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# Characterisation of a novel monoclonal antibody, XC10, that recognises phosphocholine expressed on the surface of bacterial pathogens.

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**Summary**

Phosphocholine (PC) is commonly expressed on the surface of pathogens and can act as a hapten, generating an immune response. The antibodies recognising PC can be used for diagnostics and treatment purposes. Here, a novel antibody, XC10, is described. It is specific for PC but less expensive to use than the commercial antibody Tepc15.

The two gram negative bacteria *Neisseria meningitidis* and *Haemophilus influenzae* were used in the experiments. Both express PC on their surface; *N. meningitidis* on its pili and *H. influenzae* on the cell wall.

The XC10 antibody was compared to Tepc15 via ELISA assays and was found to have very similar affinity for PC as Tepc15. None of the antibodies could distinguish between how many copies of PC were present or where on the surface the PC was expressed. Since the XC10 antibody was derived from mouse serum, a major task in its characterisation was to remove the serum albumin from the sample. Two types of centrifugational separation devices were used with varying results.

## Introduction

*Neisseria meningitidis* and *Haemophilus influenzae* are both gram negative bacteria that colonize the upper respiratory tract of humans and have the potential to cause severe diseases such as meningitis, septicaemia (blood poisoning), otitis media (ear infection), conjunctivitis (eye infections) and sinusitis (Dixon *et al* 2007).

Gram negative bacteria are characterized by having a thin cell wall containing petidoglycans protected by an intricate outer membrane comprised of phospholipids and lipopolysaccharides (LPS). The LPS is the major surface glycolipid of *H. influenzae* and consists of lipid A, the membrane anchor, attached via 2-keto-3-deoxyoctosonic acid (kdo) to the core oligosaccharide, which includes a triheptosyl backbone (Fox *et al* 2008). This backbone has oligosaccharide extensions, mainly comprised of hexose sugars, which can be subject to substitution with additional groups such as phosphocholine (PC) (Lysenko *et al* 2000, Wieser *et al* 1997).

*N. meningitidis* also expresses PC but on its pili. Pili are hairlike appendages expressed on the surface of bacteria and aid the attachment of the bacteria to other bacteria or host organisms. The expression of PC on the surface of these two pathogens is subject to phase variation. Phase variable expression is an immune evasion technique that allows pathogen to vary their surface moieties by switching genes on and off. This is a common feature in bacteria, and other molecules on the cell surface are also subject to phase-variable expression (Dixon *et al* 2007, Long *et al* 1998). Phase variation is thought to increase the fitness of the organism by helping the organism avoid antigen-specific host immune defences that can be induced by molecules such as PC (Lysenko *et al* 2000). When PC is attached to a carrier such as LPS or pili it can act as a hapten, a small molecule that induces an immune response (deVos and Dick 1993). A large number of pathogens are known to express PC on their surface. This assists in the process of adhesion and colonization of the host by mimicking the mechanisms of the host cell signalling. The host immune system in turn has evolved processes that recognise this feature (Huteau *et al* 2004).

The incorporation of PC into the LPS of *H. influenzae* is controlled by the *lic1* locus comprised of *lic1A* to *lic1D*. The phase variable expression of PC is caused by tetranucleotide repeats within the *lic1A* gene. A mutation in any of the four genes will disrupt the addition of PC to LPS. Recent work has shown that a subgroup of non-typable *H. influenzae* strain contain two copies of the *lic1* locus which leads to addition of two PC to LPS. A mutation in one of the two copies of the gene *lic1D* will give an addition of only one PC to LPS (Fox *et al* 2008) instead of two (Lysenko *et al* 2000). The PC residues can be linked to the hexose residues extended from any of the three heptose residues of LPS, Hep I, II and III (Fig1). This causes a slight variation of the epitope recognised by antibodies (Lysenko *et al* 2000).

