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(54) Title: ANTIBODY PRODUCTION METHOD

(57) Abstract: A method of inducing the sustained release of antibodies in milk comprising the step of: a) implanting at least one antigen releasing device adjacent to, within close proximity of or within at least one supramammary lymph node, wherein in use the antigen releasing device releases an antigen into the tissue area around the supramammary lymph node which stimulates antibody secretion into a mammary gland.



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"Antibody Production Method"

Field of the Invention

The present invention relates to a method for inducing a sustained production of antibodies or immunoglobulins. More particularly, it relates to a method of
5 inducing a sustained production of antibodies in the milk of a mammal.

Background Art

The advantage of producing antibodies in milk over the traditional source of antibodies from blood lies in the constancy of supply through the daily access to milk. After blood products are harvested, the production animal is allowed to
10 recover for extended periods (for example, up to one month). Hence, the yield of antibodies is limited (approximately 3 to 30 mg per month) due to the finite amount of blood that can be obtained. In contrast, for example, dairy animals milked daily results in significant increases in yield (eg 2 mg per day multiplied by 30 days, gives 150 mg per month).

15 Injections or infusions directly into the teat of the mammal have previously been used to stimulate the production of antibodies in milk. Such examples are set out in the following literature: J.L. Smith, J.S Hogan & K.L Smith (1999) "Efficacy of intramammary immunization with an Escherichia coli JS bacteria." Journal of Dairy Science, 82:2582-2588; J.S Hogan, K.L Smith, P.Schoenberger, S. Romig
20 & L Thompson (1997) "Response of antibody titres to intramammary immunization with Escherichia coli JS bacteria." Journal of Dairy Science, 80: 2398-2402; F.J Bourne, T.J Newby & J.W Chidlow (1975) "The influence of route of vaccination on the systemic and local immune response in the pig." Research in Veterinary Science, 18:244-248. Such injections are time consuming as they must be
25 repeated to re-stimulate antibody production. Furthermore, injection directly into the teat of a mammal frequently results in infections such as mastitis.

Intramammary immunisation techniques have generally not been preferred as a route for vaccination under field conditions due to the high chance of mammary

infection (R. F. Sheldrake (1987) Australian Journal of Dairy Technology, 42:30-32) and often requires application by highly skilled practitioners.

5 It should be noted that much of the published literature concerning immunoglobulin production in mammary gland secretions is directed to disease prevention (that is, vaccination) in animals or their offspring. Few are directed to the production of immunoglobulin enriched milk for the purposes of obtaining the immunoglobulins themselves.

Summary of the Invention

10 In a first aspect of the invention, there is provided a method for inducing the sustained release of antibodies in milk comprising the step of: implanting at least one antigen releasing device adjacent to or within at least one supramammary lymph node, wherein in use the antigen releasing device releases an antigen into the tissue area around the supramammary lymph node which stimulates antibody
15 secretion into a mammary gland.

According to a second aspect the invention also provides antibodies produced by any one of the methods of the present invention.

According to a third aspect the present invention relates to a method for the production of milk containing antibodies which method comprises induction of
20 antibodies according to the method detailed above and then collecting the antibody containing milk from the mammal. The collection of milk may be effected using normal milking processes.

Other objects, features, and advantages of the instant invention, in its details as seen from the above, and from the following description of the preferred
25 embodiment.

Brief Description of the Drawings

Figure 1 shows a photograph of the supramammary gland stained blue due to migration of dye inoculated in the groin area of the animal (photo courtesy of Dr Martin CAKE, Anatomy Department Murdoch University).

5 **Figure 2** shows a photograph of the intranasal immunisation of a goat.

Figure 3 shows a photograph of the implantation of an antigen releasing device in accordance with an aspect of the present invention into the groin of a sheep.

Figure 4 shows the location of the implanted antigen releasing device of Figure 3 in the sheep.

10 **Figure 5** shows a schematic diagram of the apparatus used to implant the antigen releasing device.

Figure 6 shows a photograph of the diffusion of a lipase protein from an antigen releasing device in accordance with an aspect of the present invention into a milk agar plate. As the enzyme diffuses from the pores of the tube, it hydrolyses the
15 lipids in the milk agar, as evident from the dark zones around the rod. The lipase protein was used as the model antigen for the development of the invention.

Figure 7 shows a graph of the level of anti-lipase antibodies in milk from goats immunised with different protocols. The level of anti-lipase antibodies in milk from goats immunised with different protocols. The mean absorbance values of two
20 animals were plotted over 28 days for each protocol. Maximum antibody levels were achieved with the procedure using an antigen releasing device (CRD) and intramuscular injection.

Figure 8 shows a graph of the individual absorbance levels of anti-lipase antibodies in two separate animals implanted with an antigen releasing device
25 (ARD) and given intramuscular injections. The levels of anti-lipase antibodies in two separate animals implanted with an antigen releasing device (CRD) and given

intramuscular injections. The two results highlight the reproducible nature of the immunisation procedure

Figure 9 shows a graph of the level of anti-lipase antibodies in serum from goats immunised with different protocols. The mean absorbance values of two animals
5 were plotted over 28 days for each protocol.

Figure 10 shows a comparison of mean anti-lipase anti-body levels in milk (♦) and serum (■) produced by immunisation of goats with an antigen releasing device (ARD) and intramuscular injection.

Figure 11 shows a graph of anti-lipase antibody production in milk (♦) and serum
10 (■) in goats implanted with an antigen releasing devices. The level of anti-lipase antibody levels in milk and the absence of anti-lipase antibodies in serum suggest that the antigens in the antigen releasing device implanted in the groin area are diffusing into the supramammary lymph node.

Figure 12 shows photographs of a diffusion assay of milk agar on glass slides
15 illustrating the inhibitory effects of anti-lipase antibodies on the lipolytic activity of the lipase enzyme. Slide 1: PBS or saline was added to the wells. Slide 2: 5mg/ml lipase from *P. fluorescens*. Slide 3: 5mg/ml of lipase with an antibody negative serum (1:1 dilution). Slide 4: 5mg/ml of lipase with serum that was positive for anti-lipase antibodies (1:1 dilution).

Figure 13 shows a graph of the levels of IgG, IgA and IgM in the milk of goats
20 inoculated with lipase from *Pseudomonas fluorescens*. Two groups of animals were inoculated in different locations (Group 1 (G1) into the flank and Group 2 (G2) in the region adjacent to the supramammary lymph nodes) on Days 0, 10 and 19. The relative levels of IgG, IgA and IgM were determined by ELISA. The
25 levels of all three classes of immunoglobulins were higher in the milk of Group 2 animals when compared to Group 1 animals.

Disclosure of the Invention

General

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described.

5 It is to be understood that the invention includes all such variation and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

10 Each document, reference, patent application or patent cited in this text is expressly incorporated herein by reference, which means that it should be read and considered by the reader as part of this text. That the document, reference, patent application or patent cited in this text is not repeated in this text is merely for reasons of conciseness.

15 The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

20 Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Through this specification the acronyms CRD and ARD are used interchangeably. Both refer to the antigen releasing device described, disclosed and claimed
25 herein.

Other definitions for selected terms used herein may be found within the description of the invention and apply throughout. Unless otherwise defined, all

other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

Detailed Disclosure of the invention

5 This invention is based on the unexpected discovery that a sustained release of antibodies in milk can be achieved by stimulating the supramammary lymph node over a long period with antigen released from an implanted antigen releasing device implanted adjacent to, within close proximity of or within the supramammary gland. The advantage of stimulating the supramammary lymph
10 node lies in the proximity of the node to the mammary glands. Antibodies produced by the supramammary lymph node are secreted into the mammary glands and therefore enter the milk of the mammal. The present method causes stimulation of antibody production that can be maintained over long periods by the slow release of antigen from the antigen releasing device. The method stimulates
15 the production of different subclasses or isotypes of immunoglobulin, such as IgA, IgG and IgM.

Thus, in a first aspect of the invention, there is provided a method for inducing the sustained release of antibodies in milk comprising the step of: implanting at least one antigen releasing device adjacent to, within close proximity of or within at
20 least one supramammary lymph node, wherein in use the antigen releasing device releases an antigen into the tissue area around the supramammary lymph node which stimulates antibody secretion into a mammary gland.

According to this invention the distance of the implant from the supramammary gland should be at least close enough that the release of antigen from the antigen
25 releasing device causes the antibody response of the mammal (into which it is implanted) to be maintained at a level that facilitates the production of antibodies in milk at levels that are therapeutically or anti-microbially suitable. For examples, the ARD may be implanted in the udder. Alternatively, by way of illustration the antigen releasing device is preferably implanted at a distance of up to 100 mm
30 from at least one supramammary lymph node, wherein in use the antigen

releasing device releases an antigen into the tissue area around the supramammary lymph node which stimulates antibody secretion into a mammary gland. Preferably, the antigen releasing device is implanted adjacent to or at a distance of between about 1 mm and 100 mm from the supramammary lymph node. Most preferably, the distance is between about 50 mm and 100 mm.

It will be appreciated that the present invention may be used to stimulate antibody production in a number of glands in an animal. In this respect immunisation at one gland has been shown to result in antibody activity in secretions by other mammary glands (see, for example, F.J Bourne et al (1975) Research in Veterinary Science 18:244-248)

The method of the present invention is performed on mammals. Preferably, the mammals used in the method are rodents or ruminants. Most preferably, the mammals are goats, sheep or cattle. Desirably the mammals are dairy cattle breeds; however dairy goat or sheep breeds may also be used.

The term "milk" used herein refers to both milk and colostrum in the form in which it is produced by the mammal or any derivative of whole milk, such as skimmed milk or whey, in liquid or in solid form.

The term "antigen" as used herein refers to any material capable of inducing an antigenic response in a treated mammal. Antigens may be selected according to the ultimate utility of the antibodies. That is, if the antibodies are to be used for generating passive immunity, the antigen against which such immunity is sought should be used may be derived from bacteria, viruses, yeasts, mycoplasmas, proteins, haptens, peptides, animal tissue extracts, plant tissue extracts, fungi, pollens, dust, chemical antigens intact mammalian cells (including spermatazoa) and fractions of cells. Where haptens or peptides are to be used as antigens these should first be conjugated to carrier substances such as proteins using chemistry well known to people versed in the art (Hanly et al; Review of Polyclonal Antibody Production Procedures, ILAR Journal (1995), 37:3, 93-118).

In one embodiment of the invention any bacterial antigen may be used in the invention. Preferably, the bacterial antigens are desirably selected from the bacterial species selected from, but no limited to: *Escherichia*, *Staphylococcus*, *Streptococcus*, *Salmonella* and *Helicobacter*. Particularly preferred bacterial
5 species are *Escherichia coli*, *Clostridium difficile*, *Vibrio cholerae* and *Helicobacter pylori* and *Pseudomonas fluorescens*. Most preferably, the antigen is lipoprotein lipase from *Pseudomonas fluorescens*.

Implantation of the antigen releasing device adjacent to or within at least one supramammary lymph node causes the antigen contained in the antigen releasing
10 device to be released into the tissue near and within the node (Figure 1). This in turn stimulates the node to generate antibodies to the antigen. These antibodies are secreted into the mammary glands and therefore enter the milk of the mammal.

The size, characteristics and choice of antigen releasing device is dependant on
15 the size and properties of the antigen of interest. It is desirable that the choice of antigen releasing device allows the antigen contained therein to be released from the device at a rate which causes the antibody response of the mammal into which it is implanted to be maintained at a desirable level.

