, a usually sublethal dose is delivered to the systemic circulation. Other pathological findings in SIDS include liquid (unclotted) blood within the chambers of the heart, elevated cross-linked fibrin degradation products (seen in toxaemia/sepsis) and an empty bladder' suggesting decreased renal perfusion (toxaemic shock) during the last sleep. Several organs are significantly heavier in SIDS compared with other causes of death. These include the lungs, heart, thymus, liver spleen and brain (Seibert and Haas, 1994). This finding is consistent with capillary leakage common in bacterial toxaemias. A model for SIDS is infant botulism, a disease in which colonising bacteria produce toxin which is absorbed from the gut and distributed in the systemic circulation, resulting finally in profound paralysis and death (if untreated). In infant botulism, toxin is demonstrated in the blood and the organism is isolated from faeces. A number of cases of infant botulism have been misdiagnosed as SIDS.

As mentioned above, support for a toxaemic mode of death in SIDS comes from memory-monitored deaths. The picture conveyed from these SIDS deaths "captured" on monitors, is of an initial pyrexia, tachycardia and tachypnoea followed by profound bradycardia and hypotension (a shock-like state). Cardiac standstill is usually followed by gasping and then cessation of respiration. There was no evidence of asphyxia.

In summary, the epidemiological risk factors for SIDS are consistent with an infectious aetiology and exposure to an infectious agent and/or a susceptibility to infection. The

toxicity of SIDS sera and the potentiating effects of virus infection and nicotine are consistent with a bacterial toxin causation. Enteric toxigenic bacteria (*E. coli* and/or *Cl. perfringens and/or S. aureus*) are good candidates and are found in a large proportion of SIDS cases but to a much lesser extent in living and dead controls. The consistent pathological findings suggest a specific toxin. The prone position may enhance toxin delivery to the systemic circulation and the heart. The above hypothesis also accommodates deaths occurring in the supine and lateral positions reinforcing the point that proneness is not a prerequisite for SIDS. Memory-monitored deaths indicate that toxaemic shock and cardiac standstill are the final events.

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CLAIMS

1. A method of diagnosing a person as SIDS by detecting the presence of curli protein in a biological sample taken from the person, the method including the steps of:

taking the biological sample from the person,

contacting the sample with an agent for specifically

binding curli protein under conditions for formation of an agent:protein complex, and

assaying for the formation of the agent:protein complex to detect the presence of the curli protein.

2. A method of diagnosing a person for SIDS as in claim 1 wherein the person is dead and the method is used to assist in determining if SIDS was a cause of death.

3. A method of diagnosing a person for SIDS as in claim 1 wherein the method is used as a pre- or antenatal screen for susceptibility to SIDS.

4. A method of diagnosing a person for SIDS as in claim 1 wherein the method is used as an antenatal screen for susceptibility to SIDS.

5. A method of diagnosing a person for SIDS as in claim 4 wherein the sample is taken within 6 months of birth.

6. A method of diagnosing a person for SIDS as in claim 4 wherein the sample is taken within the period between about 2 and 4 months after birth.

7. A method of diagnosing a person for SIDS as in claim 1 wherein the sample is a body fluid.

8. A method of diagnosing a person for SIDS as in claim 1 wherein the sample is blood.

9. A method of diagnosing a person for SIDS as in claim 1 wherein the sample is material from the person's gastrointestinal tract.

10. A method of diagnosing a person for SIDS as in claim 1 wherein the curli protein originates from an organism of the species *Escherichia coli*.

11. A method of diagnosing a person for SIDS as in claim 1wherein the agent is an antibody, or fragment thereof.

12. A method of diagnosing a person for SIDS as in claim 11 wherein the antibody is polyclonal.

13. A method of diagnosing a person for SIDS as in claim 11 wherein the antibody is monoclonal.

14. A method of diagnosing a person for SIDS as in claim 1 wherein the assay for the formation of antibody:protein complex is a direct assay and involves adding a labelled antibody specific for the curli protein and assaying for the presence of the labelled antibody.

15. A method of diagnosing a person for SIDS as in claim 14 wherein the assay is an enzyme linked immuno assay.

16. A method of diagnosing SIDS by detecting the presence of antibodies to curli protein, the method including the steps of:

obtaining a sample of antibody containing fluid from a person,

contacting the sample with a protein capable of reacting specifically with the antibodies, and

assaying for the formation of the protein:antibody complex to detect the presence of antibodies to the curli protein.