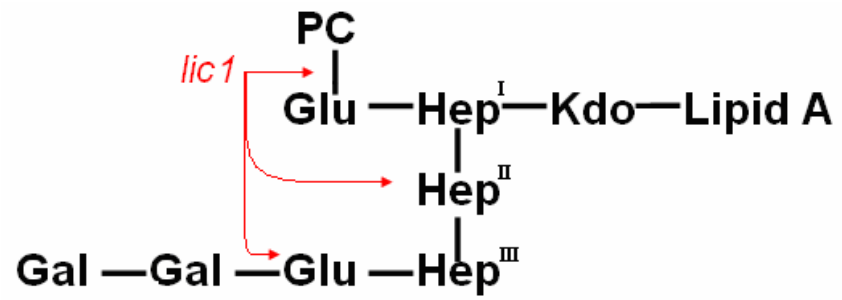


Fig 1. **Structure of the LPS of *H. influenzae.*** PC can be attached to any hexose sugar residue (Glu [glucose] or Gal [galactose]) attached to the three heptoses (Hep I, II and III). In this figure, PC is attached to the glucose residue extended from Hep I. The core oligosaccharide is attached to the membrane anchor, Lipid A, via a kdo (2-keto-3-deoxyoctlosonic acid) moiety.

The pili of *N. meningitidis* are made from an assembly of many pilin monomers and extend from the bacterial surface assisting with adhesion and cytotoxicity. Pili play an essential role in the course of colonisation of the host. The gene *pptA* (phosphoryltransferase A) is involved in the post translational attachment of PC to pili. The *pptA* gene contains a poly-G tract that leads to the phase variable expression of PC on pili of *N. meningitidis.* Inactivation of the *pptA* gene gives a complete loss of PC attachment to pili (Warren and Jennings 2003).

Since both *N. meningitidis* and *H. influenzae* have phase variable expression they are genetically and phenotypically variable and the existing vaccines are far from satisfactory. Antibodies for fast detection of the pathogen, as treatment or therapy and for research purposes, are therefore of increasing interest (Huteau *et al* 2004). On the market today, Tepc15 is the most widely used monoclonal antibody that recognises phosphocholine expressed on the surface of these organisms. When this antibody is used in assays to detect pathogens it is most often used with a secondary antibody, marked for easy detection. What secondary antibody is used is determined by how well it recognises the primary antibody (Tepc15), it has no further relevance for the study. The secondary antibody commonly used in experiments with Tepc15 is immunoglobulin A (IgA). IgA is expensive and rarely used in laboratories for other purposes. Therefore it would be beneficial to find a monoclonal antibody specific for PC but recognised by a more frequently used and cheaper secondary antibody. The antibody XC10 is a nice candidate since it is specific for PC and can be recognised by immunoglobulin G (IgG), which is cheaper and more frequently used for other purposes compared to IgA.

**Aims**

The aim of these experiments was to characterise a novel monoclonal antibody, XC10 and determine if it had similar specificity for PC as Tepc15.

## Results

### Comparison of the binding specificity of XC10 and Tepc15

The presence of pili and LPS on the *N. meningitidis* and *H. influenzae* strains used in the experiments had to be verified in order to draw conclusions of the presence of the antigen phosphocholine (PC).

The presence of pili in both strains (C311 and C311*pptA)* of *N. meningitidis* was confirmed by a Western blot probed with anti-pilin antibody (Fig 2a). The increased migration of the pili from strain C311*pptA* reflected the loss of PC in this mutant strain (Fig 2a).

The specific binding to phosphocholine was confirmed for both Tepc15 and XC10 as both bound to the pili expressing PC in strain C311, but not to the pilin of strain C311*pptA* which lack PC (Fig 2b, c). The Western blot analysis revealed that the two antibodies had the same binding affinity for PC expressed on nesserial pili.

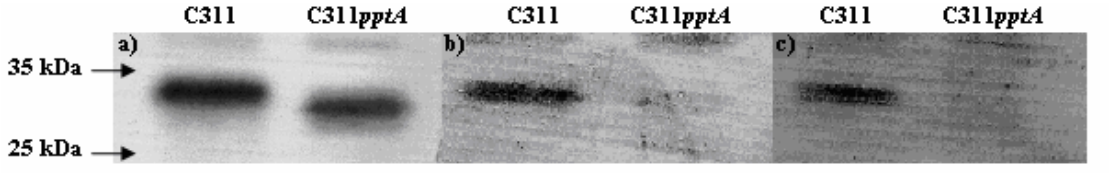


Fig. 2. **Assay of the XC10 binding to PC on *N. meningitidis*** a) Western blot with anti-pilin antibody b) Western blot with Tepc15 c) Western blot with the novel XC10