Devices for slow release of compositions are described in, for example, US Patent
20 No. 3,279,996, whilst immunopotentiating devices for the sustained release of antigen are described, for example, in Australian Patent No. 740133

A porous silicon implant impregnated with a beneficial substance is described in Patent No. DE69917625D. An implantable device for molecule delivery is described in U.S Patent No. 6,716,208. Other suitable examples of sustained-
25 release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g., films, or microcapsules compressed into delivery devices. Examples of sustained-release matrices include polyesters, hydrogels [e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer et al., J. Biomed. Mater. Res.
30 15:167-277 (1981) and Langer, Chem. Tech. 12:98-105 (1982) or

- poly(vinylalcohol)], polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers 22:547-556 [1983]), non-degradable ethylene-vinyl acetate (Langer et al., supra), degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™
- 5 (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Another reference is B.Baras, M.A. Benoit & J.Gillard (2000) "Parameters influencing the antigen release from spray dried poly (DL-lactide) microparticles." International Journal of Pharmaceutics, 200:133-145.
- 10 While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antigens remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity.
- 15 Rational strategies can be devised for antibody stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular SS or disulfide bond formation through thio-disulfide interchange, stabilisation may be achieved by modifying sulfhydryl residues, lyophilising from acidic solutions, controlling moisture content, using appropriate
- 20 additives, and developing specific polymer matrix compositions.

Sustained-release fragment compositions also include liposomally entrapped fragments. Liposomes containing the antibody are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. USA 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA 77:4030-4034 (1980); EP

25 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30% cholesterol, the selected proportion being adjusted for the optimal antibody therapy. Other devices for the

30 slow release of antigen into the tissue near the supramammary lymph node are encompassed within the present invention.

An effective amount of antigen to be employed therapeutically will depend, for example, upon the objectives, the route of administration, the type of antigen and/or adjuvant and the condition of the animal. Accordingly, it will be necessary for the therapist to titre the dosage and modify the mode of administration as required to obtain the optimal effect. Typically, the operator will administer an antigen until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

In the further aspect of the invention, there is provided a method for inducing the sustained release of antibodies in milk comprising the further step of administering a primer composition by an administration route selected from intramammary, intraperitoneal, intramuscular or intranasal. Administration of a primer may take place before, during or after implanting the antigen releasing device. It is preferable that the primer composition be delivered to a mucosal surface so that antibody production on mucosal surfaces (of which the mammary gland is one) is preferentially stimulated.

The primer composition administration could be a single administration, or could comprise a number of administrations at intervals over a period of days or weeks. Timing of the administration of primer composition is generally spaced based on contemporary immunisation protocols (for example, every 2 weeks). To avoid local irritation and congestion, it is usually preferred that the primer composition not be administered to the same site more frequently than every second week. The initial exposure of this priming step stimulates the low level production of antibodies, which production is then increased and maintained by antigen released by the antigen releasing device.

The method of the present invention may also comprise the additional step of administering a booster composition comprising antigen to a mammal by an administration route selected from intramammary, intraperitoneal, intramuscular and/or intranasal after the antigen releasing device has been implanted. It is preferable that the booster composition be delivered to a mucosal surface so that antibody production on mucosal surfaces (of which the mammary gland is one) is preferentially stimulated.

Such booster compositions could be administered as a single administration, or could comprise a number of administrations at intervals over a period of days or weeks. Administration of booster compositions is generally spaced to suit the convenience of the operator. To avoid local irritation and congestion, it is usually preferred that administration of the booster composition to the same site not be more frequent than every other week.

In a preferred method, the antigen administered is the same for each step of the method. Therefore, the same antigen may be used in the antigen releasing device, the primer composition and/or the booster composition.

10 The use of adjuvants both within the antigen releasing device and in the compositions used for priming and boosting is also desirable. An adjuvant can serve as a tissue depot that slowly releases the immunogen and also as a lymphoid system activator that non-specifically enhances the immune response [Hood *et al.*, in *Immunology*, p. 384, Second Ed., Benjamin/Cummings, Menlo Park, California (1984)]. Suitable adjuvants for use with the antigens of the invention include but are not limited to the following: Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIC), TiterMax Gold™, adjuvant 65, cholera toxin B subunit, IL1-B Fragment 163-171 synthetic human adjuvant, alhydrogel; or bordetella pertussis, muramyl dipeptide, cytokines, saponin, Adju-Phos, Algal Glucan, 20 Algammuliu, Alhydrogel, Antigen Formulation, Avridine, Bay R1005, calcitriol, calcium phosphate, Gel, CRL 1005, cholera Holotoxin (CT), DDA, DHEA, DMPC, DMPG, DOC/Alum Complex, Gamma Inulin, Gerbu Adjuvant, GMDP, Imiquimod, Imuithier, Interferon-gamma, ISCOM(s), Iscoprop 7.0.3, Loxoribine, LT-OA or LT Oral adjuvant, MF59, MONTANIDE ISA51 and ISA720, MPL, MTP-PE, MTP PE 25 Liposomas, murametide, murapalmitate, NAGO, Nonionic surfactant vesicles, Pleuram, PLGA, PGA and PLA, Pluronic L121, PMMA, PODDS, Poly Ra, Polyru Polyphosphazene, Polysorbate 80, Protein Cochleates, QS-21, Quil A, Rehydrogel HPA, Rehydrogel LV, S-28465, SAF-1, Sclavo, peptide, Seudai Protodiposomes, sendai-containing lipid matrices, Span 85, speical, squalene, stearyl Tyrosine, 30 Theramide, Threonyl-MDP, Ty Particles, saponin Q521, MF59 and Alum.

In a further aspect of the invention, the method further comprises a preselection step. In this step individual animals are tested and selected for their ability to produce antibodies. Considerable between-animal variability exists for the production of antibodies. This preselection step, wherein the animals showing the best antibody titre responses are selected, assists in decreasing the between-animal variability factor. This process may similarly be used to build groups of animals particularly suited to antibody production.

In relation to the step of administering the priming composition or the boosting composition, preferably the antigenic substances are suspended in liquid medium for infusion or injection according to known protocols. Any appropriate carriers, diluents, buffers, and adjuvants known in the art may be used. Suitable suspension liquids include saline solution, water, and physiologic buffers.

If administration of the priming composition or the boosting composition is by injection, preferably prior to injection the antigens are emulsified in appropriate carriers with adjuvant using, for example, a laboratory homogeniser. In one example of such a method, aqueous antigen is mixed with 3 volumes of adjuvant and emulsified until a stable water-in-oil emulsion is formed. The presence of a stable emulsion can be demonstrated using tests well known in the art.

According to a second aspect, the invention also provides antibodies produced by any one of the methods of the present invention and fragments of such antibodies. The antibodies may be of any of the different subclasses or isotypes of immunoglobulin, eg IgA, IgG or IgM, or any of the other subclasses.

Exemplary antibody molecules and fragments that may be prepared according to this aspect of the invention include intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope. Such portion of antibodies that contain the paratope include those portions known in the art as Fab, Fab', F(ab')₂ and F(v). Fab and F(ab')₂. These portions of antibodies may be prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibodies by methods that are well known. See for example, U.S. Pat. No. 4,342,566. Fab'

antibody portions are also well known and are produced from F(ab')₂ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. Preferably the antibodies are intact antibody molecules, and are utilised as illustrative herein.

According to a third aspect the invention relates to a method for the production of mammalian milk containing antibodies, which method comprises induction of antibodies according to the method detailed above and then collecting the antibody containing milk from the mammal. The collection of milk may be effected using normal milking processes.

This milk is useful in the form obtained directly from the mammal, but may be processed if required. Examples of processing steps include heat treatment, ultra violet radiation, concentration, supplementation with food additives, drying into concentrates, milk or whey powders and the like.

As a further step to the method of the invention, the antibodies may be isolated from the milk. Isolation may be effected using separation techniques known in the art. For example, methods for the isolation of immunoglobulin rich fractions from whey (Nielson, K. (1986) Can. J. Vet. Res 50: 227-231; EP 0320152; WO 97/27757; GB2 179947) from milk (Kanamara et al (1993) Milchwissenschaft 48:247-251; U.S. Pat. No. 4,229,342), from colostrum (Kanamaru et al (1982) Agric. Biol. Chem 46:1531-1537, French Patent No. 2520235, New Zealand Patent No. 239466 and U.S. Pat. No. 4,582,580), and from milk and colostrum (U.S. Pat. No. 4,644,056) are known.

The isolated antibodies may subsequently be purified if desired. Purification may be carried out according to known techniques such as precipitation and ion exchange chromatography. Suitable techniques are disclosed in the journals and patents referenced above. Both the isolated and purified antibodies produced in accordance with the additional process steps also form part of the present invention.

Methods for producing protein concentrates containing antibodies on a commercial scale are disclosed in Swiss Patent No. 1,573,995 incorporated herein by reference. Briefly, the method comprises collecting the milk of hyperimmunised milk-bearing females; separating the cream and the impurities, 5 coagulating the clarified and skimmed milk, separating the casein, filtering, ultrafiltering and sterilising the proteins of the whey by filtration, evaporating and drying the product under conditions which do not denature the antibodies and which maintain sterility.

Examples

10 Further features of the present invention are more fully described in the following non-limiting examples. It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention. It should not be understood in any way as a restriction on the broad description of the invention as set out above.

15 **Example 1**

Preparation of Antigen Releasing Device minus adjuvant

MATERIALS

- (i) 0.15g mannitol-D (Sigma-Aldrich)
- 20 (ii) 0.15g sodium citrate (Proanalys)
- (iii) 11.25mg lipase from *Pseudomonas fluorescens* (Sigma-Aldrich)
- (iv) 0.75 ml part A Silastic (Dow Corning Q7-4850)
- (v) 0.75 ml part B Silastic (Dow Corning Q7-4850)
- (vi) 2 x 2.5 ml disposable syringes (Terumo)
- 25 (vii) 2 x 1ml syringe (Terumo)
- (viii) 2 x 12G x 4 inch stainless steel hypodermic needles
- (ix) 37 °C incubator
- (x) sterile petri dish
- (xi) sterile scalpel
- 30 (xii) sterile spachella

(xiii) 32ml glass McCartney bottle

METHODS

- (i) Remove pistons from all syringes
- 5 (ii) Part A silastic placed into 1 ml syringe using spatula. Piston replaced into syringe and 0.75ml quantity dispensed into one 2.5ml syringe. Procedure repeated for Part B.
- (iii) Lipase, mannitol and sodium citrate are combined and mixed in a small glass McCartney then placed into the 2.5ml syringe containing part A of the
- 10 silastic. Part B was then expelled from its syringe into the other 2.5ml syringe effectively 'sandwiching' the lipase, mannitol and sodium citrate between part A and part B of the silastic in one syringe. The piston was replaced in this syringe and removed from the empty syringe. The contents of the first syringe were expelled into the second syringe then its piston replaced. The procedure was
- 15 repeated 20 times to effectively mix all reagents. The reagents were finally expelled into two 12G needles and stored at 37°C for three days. The cured silastic was extracted from the needles, cut into 3cm lengths in the open petri dish and placed under UV light for 24 hours before being stored in sterile 10ml centrifuge tubes at -20°C. Each 3cm ARD contained 1mg lipase, 13mg mannitol,
- 20 13mg sodium citrate, in 250µl of total silastic.