17. A method of diagnosing a person for SIDS as in claim 16 wherein the person is dead and the method is used to assist in determining if SIDS was a cause of death.

18. A method of diagnosing a person for SIDS as in claim 16 wherein the method is used as an antenatal screen for susceptibility to SIDS.

19. A method of vaccinating a human against SIDS, the method including the step of administering a protein to the human in a form capable of eliciting an immune reaction to produce antibodies capable of neutralising a curli protein.

20. A method of vaccinating a human against SIDS as in claim 19 wherein the human is a pregnant woman or a prospectively pregnant woman.

21. A method of vaccinating a human against SIDS as in claim 19 wherein the protein is a curli protein.

22. A method of vaccinating a human against SIDS as in claim 19 wherein the protein is the CsgA protein.

23. A method of vaccinating a human against SIDS as in claim 19 wherein the protein is immuno cross reactive with the curli protein.

24. A method of vaccinating a human against SIDS as in claim 19 wherein the protein is the CsgA protein with one or more conservative amino acid substitutions.

25. A method of vaccinating a human against SIDS as in claim 21 wherein the toxicity of the curli protein is reduced.

26. A method of vaccinating a human against SIDS as in claim 19 wherein the protein is linked to a carrier protein.

27. A method of vaccinating a human against SIDS as in claim 19 wherein the curli protein has been expressed on the surface of a bacterium, and a preparation of whole live or dead bacteria are delivered orally to elicit the immune reaction.

28. A method of vaccinating a human against SIDS as in claim 19 wherein the curli protein has been expressed on the surface of a bacterium, and a preparation of fractionated bacteria are delivered orally to elicit the immune reaction.

29. A method of vaccinating a person against SIDS, the method including the step of administering a nucleic acid molecule that encodes a curli protein so that a host cell expresses the curli protein to thereby induce an immune reaction.

30. A method of vaccinating a person against SIDS as in claim 29 wherein the method includes the step of administering the nucleic acid to a host cell when present in the person.