The presence of LPS on *H. influenzae* (strains 1159, 1158*lic1D* and 1159*lic1*) was confirmed by a silver stain of the sodium dodecyl sulfate (SDS) polyacrylamide gel run simultaneously with the gel for Western blot (Fig 3c). When the LPS moiety was present on the surface it incorporated the silver ions and gave a distinct stained band on the gel. In its absence, no band could be detected. The immunoblot showed the same level of binding by both XC10 and Tepc 15 (Fig 3). Both antibodies bound to LPS-PC from the strain which had two PC attached to its LPS, and from the mutants who only had one PC attached to its LPS. Neither of the antibodies bound to the mutant who had no PC attached, which demonstrated the specific binding of the antibodies to PC. No significant relationship between the amount of PC attached to LPS and binding affinity could be distinguished.

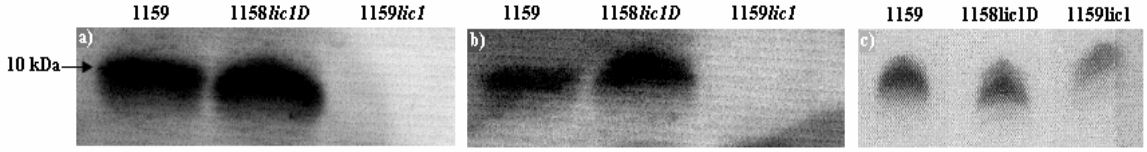


Fig. 3. **Assay of the XC10 binding to PC on *H. influenzae*** a) Western blot with Tepc15 b) Western blot with the novel XC10 c) a silverstain of corresponding SDS polyacrylamide gel showing the presence of LPS in all samples.

### Purification of XC10 to enable direct comparison with Tepc15

The XC10 antibody was extracted from mice blood samples. One of the most abundant proteins in blood is serum albumin which can be as much as half of the blood serum protein present. In order to purify the XC10 sample, and determine the concentration of antibody, the serum albumin had to be removed.

The concentration of the XC10 antibody had to be determined to efficiently compare the two antibodies. Since the XC10 antibody was in a mouse serum suspension containing large amount of serum albumin which would interfere with the XC10 purification, the albumin had to be removed. Albumin is one of the most abundant proteins in blood sera and unfortunately it had approximately the same mass as XC10.

First an albumin depletion kit (Pall) was tried. After the XC10 sample had gone through the four centrifugation tubes used, the filtrate was pooled and loaded onto the affinity gel column. The fractions collected from the affinity gel column, the XC10 starting material and the albumin reduced samples from the albumin depletion kit were analysed by an enzyme-linked immunosorbent assay (ELISA) assay. Antibody binding could be detected only in the starting material and in the albumin reduced sample, which confirmed that no antigen got stuck in the albumin depletion kit. The pH of buffers used was adjusted and the samples run through the affinity gel column twice. The new fractions were again analysed by an ELISA assay, with similar results. The samples were loaded onto a SDS polyacrylamide gel which revealed that the albumin reduction was insufficient since XC10 sample had a much higher albumin content than first thought (Fig 4).