Example 2

Preparation of Antigen Releasing Device including adjuvant

25 MATERIALS

- (i) 0.3g mannitol-D (Sigma-Aldrich)
- (ii) 0.3g sodium citrate (Proanalys)
- (iii) 22.50mg lipase from *Pseudomonas fluorescens* (Sigma-Aldrich)
- (iv) 1.2mg IL1-B Fragment 163-171 synthetic human (Sigma)
- 30 (v) 1.5 ml part A Silastic (Dow Corning Q7-4850)
- (vi) 1.5 ml part B Silastic (Dow Corning Q7-4850)
- (vii) 2 x 2.5 ml disposable syringes (Terumo)

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- (viii) 2 x 1ml syringe (Terumo)
- (ix) 2 x 12G x 4 inch stainless steel hypodermic needles
- (x) 37 °C incubator
- (xi) sterile petri dish
- 5 (xii) sterile scalpel
- (xiii) sterile spachella
- (xiv) 32ml glass McCartney bottle

METHODS

- 10 (i) Remove pistons from all syringes
 - (ii) Part A silastic placed into 1 ml syringe using spatula. Piston replaced into syringe and 0.75ml quantity dispensed into one 2.5ml syringe. Procedure repeated for Part B.
- Lipase, mannitol and sodium citrate mixed in a small glass McCartney then
- 15 placed into the 2.5ml syringe containing part A of the silastic. Part B was then expelled from its syringe into the other 2.5ml syringe effectively 'sandwiching' the lipase, mannitol and sodium citrate between part A and part B of the silastic in one syringe. The piston was replaced in this syringe and removed from the empty syringe. The contents of the first syringe was expelled into the second syringe
 - 20 then its piston replaced. The procedure was repeated 20 times to effectively mix all reagents. The reagents were finally expelled into two 12G needles and stored at 37 °C for three days. The cured silastic was extracted from the needles, cut into 3cm lengths in the open petri dish and placed under UV light for 24 hours before being stored in sterile 10ml centrifuge tubes at -20 deg C. Each 3cm ARD
 - 25 contained 1mg lipase, 13mg mannitol, 13mg sodium citrate, 50µg IL1-B, in 250µl of total silastic.

Example 3

Delivery of ARD

- 30 A device was purpose designed and built comprising 10ml disposable luer lock syringe, 10G x 4 inch stainless steel hypodermic needle and a 90mm x 10G stainless steel welding rod (see Figure 5 below). The device was assembled with

the rod attached to the piston of the syringe and passing through the needle. The piston was withdrawn about 3cm allowing for free space near the tip of the needle in which to insert the antigen releasing device. Thus, when the piston is depressed, the antigen releasing device is expelled from the needle by the rod
5 (see Figure 5 below).

The animal was placed on the floor on its back and restrained by animal handlers. An area approximately 3cm x 10cm right lateral and adjacent to the udder was swabbed with iodine. 2ml of 2% lignocaine infiltrated the cutaneous tissue
10 through a 26G hypodermic needle as a local anaesthetic. The 10G needle housing the ARD was inserted in the posterior end of this area and pushed to the anterior end subcutaneously. The piston was depressed and needle withdrawn simultaneously. The point of insertion was swabbed with iodine. In most cases the antigen releasing device could be felt *in situ*.

15

Example 4

Preparation of Lipase nasal inoculum

MATERIALS

- 20 (i) 15.5 mg lipase from *P.fluorescens* (Sigma-Aldrich)
- (ii) 38.75ml 0.85% saline (Excel laboratories)
- (iii) 1mg Cholera Toxin B Subunit (US Biological)
- (iv) 2.5ml syringes
- (v) 50ml sterile tube (Falcon)
- 25 (vi) 8-10cm length of approximate 20G plastic tube from winged infusion set (Terumo)

METHODS

- (i) All reagents were combined in 50ml tube on day of first administration.
- 30 (ii) The remainder was stored at 4°C until used.

Example 5Delivery of Nasal Inoculum

2.5ml of inoculum (containing 1mg lipase, 64.5µg Cholera Toxin B Subunit) was
5 drawn into syringe with the 20G tube from the infusion set attached. 60-80% of
tube length was placed into right nostril of animal and slowly dispensed whilst
simultaneously withdrawing the tube from the nostril (see Figure 2).

Example 6Preparation of Lipase Intramuscular injected inoculum

10

MATERIALS

- (i) 36.5mg of lipase from *P.fluorescens* (Sigma)
- (ii) 9.1 ml of 0.85% saline (Excel Laboratories)
- (iii) TiterMax Gold™ adjuvant
- 15 (iv) glass McCartney bottle
- (v) 3ml all plastic syringes (Teruma)
- (vi) stainless steel double-hub
- (vii) 23G x 1 inch hypodermic needle

20 METHODS

- (i) Lipase and saline were combined in glass McCartney giving a
concentration of 4mg/ml on the day of first administration. The remainder was
stored at 4 °C until required.

25 **Example 7**Delivery of Intramuscular Inoculum

0.5ml of 4mg/ml lipase in saline solution as drawn into syringe and emulsified with
0.5ml TiterMax Gold™ as per manufacturers instructions. Inoculum was
30 administered intramuscularly (IM) in the rear of the left hind leg using 23G x 1 inch

needle attached to the syringe. Animals received IM injections Day 7, Day 14 and Day 21.

Example 8

5 Immunisation Protocol 1

The animals were given an IM injection in the upper hind limb (rump).

10 Five animals were immunised with four different antigens. Two animals each received one type of antigen and the remaining three animals received a different antigen each. Table 1 summarises the schedule of the primary inoculums and two subsequent boosts, and the concentrations of antigen (Ag), and volumes of the TiterMax adjuvant (T/max).

Table 1: Immunogen composition and inoculation schedule for goats inoculated with a variety of antigens.

Goat Inoculation Schedule

PRIMARY INOCULATION							
Tag	Antigen	Primary	Ag (μg)	Ag(μl)	T/Max(μl)	Tot (μl)	Bleed y/n
X0247	<i>P.fluorescens</i> lipase	28/11/2003	1250	250	750	1000	Y
X0248	<i>P.fluorescens</i> lipase	28/11/2003	1250	250	750	1000	Y
X0241	Type X protease	17/03/2004	500	500	500	1000	Y
X0242	Type XVII-B protease	17/03/2004	500	500	500	1000	Y
X0243	Type VIII protease	17/03/2004	500	500	500	1000	Y

SECONDARY INOCULATION							
Tag	Antigen	S'dary	Ag (μg)	Ag(μl)	T/M(μl)	Tot (μl)	Bleed y/n
X0247	<i>P.fluorescens</i> lipase	10/12/2003	950	190	560	750	Y
X0248	<i>P.fluorescens</i> lipase	10/12/2003	950	190	560	750	Y
X0241	Type X protease	1/04/2004	500	500	500	1000	Y
X0242	Type XVII-B protease	1/04/2004	500	500	500	1000	Y
X0243	Type VIII protease	1/04/2004	500	500	500	1000	Y

TERTIARY INOCULATION							
Tag	Antigen	Tertiary	Ag (μg)	Ag(μl)	T/M(μl)	Tot (μl)	Bleed y/n
X0247	<i>P.fluorescens</i> lipase	22/12/2003	2500	500	500	1000	Y
X0248	<i>P.fluorescens</i> lipase	22/12/2003	2500	500	500	1000	Y
X0241	Type X protease	16/04/2004	500	500	500	1000	Y
X0242	Type XVII-B protease	13/05/2004	500	500	500	1000	N
X0243	Type VIII protease	16/04/2004	500	500	500	1000	Y

Example 9Immunization Protocol 2

- 5 The animals were given antigen inoculation by antigen releasing device (ARD) and/or injection.

Table 2 summarises the schedule of inoculums and refers to 6 animals and one antigen.

10

- One animal received a sub-cutaneous injection in the groin on Day 0 of the antigen and on Day 30 the same animal received an IM injection of the antigen. One animal received a sub-cutaneous injection of the antigen on Day 0 only. One animal received an implant having the antigen in the groin on Day 0. One animal
15 received an implant having the antigen in the groin on Day 0 and on Day 30 received an IM injection of the antigen in the rump. Two animals received an implant having antigen in the groin on Day 0 and on Day 15 one received a nasal and the other an IM injection boost.

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Table 2: Protocols for initial trial of antigen releasing device (ARD).

Trial CRD and S/C injection of Ag to assess Ab secretion via mammary

Date	Tag	Ag	Amount	Adjuvant	Amount	Delivery	Total Vol	Location	Milk y/n	Bleed y/n
28/04/2004	White 651 8RS	Lipase	2.5 mg	T/Max	500ul	S/C	1ml	groin	Y	Y
28/04/2004	Black 1292 BX8	Lipase	2.5 mg	T/Max	500ul	S/C	1ml	groin	Y	Y
28/04/2004	Black 1675 BX8	Lipase	250ug unsheathed silastic CRD			S/C		RH groin	Y	Y
28/04/2004	White 552 8RS	Lipase	250ug sheathed silastic CRD			S/C		RH groin	Y	Y

Date	Tag	Ag	Amount	Adjuvant	Amount	Delivery	Total Vol	Location	Milk y/n	Bleed y/n
13/05/2004	White 651 8RS	Lipase	0	0	0	0	0	0	Y	Y
13/05/2004	Black 1292 BX8	Lipase	0	0	0	0	0	0	Y	Y
13/05/2004	Black 1675 BX8	Lipase	CRD						Y	Y
13/05/2004	White 552 8RS	Lipase	CRD						Y	Y
13/05/2004	White 367	lipase	1mg / 12mg mannitol/12mg SC-CRD				30x2mm	Rh groin	Y	Y
13/05/2004	Orange 468	lipase	1mg / 12mg mannitol/12mg SC-CRD				30x2mm	Rh groin	Y	Y

Date	Tag	Ag	Amount	Adjuvant	Amount	Delivery	Total Vol	Location	Milk y/n	Bleed y/n
28/05/2004	White 651 8RS	Lipase	2.5 mg	T/Max	500ul	S/C	1ml	RH rump	Y	Y
28/05/2004	Black 1292 BX8	Lipase	0	0	0	0	0	0	Y	Y
28/05/2004	Black 1675 BX8	Lipase	0	0	0	0	0	0	Y	Y
28/05/2004	White 552 8RS	Lipase	2.5 mg	T/Max	500ul	S/C	1ml	RH rump	Y	Y
28/05/2004	White 367	lipase	2.5 mg in 2.5 ml saline				2.5ml	nasal	Y	Y
28/05/2004	Orange 468	lipase	2.5 mg	T/Max	500ul	S/C	1ml	Lhrump	Y	Y

Date	Tag	Ag	Milk y/n	Bleed y/n
1/07/2004	White 367	lipase	Y	Y
1/07/2004	Orange 468	lipase	Y	Y

Example 10**Immunisation protocol 3**

5

Protocols used 2 animals per group with 12 goats used in total.

Six different protocols were evaluated in two separate animals to determine an optimal procedure to produce sustained antibody levels in milk, summarised in

10 Table 4 and 5.

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Table 4: The immunisation protocols used to stimulate the production of antibodies in milk.

Group	Description	Adjuvant	Innoculatio Protocol				Sampling Frequency	
			Day 1	Day 7	Day 14	Day 21	Milk Collection	Blood Collection
1	Intramuscular (IM) injection only	YES		IM injection	IM boost	IM injection	Daily	3-4 day intervals
2	ARD only	YES	Implant ARD				Daily	3-4 day intervals
3	Intranasal (IN) spray only	YES		IN inoculati	IN boost	IN boost	Daily	3-4 day intervals
4	Intramuscular (IM) injection + ARD	YES	Implant ARD				Daily	3-4 day intervals
5	Intranasl (IN) sprlay + ARD	YES		IM injection	IM boost	IM injection	Daily	3-4 day intervals
6	ARD only	NO	Implant ARD				Daily	3-4 day intervals

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Table 5: The immunisation protocols used to stimulate the production of antibodies in milk.