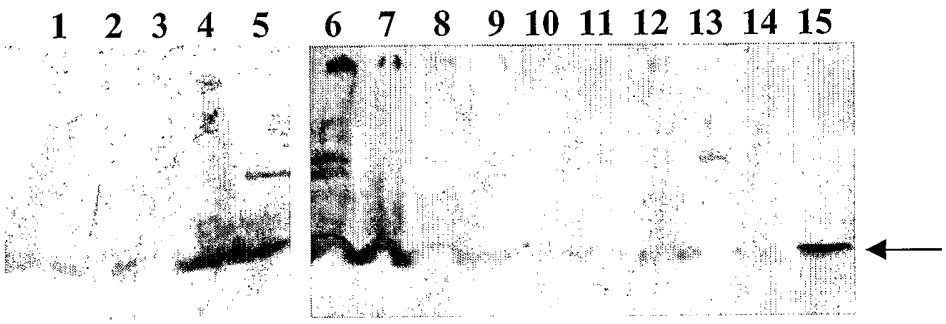

FIGURE 1A

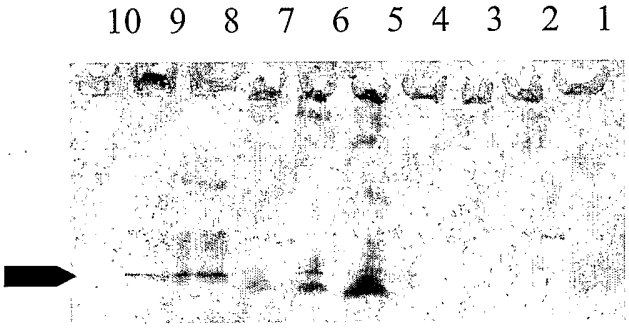

FIGURE 1B

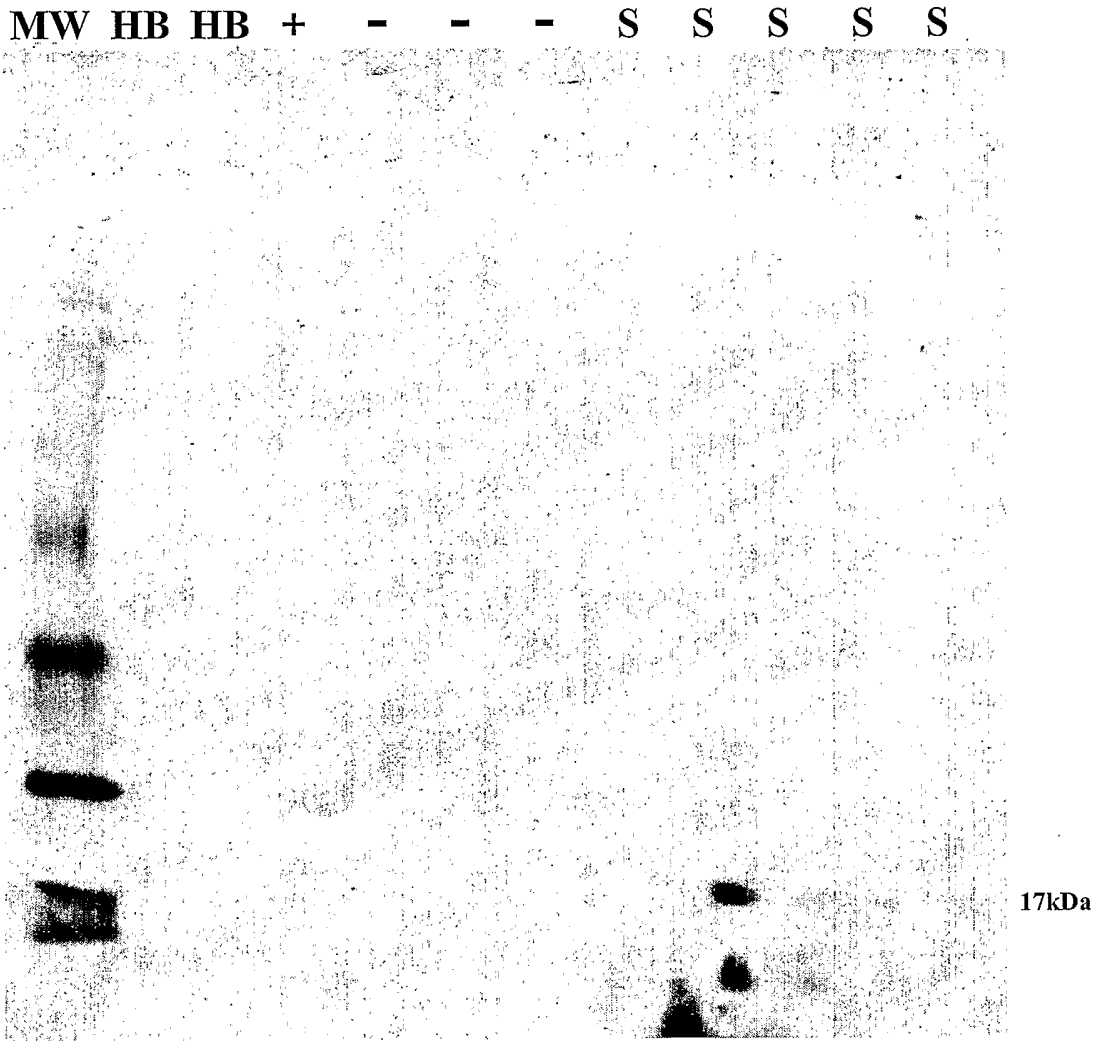

FIGURE 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01085

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 39/395 A61P 31/04, 37/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Electronic database as indicated below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, WPAT: curli protein, CsgA, AgF, fibronectin binding protein, ZB-AIII, SIDS and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Bettiol, S et al (1994), Epidemiology & Infection, 112/2 pages 275-284 "Bacterial flora of Tasmanian SIDS infants with special reference to pathogenic strains of *Escherichia coli*" -see Abstract, Table 1, Discussion	1-30
Y	Bettelheim, K et al (1990), Scand. J. Infect. Diseases 22/4 pages 467-476, "Toxigenic Escherichia coli Associated with Sudden Infant Death Syndrome" -see Abstract, page 469 and Discussion	1-30
Y	EP-A-0342173 (Normark, S) 15 November 1989 -see Abstract, page 8 lines 5-13, page 8 line 42-page 9 line 3, claims 14, 15	1-30

☐ Further documents are listed in the continuation of Box C ☒ See patent family annex

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU02/01085

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member					
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	US	2002081722	US	6030805	AU	55907/94
	CA	2148479	FI	952108	NO	951679
	WO	9410330				

END OF ANNEX