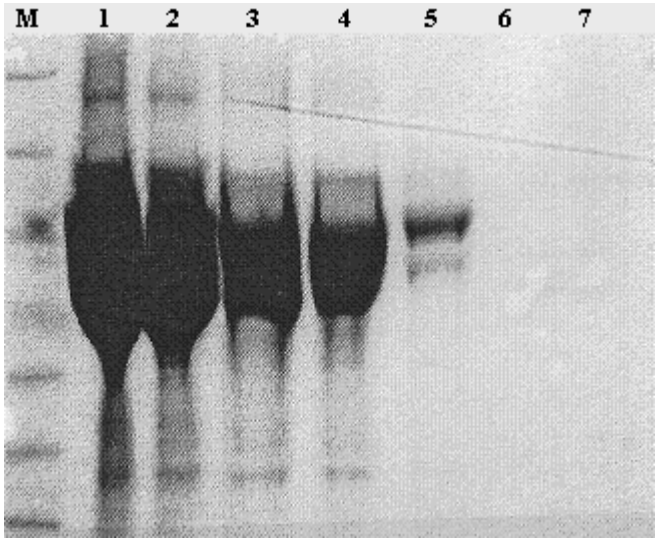


Fig 4. **An SDS polyacrylamide gel of the samples after the albumin reducing process**. Lane 1: XC10 starting material, lane 2: albumin reduced sample from the albumin depletion kit, lane 3: first flow through off the affigel column, lane 4: second flow through off the affigel column, lane 5: wash off the affigel column, lane 6: fraction 1 off the affigel column, lane 7: fraction 2 off the affigel column.

To increase the amount of albumin removed from the serum, the sample was run through a bench column which contained 15 albumin depleting discs (Albumin depleting kit, Pall) and the filtrate was run through the affinity column. The fractions off the column were analysed by an ELISA assay (data not shown). The ELISA assay showed some binding of the antibodies to the starting material as well as in the flow through which indicated that the albumin bound to the column blocking XC10 from binding, further stressing the importance of succeeding in removal of the albumin from the sample. A SDS polyacrylamide gel confirmed the results as it showed a significant amount of albumin still present in the sample (Fig 5).

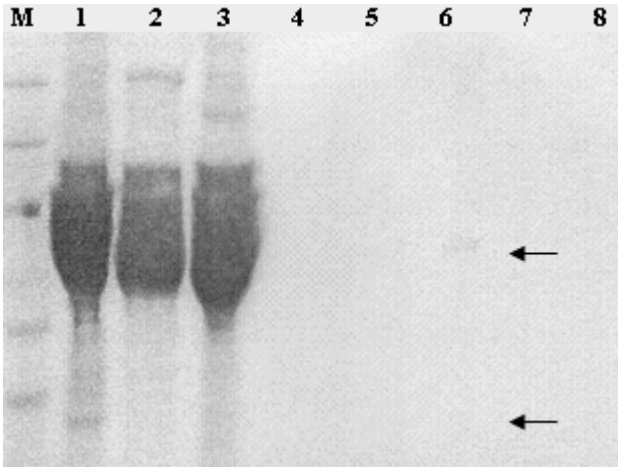


Fig 5. **SDS polyacrylamide gel of the samples from the albumin reduction process**. Lane 1: XC10 starting material, lane 2: albumin reduced sample, lane 3: wash off the albumin removing column, lane 4: the wash of the Affigel column, lane5: fraction 1 off the Affigel column, lane 6: fraction 2 off the Affigel column. Faint bands at the position expected for XC10 can be seen in lane 6.

There was a small reduction in the amount of albumin present in the sample after going through the albumin depleting column (lane 1 and 2 in Fig 5) but not nearly enough for a successful albumin removal. Lane 3 (Fig 5) showed that the column managed to remove a substantial amount of albumin from the starting material even though a lot was still present in the sample afterwards.

To further increase the reduction of the albumin content in the XC10 sample, the sample was run through a 30 K red micro separation centrifugal tube (Pall). The flow through was discharged and the top layer was loaded on to the affinity column and left to run in a loop over night hoping that more of the antibody would have a chance to bind to the column. Fractions were collected from all steps and loaded on to a gel (data not shown). There was still a significant amount of albumin in the sample, but two bands corresponding to the light and heavy chain of the XC10 antibody could be seen in the second fraction off the column. However, the amount was too small to purify and the albumin content needed to be further reduced. The ELISA assay of the same samples further confirmed the results of the SDS polyacrylamide gel (Fig 6). The antibody activity seemed to disappear after the sample had been applied to the affinity gel column indicating that only a small amount of the antibody bound to the column.