Group 1 - inject.					
S/Mark	Inoculum	Amount	Delivery	Site	Freq
	lipase	2.0mg	I.M	R/h quart	Day 7
	TiterMax	500ul			Day 14
	saline	500ul			Day 21

Group 2 - CRD inc Adj					
S/Mark	Inoculum	Amount	Delivery	Site	Freq
	lipase	1mg	CRD	RH groin	Day 0
	mannitol	13mg			
	sod citrate	13mg			
	IL-1B	50ug			
	silastic	250ul			

Group 3 - nasal only					
S/Mark	Inoculum	Amount	Delivery	Site	Freq
	lipase	1mg	nasal	nostril	Day 7
	C/ Tox				Day 14
	saline	2.5ml			Day 21

Group 4 - CRD & inj					
S/Mark	Inoculum	Amount	Delivery	Site	Freq
	lipase	1mg	CRD	RH groin	Day 0
	mannitol	13mg			
	sod citrate	13mg			
	IL-1B	50ug			
	silastic	250ul			
	lipase	2.0mg	I.M	R/h quart	Day 7
	TiterMax	500ul			Day 14
	saline	500ul			Day 21

Group 5 - CRD & nasal					
S/Mark	Inoculum	Amount	Delivery	Site	Freq
	lipase	1mg	CRD	RH groin	Day 0
	mannitol	13mg			
	sod citrate	13mg			
	IL-1B	50ug			
	silastic	250ul			
	lipase	1mg	nasal	nostril	Day 7
	C/ Tox				Day 14
	saline	2.5ml			Day 21

Group 6 - CRD minus Adj					
S/Mark	Inoculum	Amount	Delivery	Site	Freq
	lipase	1mg	CRD	RH groin	Day 0
	mannitol	13mg			
	sod citrate	13mg			
	silastic	250ul			

A total of 12 goats were studied, with two goats dedicated to each inoculation regime. The presence of anti-lipase antibodies was evaluated with Enzyme-Linked Immunosorbent Assay (ELISA). The mean absorbance value less than that of the blank control of the daily milk samples were plotted (Figure 6). Results were
5 reproducible between animals, as shown in Figure 7.

All six regimens were successful in raising antibodies. However, the relative concentrations of antibodies for each group varied. The highest mean absorbance value; which is indicative of the greatest concentration of antibodies
10 produced; was recorded for the Group 4 animals. The Group 4 animals received an ARD implant on Day 0 of the program and 3 subsequent injections to the back flank area on Days 7, 14 and 21.

The mean absorbance value of Group 4 was greater than the value produced by
15 the Group 1 goats, who only received IM injections at day 7, 14 and 21.

Both Group 1 and 4 animals showed some response up to approximately Day 14. At Day 15, the levels of anti-lipase antibodies increased substantially, presumably a consequence of the secondary immune response. The higher levels of
20 antibodies were sustained for the duration of the study, in this case up to Day 28.

The levels of anti-lipase antibodies in the serum of the inoculated animals was also measured, as shown in Figures 8, 9 and 10. Figure 8 shows results of the individual absorbance levels of anti-lipase antibodies in two separate animals
25 implanted with an antigen releasing device (ARD) and given intramuscular injections. Figure 9 shows results of the level of anti-lipase antibodies in serum from goats immunised with different protocols. Figure 10 shows results of a comparison of mean anti-lipase antibody levels in milk and serum produced by immunisation of goats with an antigen releasing device (ARD) and intramuscular
30 injection. Figure 11 shows the anti-lipase antibody production in milk and serum in goats implanted with an antigen releasing devices. The level of anti-lipase antibody levels in milk and the absence of anti-lipase antibodies in serum suggest

that the antigens in the antigen releasing device implanted in the groin area are diffusing into the supramammary lymph node.

Example 11

Immunization Protocol 4

- 5 The protocol used two lactating goats (*Capra hircus*) for each group, with four goats used in total. The antigen used was lipase from *Pseudomonas fluorescens*.

- Preparation for immunisation protocol

1. Emulsify 30mg of lipase in 15ml 0.85% saline.
2. Emulsification with Titermax Gold was according to manufacturer's
10 specification. The lipase in saline solution was mixed with an equal volume of Titermax Gold (1:1).
3. Dispense 1ml into 2.5ml syringe for administration. (Each 1ml dose contains 1mg lipase)
4. Two groups of animals were inoculated on Day 0, 10 and 19.
- 15 5. Group 1 was inoculated in the left flank and Group 2 was inoculated adjacent to the supramammary lymph node.
6. Milk and serum was collected.

- Coating plates for Enzyme Linked Immunosorbent Assay (ELISA)

1. Prepare 2.5µg/ml of antigen in coating buffer.
- 20 2. 100µl of the mixture was dispensed into each well of a 96 well ELISA tray.
3. The plates were covered and were left to stand overnight at 4°C.

- Enzyme Linked Immunosorbent Assay (ELISA) protocol

1. Prior to use, the coated plates were washed 3 times with PBS Tween.
2. A serum diluent of 1% Human serum in PBS Tween.
- 25 3. Load 100µl of the serum diluent into each well.
4. Load 1µl of sample of interest to well.
5. Plates were incubated at 37°C for 2 hours.

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6. Plates were washed 3 times with PBS Tween.
7. 1/1000 dilutions of mouse α -sheep IgG, mouse α -sheep IgA and mouse α -sheep IgM in 1% serum diluent (1% Human serum in PBS Tween) were prepared.
- 5 8. Load 100 μ l into respective wells.
9. The plates were placed in 37°C for 2hrs.
10. The plates were washed 3 times with PBS Tween.
11. 1/2500 dilution of rabbit α -mouse IgG (H+L) in 1% serum diluent was prepared.
- 10 12. Load 100 μ l per well.
13. Incubate at 37°C for 2hrs.
14. The plates were washed 3 times with PBS Tween.
15. A 1/100 dilution of 250mg/ml Nitrophenyl phosphate in Diethanolamine Buffer was prepared.
- 15 16. Load 100 μ l per well.
17. Incubate at room temperature for approximately 20 to 30 minutes.
18. The reaction was terminated with 50 μ l of 3.75M NaOH.
19. ELISA plates were read at 405nm.

- Results

- 20 Milk collected was analysed by ELISA for levels of IgG, IgA and IgM. Results from Group 1 (intermuscular into the flank – designated G1) and Group 2 (stimulation of the supramammary lymph node – G2 animals) in Figure 13 shows higher levels of all three classes of immunoglobulin produced in the milk of Group 2 animals when compared to Group 1 animals.

- 25 **Example 12**

Collection and storage of milk samples

MATERIALS

- (i) Beckman Acuspin reffridgerated centrifuge

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- (ii) Rennet Type II from Mucor meihei (Sigma) in Hp water at a concentration of 2mg/ml
- (ii) 10ml sterile centrifuge tubes
- (iv) P1000 pipetteman
- 5 (v) 1ml disposable transfer pipettes
- (vi) 5ml plastic storage tubes
- (vii) 37°C incubator

METHOD

- 10 Milk was collected by hand milking into 32ml glass McCartney bottles in the absence of any chemical or mechanical stimuli. Milk was generally collected in the morning without prior separation from kids. Samples were stored on ice after collection and transferred as soon as practicable thereafter. Milk was transferred to sterile 10ml centrifuge tubes and centrifuged at 2000rpm for 15mins at 4°C.
- 15 Milk was aspirated from under the solid fat layer using disposable pipette and placed in fresh 10ml tube. The pipette was carefully plunged through the fat layer into the milk layer below. 2mg/ml Rennet solution was added to the milk at the ratio of 0.4ml rennet to 5ml milk, tubes were shaken then incubated at 37°C for 1 to 2 hours. Tubes were centrifuged at 5000rpm x 15mins at 4°C. Supernatant
- 20 was removed by transfer pipette and stored at -20°C.

Antibodies in the milk were quantified by the Enzyme-Linked Immunosorbent Assay (ELISA). The absorbance values, which are indicative of relative concentrations of the anti-lipase antibody, for serum were lower when compared

25 to milk. The levels of anti-lipase antibodies produced in the milk were higher when compared to antibody levels in serum.

Example 13

Collection and storage of blood samples

30 MATERIALS

- (i) 7ml of 9ml Vacutainers™ (Bectco Dickinson) for serum collection
- (ii) 20G x 1.5inch Vacutainer™ (Bectco Dickinson) needles and holder

METHODS

Blood samples were taken from the jugular vein using Vacutainer™ collection tubes, holder and needle. Tubes were stored at 4°C. Samples were centrifuged
 5 at 4000rpm x 15min at 4°C. Upper serum layer was removed using a transfer pipette and stored at -20 °C.

Example 14

Enzyme-Linked Immunosorbent Assay (ELISA)

10 MATERIALS

- (i) 10 x Phosphate Buffer Saline (PBS); 1L double distilled water, 1.91g KH₂PO₄ (BDH Chemicals Aust Pty Ltd), 6.1g Na₂HPO₄ (ASAX Chemicals), 2g KCl (BDH Chemicals Aust Pty Ltd),
- (ii) 80g NaCl, 1.95g NaN₃ (Sigma Aldrich), pH to 7.4
- 15 (iii) 200ml Carbonate coating buffer pH 9.6 containing; 200ml double distilled water , 3.18g Na₂CO₃ (BDH), 5.88g NaHCO₃ (BDH), 0.39g NaN₃ (Sigma).
- (iv) 0.85% saline (Excel Laboratories)
- (v) 0.25 mg/ml lipase from *P.fluorescens* (Sigma Aldrich) in carbonate coating buffer
- 20 (vi) PBS-TW20 plate washing solution (BDH); 200ml 10 x PBS (as above), 1800ml distilled water, 1ml Tween-20 (Labchem)
- (vii) Serum diluent; 200ml glycerol (BDH), 29g NaCl (BDH), 0.2g KH₂PO₄ (BDH), 0.61g Na₂HPO₄ (BDH), 0.2g KCl (BDH), 1.95g NaN₃ (Sigma), distilled water to 1L, 1.5ml Tween-20 (Labchem), pH to 7.4. Store 4°C.
- 25 Dessicated BSA (CSL) added to desired aliquot at 1% concentration, prior to use.
- (viii) Saline 0.85% (Excel Laboratories)
- (ix) Donkey anti-goat IgG-Horse Radish Peroxidase (HRP) (Promega)
- (x) 1M H₂SO₄ (AJAX Finechem)
- 30 (xi) TMBS EIA solution (BioRad) Part A & B
- (xii) P200 pipetteman and tips
- (xiii) P20 pipetteman and tips
- (xiv) nunc™ polsorb 96 well ELISA plates

(xv) BioRad™ 96 well plate spectrophotometer model 450

METHODS

100µl of lipase in carbonate buffer added to wells of ELISA plate and stored at
5 4°C overnight. Plates were washed 3 times with PBS-TW. 100µl of serum diluent
was added to wells for serum analysis, and 90µl were added to wells for milk
analysis. 1µl of serum sample and 10µl of milk sample added to the serum
diluent. The mixture was created by gentle tapping the plate. The plates were
stored at 4°C overnight. The plates were washed 3 times with PBS-TW. 100µl of
10 a 1/2500 dilution of Donkey anti-goat IgG-HRP in 0.85% saline added to each
well. The plates were incubated at room temperature for 1 hour. The plates were
washed 3 times with PBS-TW. 9 Part A and 1 part B of TMBS were mixed in a
glass Schott bottle and 100µl was added to each well. The plates were incubated
at room temperature for 10 minutes. 100µl 1M H₂SO₄ was added to each well
15 and plate read on spectrophotometer at 450nm. A printout of the absorbance
results was obtained. The absorbance values of each milk and blood sample
collected from all animals were measured. Plots of absorbance values on the y-
axis against time (days) on the x-axis were prepared for individual animals and
the average absorbance value for each group (comprising two test subjects).

20

Example 15

Lipase diffusion in milk agar slide

- 25
- (i) Prepare 1% (1g/100ml) agar (Oxoid Cat No L13, Basingstoke, Hampshire, England) in Phosphate Buffer Saline (pH 7.4).
 - (ii) Add 100µl of whole milk (Masters, Perth, Australia) to 5ml 1% agar.
 - (iii) For slide format, 2.5ml of 'milk agar' is poured over the glass and allowed to set for 10 minutes (2% milk in 1% agar).
 - (iv) Five 1.5mm diameter holes in the milk agar gel were prepared with an agar punch and the agar removed by vacuum.
 - 30 (v) The agar film was incubated over night with lipase from *P. fluorescens* (Aldrich, Milwaukee, WI, USA). 5mg/ml of lipase was prepared in 0.85% saline.

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(vi) The diffusion zone, indicative of lipid degradation of the lipase test was compared to plate with (a) saline, (b) lipase + antibody negative serum and (c) lipase + anti-lipase positive serum.

- 5 Results are present in Figure 11. On each milk slide, 5 wells were filled with saline (slide 1), the lipase enzyme (slide 2), lipase with an antibody negative serum (slide 3) and lipase with an anti-lipase antibody positive serum (slide 4). In slide 2, a zone of hydrolysis is visible as the lipase enzyme hydrolyses the lipids in the milk film. The negative control (saline in slide 1) confirms that the enzyme is
- 10 responsible for the zone of hydrolysis. The hydrolysis activity of the lipase enzyme can be inhibited by an anti-lipase antibody, as evident in slide 4. Slide 3 which contains an antibody negative serum confirms that it is the antibody and not other components of serum that is inhibiting the enzyme.
- 15 Although the invention has been described with reference to certain preferred embodiments, it will be appreciated that many variations and modifications may be made within the scope of the broad principles of the invention. Hence, it is intended that the preferred embodiments and all of such variations and modifications be included within the scope and spirit of the invention.

The claims defining the invention are as follows:

1. A method for inducing the sustained release of antibodies in milk comprising the step of:
 - a) implanting at least one antigen releasing device adjacent to, within close
5 proximity of or within at least one supramammary lymph node,

wherein in use the antigen releasing device releases an antigen into the tissue area around the supramammary lymph node which stimulates antibody secretion into a mammary gland.
2. The method of claim 1 wherein the implant is located in sufficient proximity to
10 the supramammary gland such that the release of antigen from the antigen releasing device induces the production of antibodies in milk over the life of the antigen releasing device.
3. The method of claim 2 wherein the antigen releasing device is implanted at a distance of up to 100 mm from at least one supramammary lymph node.
- 15 4. The method of claim 3 wherein the antigen releasing device is implanted at a distance of between about 1 mm and 100 mm from at least one supramammary lymph node.
5. The method of claim 4 wherein the antigen releasing device is implanted at a distance of between about 50 mm and 100 mm from at least one
20 supramammary lymph node.
6. The method of any one of claims 1-5 wherein the antigen releasing device is implanted in a mammal selected from the group comprising: rodents and ruminants.

7. The method of any one of claims 1-5 wherein the antigen releasing device is implanted in a mammal selected from the group comprising: goats, sheep and cattle.
8. The method of claim 7 wherein the device is implanted in a dairy cattle, dairy
5 goat or dairy sheep breed.
9. The method of any of the above claims wherein the antigen is a bacterial antigen.
10. The method of claim 9 wherein the bacterial antigen is from a bacterial genus
10 selected from: *Escherichia*, *Staphylococcus*, *Streptococcus*, *Salmonella* and *Helicobacter*.
11. The method of claim 9 wherein the bacterial antigen is from a bacterial species
selected from: *Escherichia coli*, *Clostridium difficile*, *Vibrio cholerae* and *Helicobacter pylori* and *Pseudomonas fluorescens*.
12. The method of claim 9 wherein the bacterial antigen is lipoprotein lipase from
15 *Pseudomonas fluorescens*.
13. The method of claim 1 wherein the antigen releasing device allows the antigen
contained therein to be released from the device at a rate which causes the
antibody response of the mammal into which it is implanted to be maintained
at a desirable level.
- 20 14. A method for inducing the sustained release of antibodies in milk
comprising the steps of:
 - a) administering a primer composition; and
 - b) implanting at least one antigen releasing device adjacent to, within
close proximity of or within at least one supramammary lymph node,

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wherein in use the antigen releasing device releases an antigen into the tissue area around the supramammary lymph node which stimulates antibody secretion into a mammary gland.

15.A method for inducing the sustained release of antibodies in milk comprising
5 the step of:

- a) implanting at least one antigen releasing device adjacent to, within close proximity of or within at least one supramammary lymph node, and
- b) administering a booster composition comprising antigen to a mammal after the antigen releasing device has been implanted

10 wherein in use the antigen releasing device releases an antigen into the tissue area around the supramammary lymph node which stimulates antibody secretion into a mammary gland.

16.A method for inducing the sustained release of antibodies in milk comprising the step of:

- 15 a) administering a primer composition;
- b) implanting at least one antigen releasing device adjacent to, within close proximity of or within at least one supramammary lymph node; and
 - c) administering a booster composition comprising antigen to a mammal after the antigen releasing device has been implanted

20 wherein in use the antigen releasing device releases an antigen into the tissue area around the supramammary lymph node which stimulates antibody secretion into a mammary gland.

17.The method of claim 14 or 16 wherein the primer is administered by an administration route selected from: intramammary, intraperitoneal,
25 intramuscular or intranasal.

18. The method of claim 14 or 16 wherein administration of a primer takes place before implanting the antigen releasing device.
19. The method of claim 14 or 16 wherein administration of a primer takes place during implantation of the antigen releasing device.
- 5 20. The method of claim 14 or 16 where the primer composition is delivered to a mucosal surface.
21. The method of claim 14 or 16 wherein the primer composition is administered in a single administration.
22. The method of claim 14 or 16 wherein the primer composition is administered
10 in a number of administrations at intervals over a period of days or weeks.
23. The method of claim 15 or 16 wherein the booster is administered by an administration route selected from: intramammary, intraperitoneal, intramuscular and/or intranasal.
24. A method according to claim 15 or 16 wherein the booster composition is
15 delivered to a mucosal surface.
25. The method of claim 15 or 16 wherein the booster composition is administered in a single administration.
26. The method of claim 15 or 16 wherein the booster composition is administered in a number of administrations at intervals over a period of days or weeks.
- 20 27. The method of any one of claims 14 to 26 wherein the antigen administered is the same for each step of the method.
28. The method of any one of claims 14 to 27 wherein the antigen releasing device, the priming composition and the booster composition further comprise an adjuvant.

- 29.A method according to any one of claims 1 to 28 further comprising a preselection step prior to administration of the antigen releasing device wherein animals showing the best antibody titre responses are used in the methods of any one of claims 1 to 28.
- 5 30.A method for the production of mammalian milk containing antibodies comprising the steps of:
- a) induction of antibodies according to the method of any one of claims 1 to 29; and
 - b) collecting the antibody containing milk from the mammal.
- 10 31.The method of claim 30 wherein the milk is used in the form obtained directly from the mammal.
- 32.The method of claim 30 wherein the milk is processed prior to use.
- 33.The method of claim 32 wherein the method of processing is selected from the list comprising: heat treatment, ultra violet radiation, concentration,
- 15 supplementation with food additives, drying into concentrates or milk powders.
- 34.The method of claim 30 comprising the further step of isolating the antibodies from the milk.
- 35.The method of claim 34 wherein the antibodies are purified after isolation.
- 36.Antibodies isolated in accordance with the method of claim 34 or 35.
- 20 37.Antibodies according to claim 36 wherein the antibodies isolated are chosen from the list comprising: intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of the immunoglobulin molecule that contain the paratope.

- 37 -

38. Antibodies according to claim 37 wherein the immunoglobulin molecule portion is selected from the list comprising: Fab, Fab', F(ab')₂ and F(v) portions.

39. A method for producing protein concentrates containing antibodies comprising the steps of:

- 5 a) collecting the milk of milk-bearing female mammals implanted with antigen releasing device according to the method of any one of claims 1 to 29;
- b) separating the cream and the impurities;
- c) coagulating the clarified and skimmed milk;
- d) separating the casein;
- 10 e) filtering, ultrafiltering and sterilising the proteins of the whey;
- f) evaporating and drying the proteins under conditions which do not denature the antibodies and which maintain sterility.

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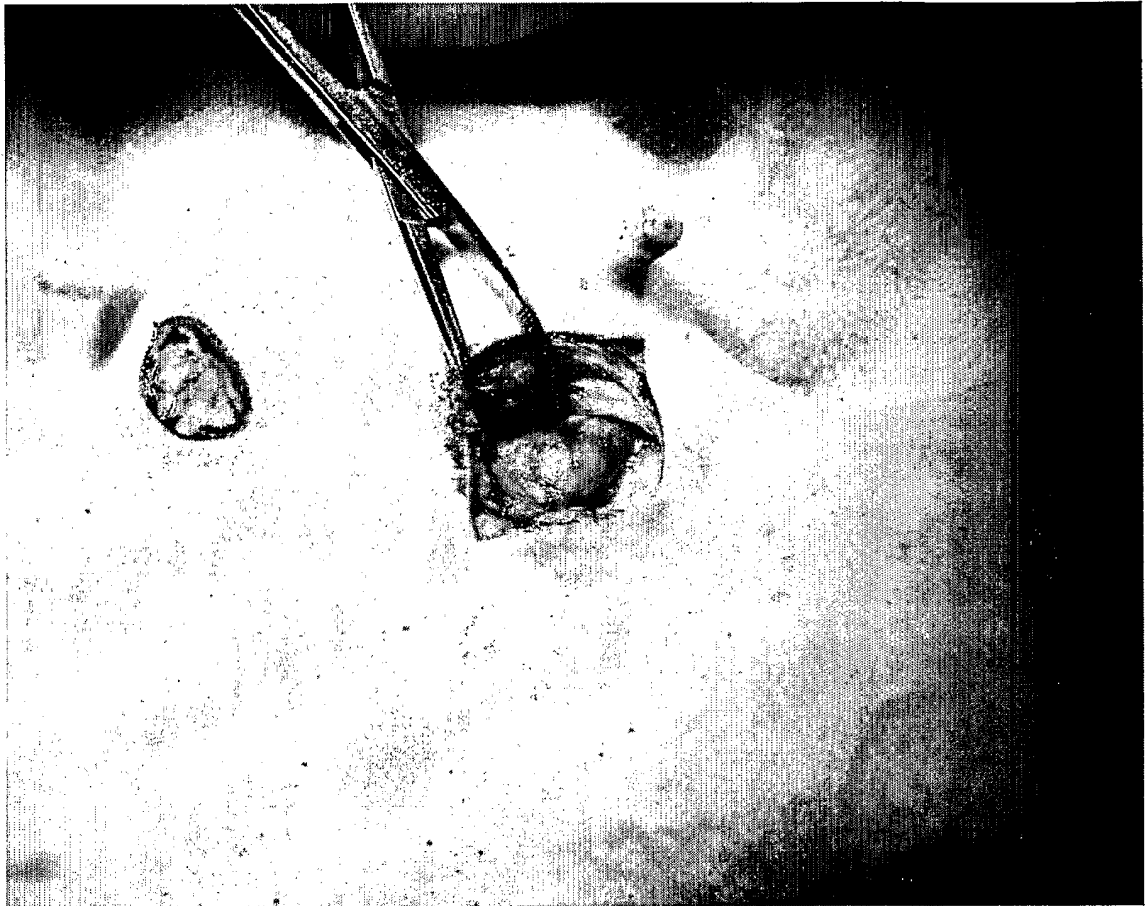