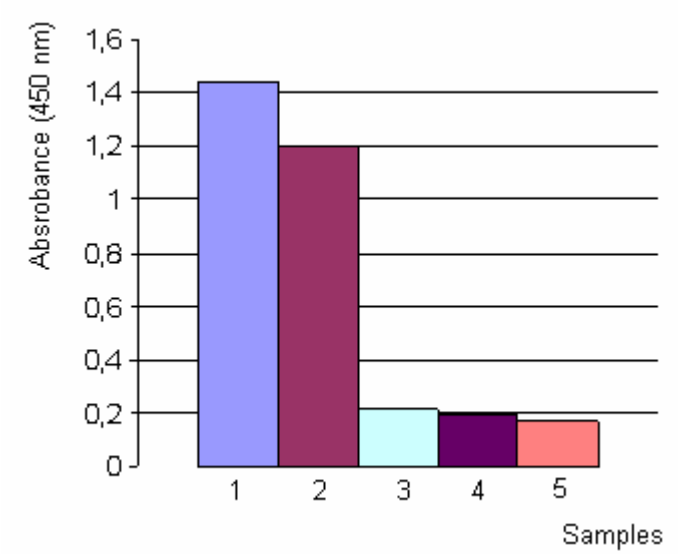


Fig 6. **ELISA assay of XC10 activity.** The amount of XC10 in the samples was determined by an ELISA assay. Bar 1: XC10 starting material, bar 2: top layer (micro sep. centrifugal tube), bar 3: over night flow through (affinity column), bar 4: fraction 1 (affinity column), bar 5: fraction 2 (affinity column).

A Millipore centrifugal device with a larger cut off value (100 K) was tried. The hypothesis was that the larger cut off value would separate the albumin from the antibody with greater success. The albumin reduced sample was loaded onto the affinity gel which was left to run in a loop over night. The fractions collected were loaded onto an SDS polyacrylamide gel and an ELISA plate with the same poor results as above. On the gel, faint bands could be seen in all fractions where the light chain of the antibody would be expected, but since there were no bands representing the heavy chain, this was most likely albumin coming of the column instead of the antibody. The ELISA results confirmed that in fact it was albumin giving rise to the bands, since there was no antibody activity in any of the fractions (Fig 7). The ELISA assay also confirmed that the antibody did not bind effectively to the column since such high antibody concentration was present in the wash.

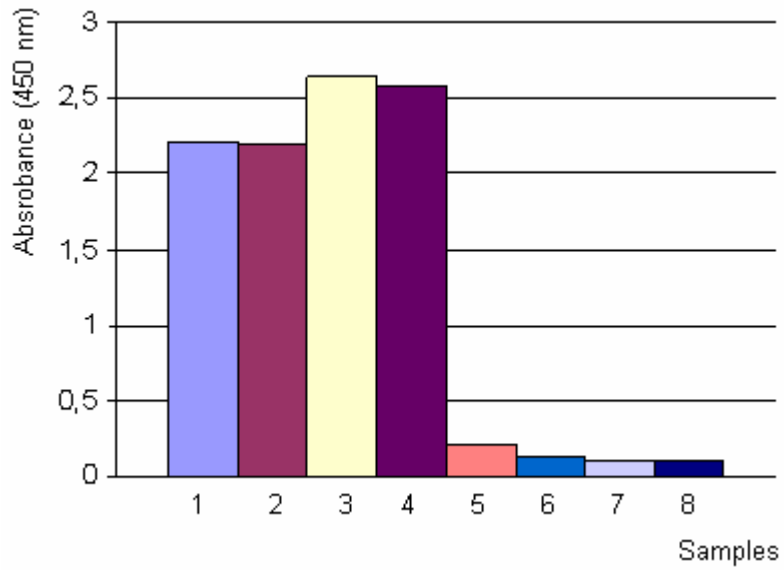


Fig 7. **XC10 activity of the albumin reduced samples.** The XC10 activity of the samples was analysed by an ELISA assay. Bar 1: XC10 starting material, bar 2: Albumin reduced sample, bar 3:

Flow through (affinity column), bar 4: Wash (affinity column), bar 5: Fraction 1 (affinity column), bar 6: Fraction 2 (affinity column), bar 7: Fraction 3 (affinity column), bar 8: Fraction 4 (affinity column).

**Comparison of the two antibodies by individually determining their PC specificity.**

Since the concentration of the XC10 sample could not be determined, a direct comparison between the two antibodies was not possible. However, the activity of each of the two antibodies could be assessed separately by ELISA assays and indirectly compared. The comparison was made through the ratio of antibody bound to *H. influenzae* with PC present and antibody bound to *H. influenzae* without PC present. The ratio was determined independently for both antibodies. The assays showed that the two antibodies had similar affinities for PC (Fig 8 and 9).