Figure 1

- 2/11 -



Figure 2

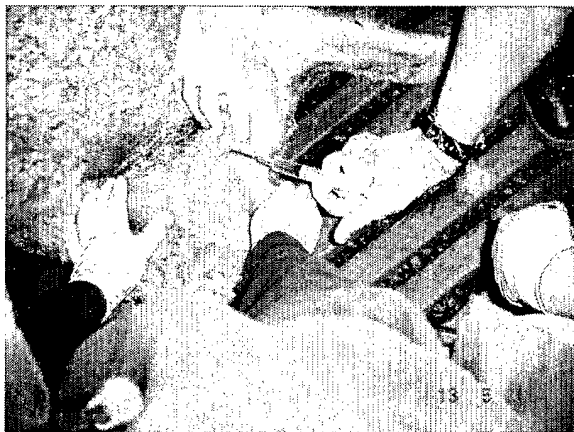


Figure 3



Figure 4

- 3/11 -

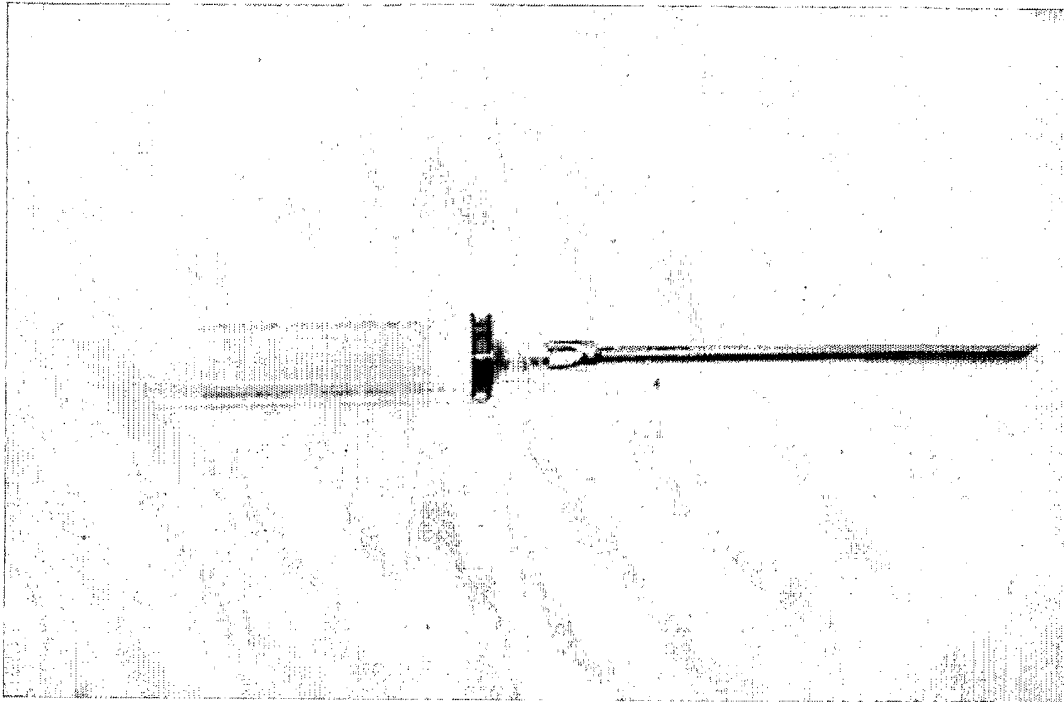


Figure 5

- 4/11 -

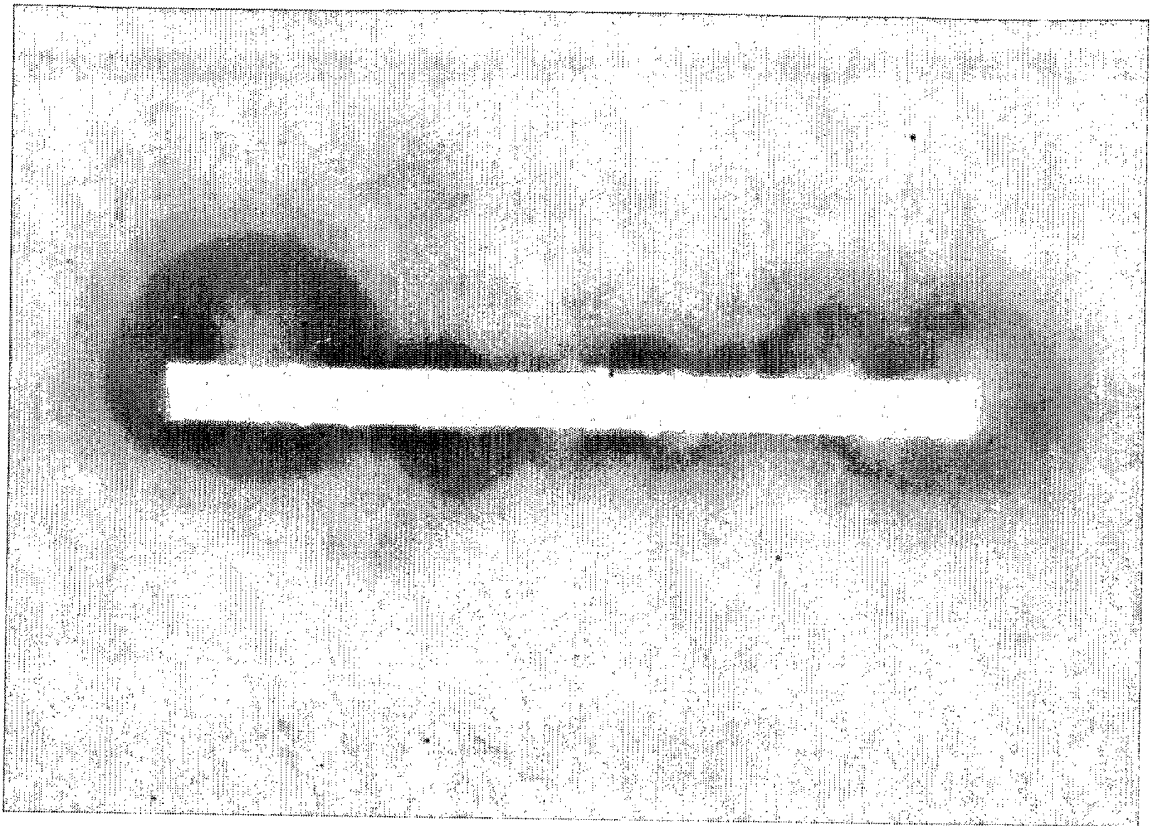


Figure 6

- 5/11 -

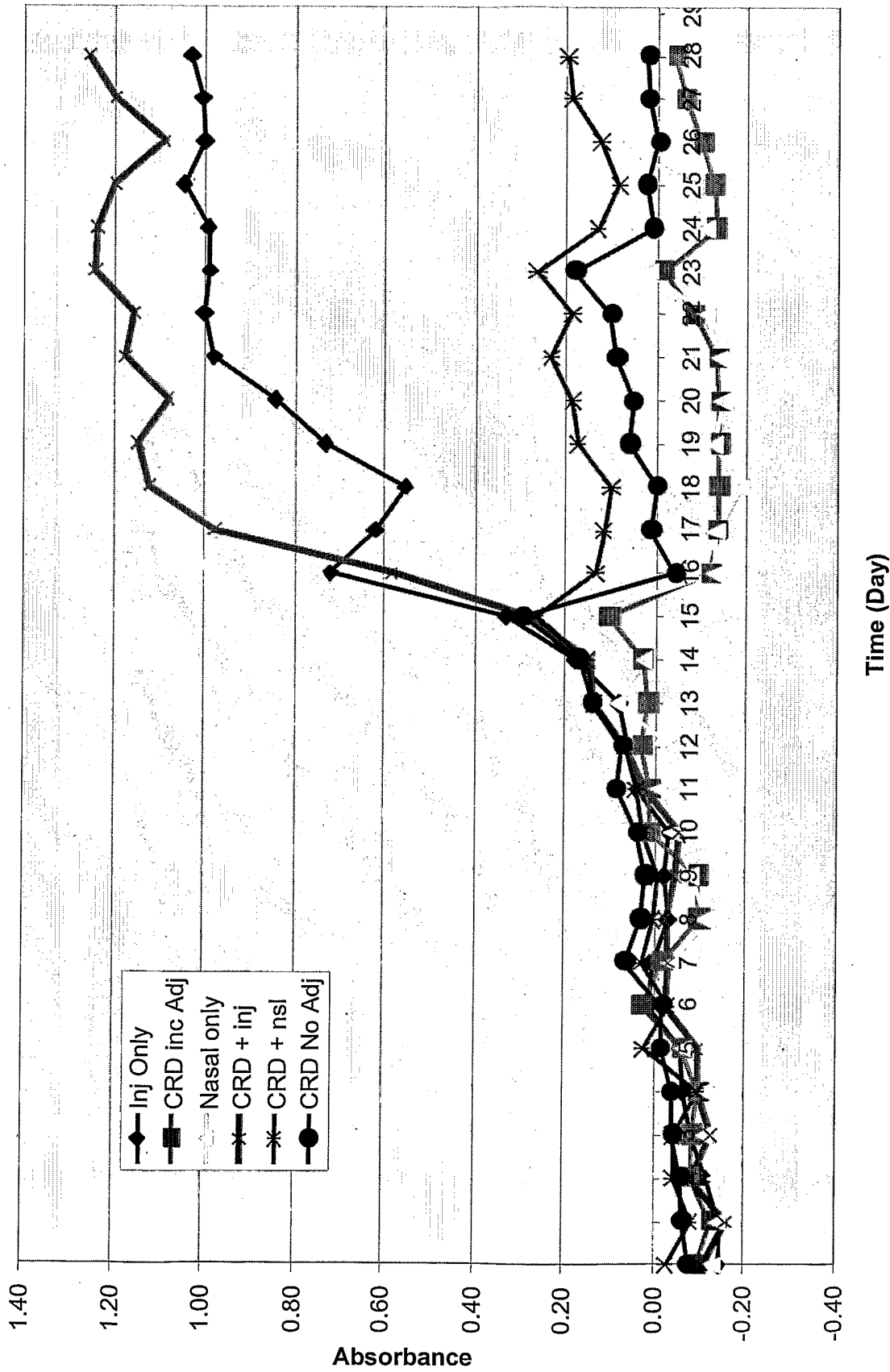


Figure 7

- 6/11 -

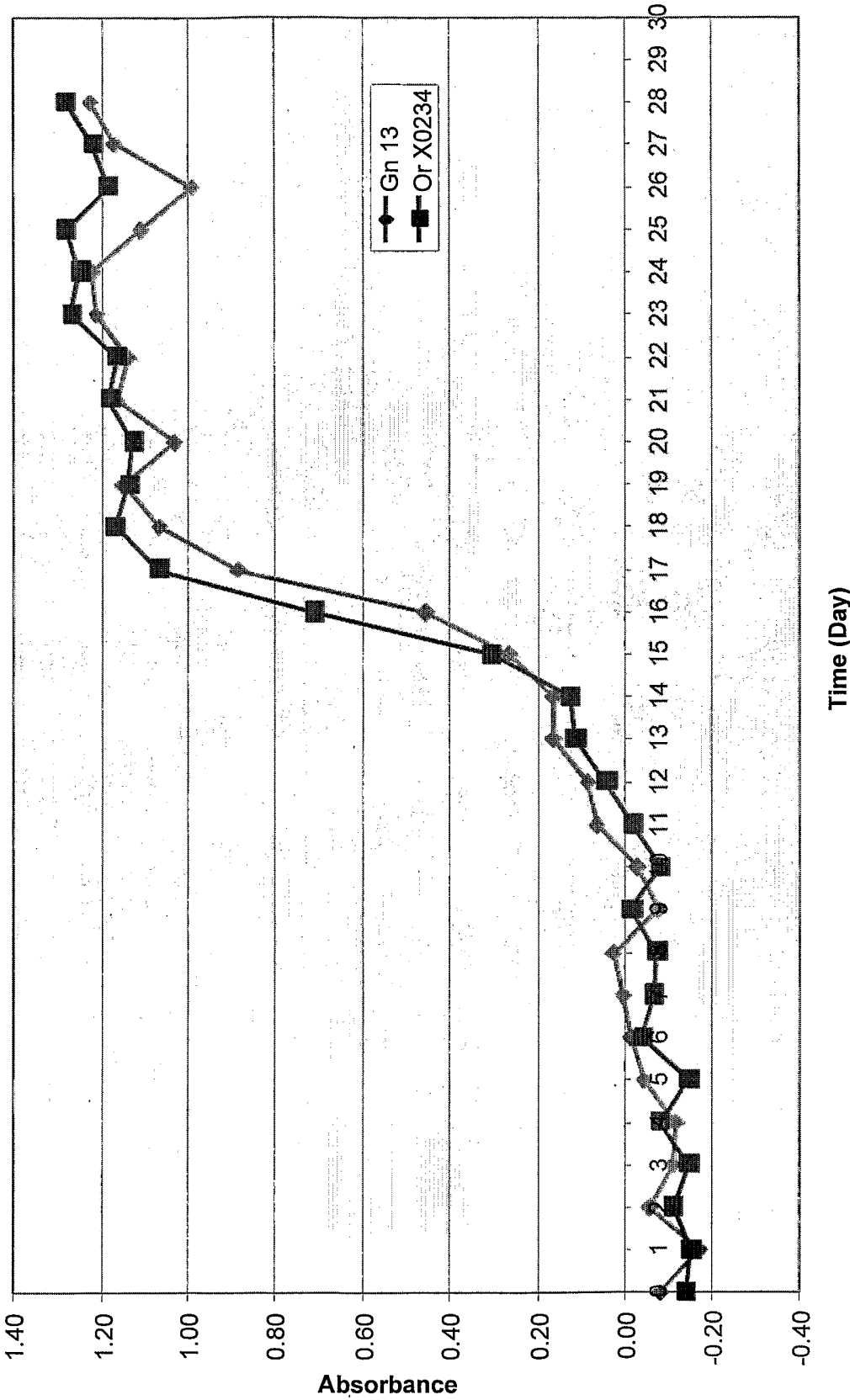


Figure 8

- 7/11 -

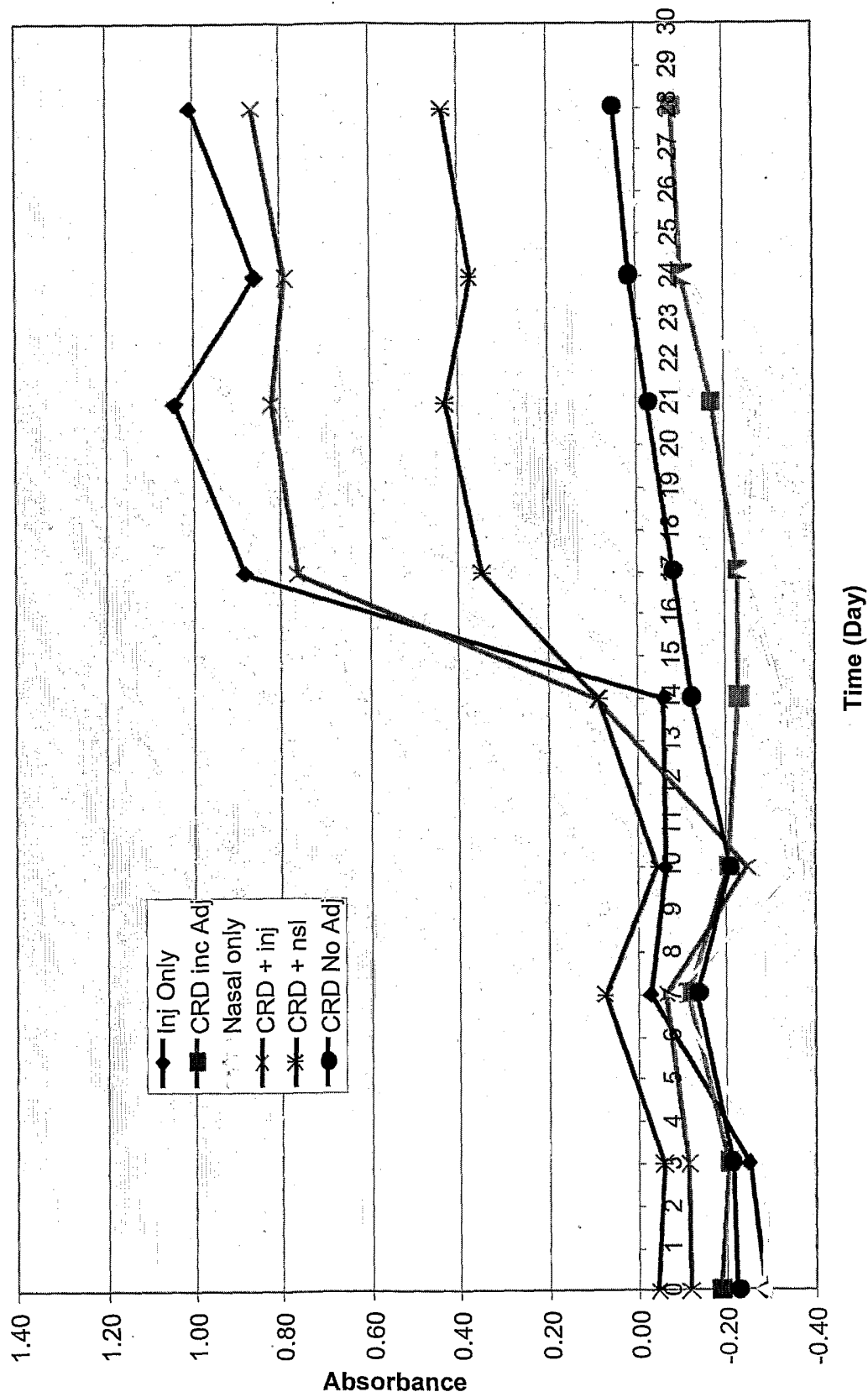


Figure 9

- 8/11 -

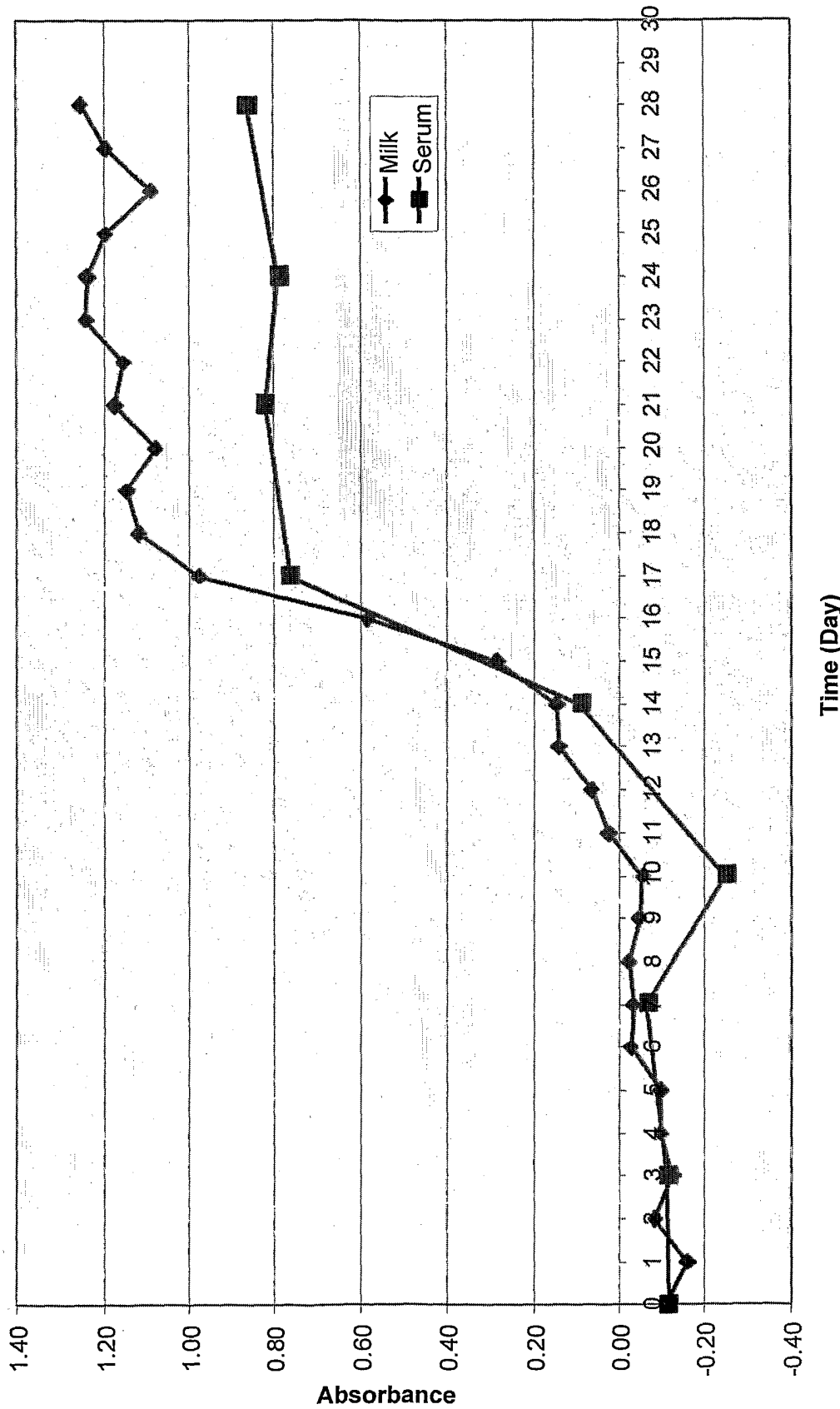


Figure 10

- 9/11 -

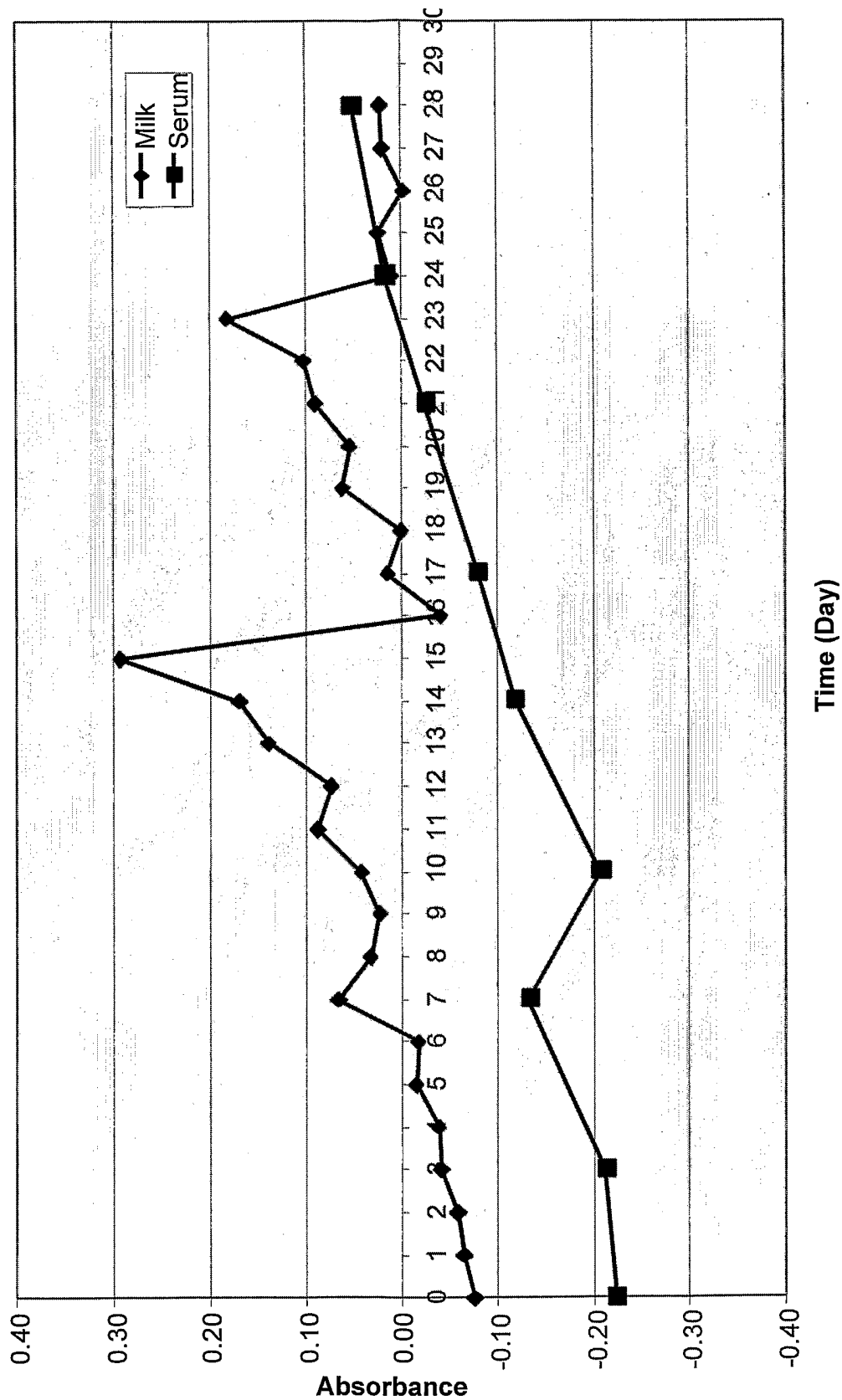


Figure 11

- 10/11 -

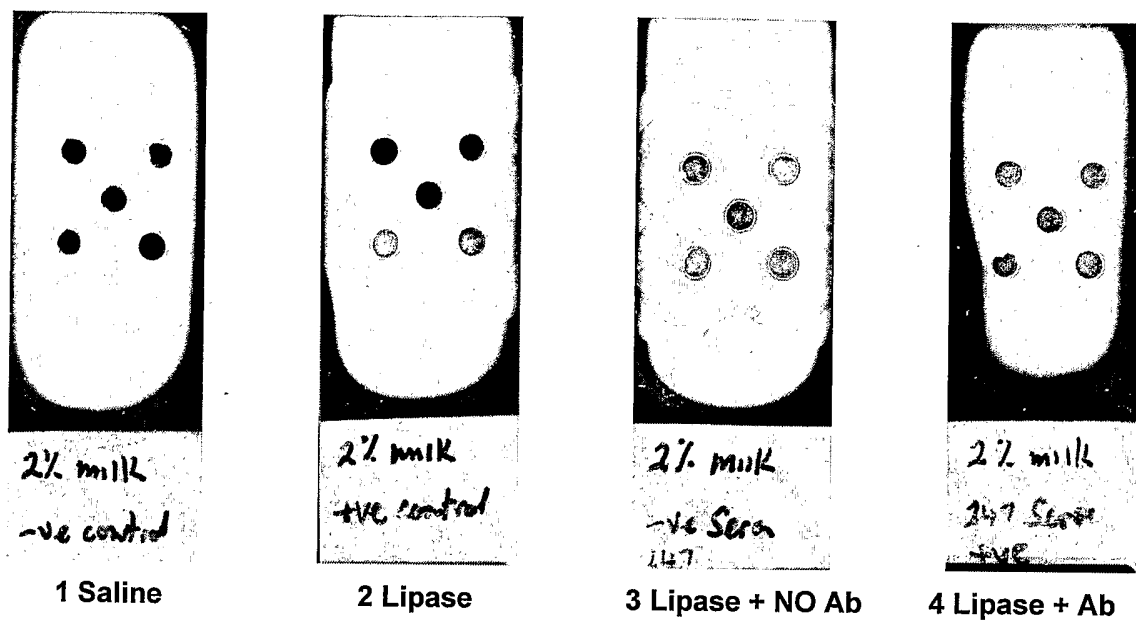
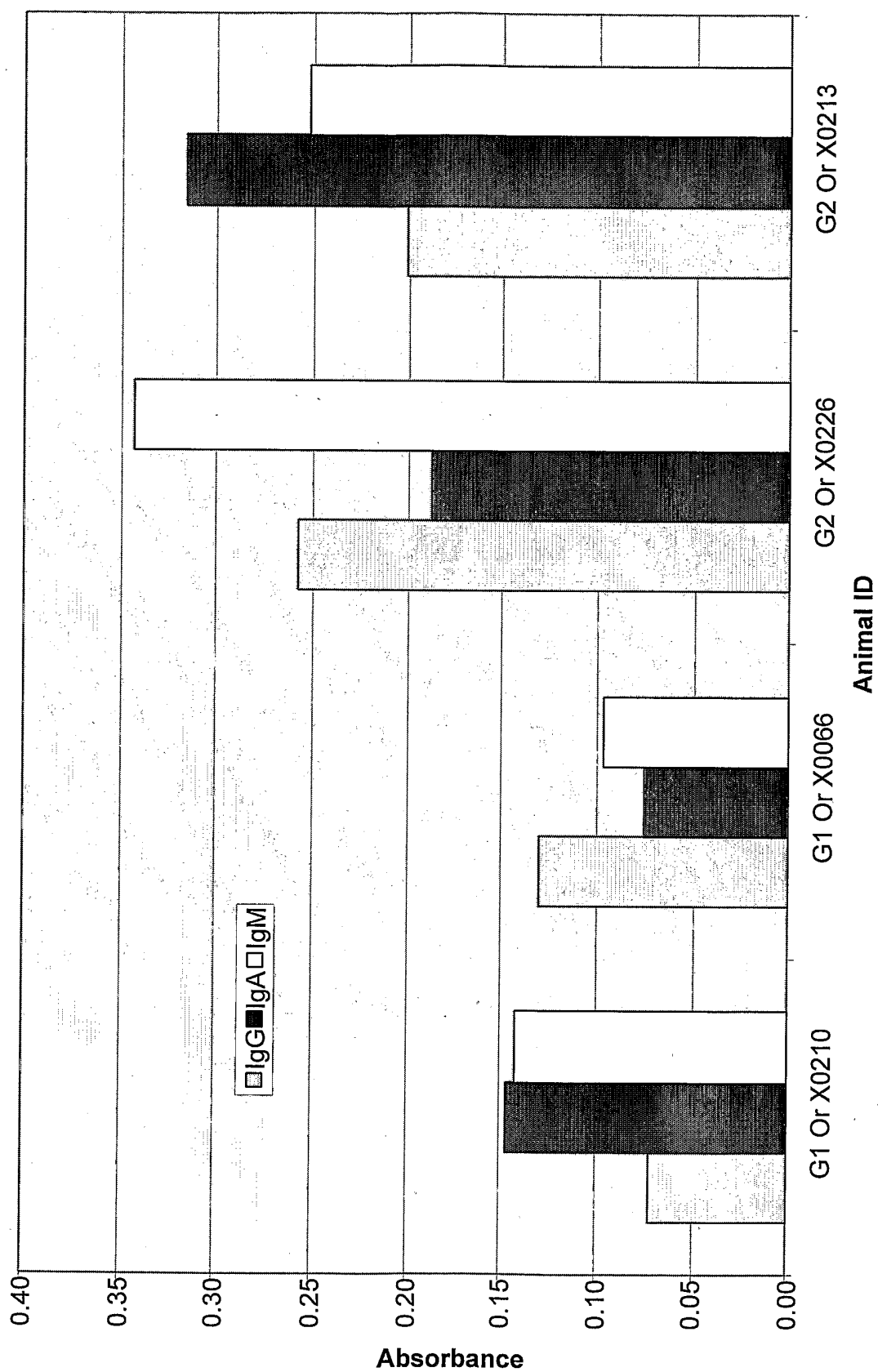


Figure 12

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**Figure 13**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2005/001540

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ⁷: A61K 39/00, 39/104, C07K 16/04, A23C 9/152, 9/158, A23L 1/305, A61P 31/04