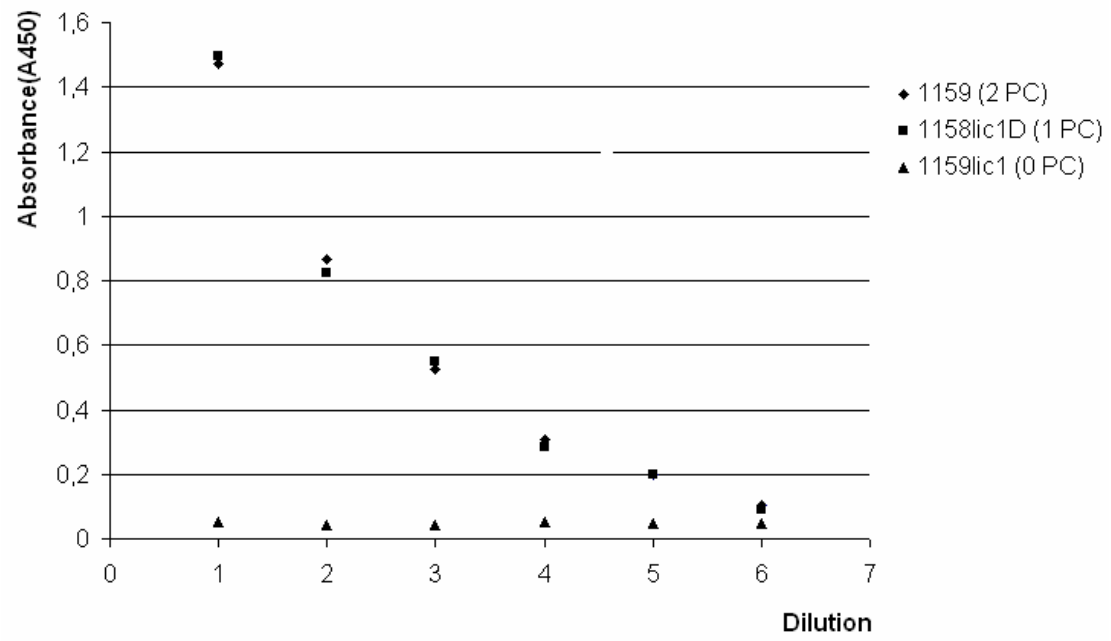


Fig 8. **Binding intensity of XC10 to *H. influenzae*.** The binding intensities of XC10 towards PC was analysed by an ELISA assay. Three strains were used; one with 0, 1 and 2 PC attached and the binding intensity was measured by the absorbance at 450 nm. The sample was diluted 1:2 between each reading.