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)

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)

DWPI, Medline & keywords: supramammary, lymph node, antibody, antigen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 20020110555 A1 (Lee) 15 August 2002 Entire document	1-39
X	Nordhaug M L et al., A field trial with an experimental vaccine against <i>Staphylococcus aureus</i> mastitis in cattle. 2. Antibody response, <i>J Dairy Sci.</i> , May 1994, 77(5):1276-84. Abstract	1-10, 13-29
A	O'Brien C N et al., Immunization with <i>Staphylococcus aureus</i> lysate incorporated into microspheres, <i>J Dairy Sci.</i> , August 2001, 84(8):1791-9. Abstract, page 1792, left column, paragraph 3	1-10, 13-29

☒ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:	
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"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
2 November 2005

Date of mailing of the international search report
15 NOV 2005

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2005/001540

C (Continuation).

DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Tollersrud T et al., Antibody responses in sheep vaccinated against <i>Staphylococcus aureus</i> mastitis: a comparison of two experimental vaccines containing different adjuvants, <i>Vet Res Commun.</i> , December 2002, 26(8):587-600 Abstract	1-10, 13-29

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2005/001540

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					
US	2002110555	AU	17390/01	CA	2389582	EP	1226176
		NZ	518638	US	2005220783	WO	0132713
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. END OF ANNEX							