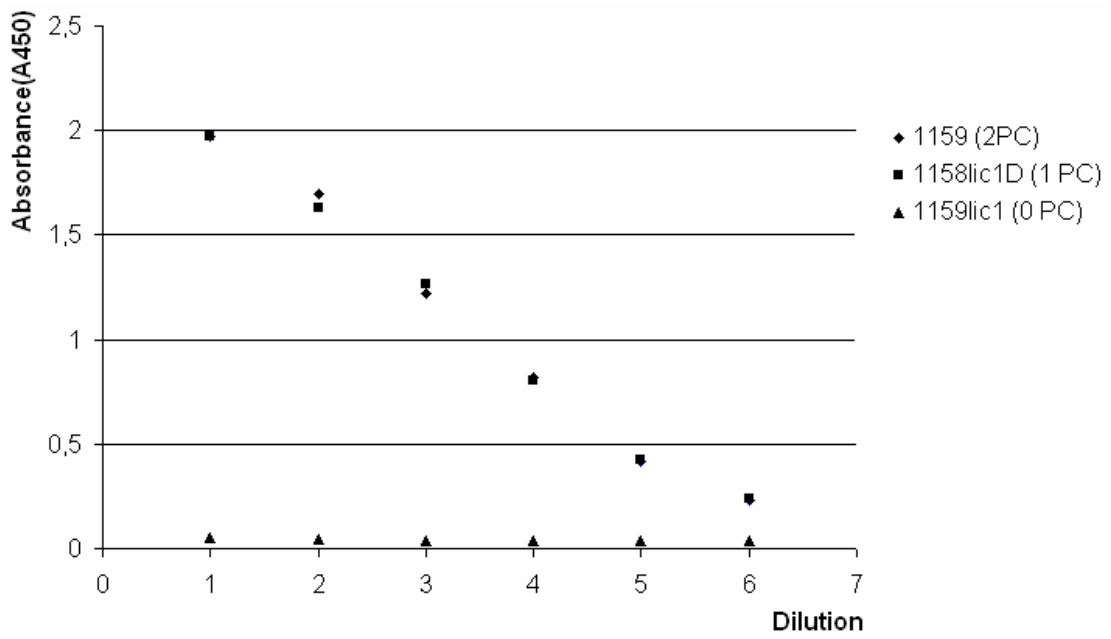


Fig 9.**Binding intensity of Tepc15 to *H. influenzae***. The intensity of binding towards PC was analysed by an ELISA assay. Three strains were used, one with 0, 1 and 2 PC attached and the binding intensity was measured by the absorbance at 450 nm. The sample is diluted 1:2 between each reading.

The graphs are very similar, and neither of the antibodies bound to *H. influenzae* without PC expressed on its surface. Neither of the two antibodies appeared to distinguish between how many PC were bound per LPS or where the PC was attached to LPS. Whether the position of PC attached to LPS had importance for binding of the antibody was further analyzed in an ELISA assay with strains 375 (PC attached to HepI), 486 (PC attached to HepII) and Eagan (PC attached to HepIII). Initial results indicated that XC10 had a higher affinity for the strain 486 than Tepc15, but when repeating the experiment this could not be confirmed. Due to the phase variable nature of these strains further analysis confirmed that 375 and Eagan were in the inactive phase, ie the expression of the genes for PC attachment was switched off, which explained why none of the antibodies bound to their LPS in the initial study (data not shown).

**Creating the fourth 1158*lic1D* mutant strain to complete the set of isogenic strains of *H. influenzae***

In order to study the relationship between the position of PC attached to LPS, and the importance of the PC's position on LPS, efforts were made to create the full set of isogenic strains of *H. influenzae* with 0, 1 and 2 PC attached at different positions of LPS.

The *lic1D* gene allocates PC to a distinct site on LPS. In these experiments *H. influenzae* strains which expressed one, two and no PC was used. However, there was another variant of the strain only expressing one PC that had not been made at the time (Fig 10 d). In order to asses the effect of the position of PC upon binding of the antibody, this mutant had to be created.

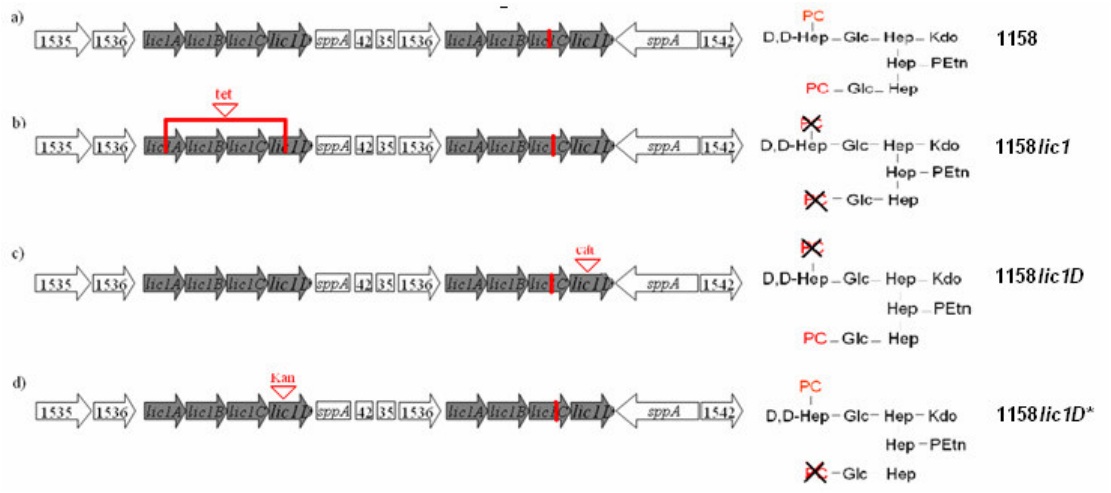


Fig 10. **Arrangement of the *lic1* locus and corresponding LPS structure of the *H. influenzae* strains.** The strain 1158 had a duplication in the *lic1* locus. The 2nd copy of *lic1C* gene, which has a vital role in PC attachment, had a mutation inactivating the gene leaving the bacteria with only one functional *lic1C* gene. The *lic1D* gene is responsible for allocation the PC molecule. a) Strain 1158 with two functional copies of the *lic1D* gene inserts two PC on the LPS. b) Strain 1158*lic1*, with an insertion of a *tet* cassette in the first *lic1* locus, which leaves the mutant with no PC attached. c) Strain 1158*lic1D* had an insertion of a *cat* cassette into the second *lic1D* gene attaches only one PC. d) Strain 1158*lic1D** with an insertion of a *kan* cassette into the first *lic1D* inserts only one PC, but at a different position on LPS than 1158*lic1D.*

The first copy of the *lic1D* gene was amplified by PCR, with primers specific for this copy, and inserted into a pGEM-Teasy plasmid. Transformants containing the insert were identified by blue/white colony screening. The white colonies were re-streaked and confirmed to contain the correct insert by PCR and agarose gel electrophoresis. The gene was interrupted by a kanamycin resistance cassette and the gene transferred into the wild type 1158 strain as seen in the strategy described in Fig 11. However, due to time constraints, this last step was not completed.

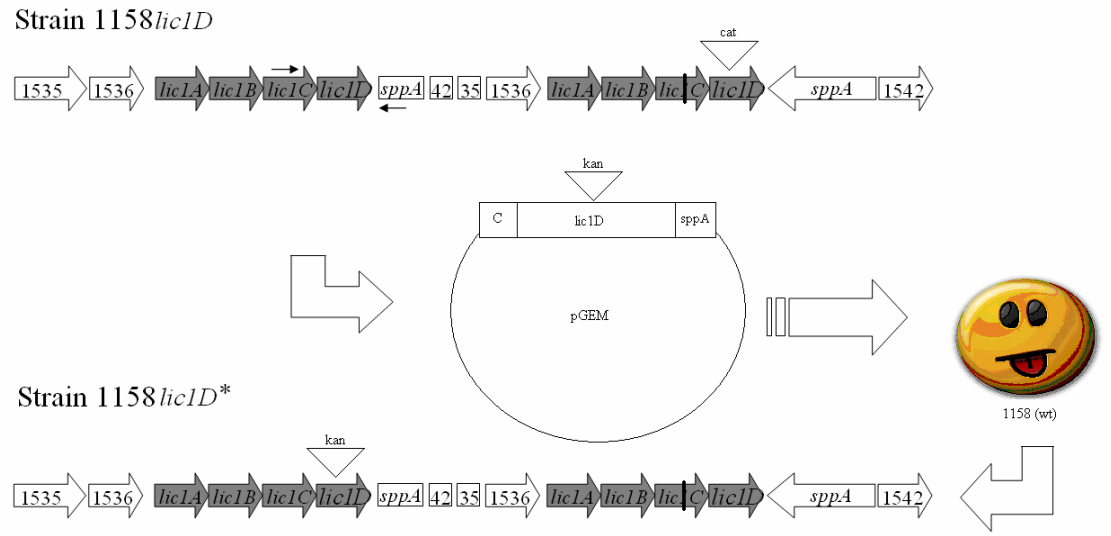


Fig 11. **The strategy for creating the 1158*lic1D** mutant**. The first copy of the *lic1D* gene was amplified and inserted to a pGEM-Teasy plamid. The gene was interrupted by a *kan* cassette and inserted to the wild type 1158 which then would become 1158*lic1D** expressing one PC but at a different position than 1158*lic1D*.

## Discussion

### The importance of sufficient albumin removal

Although it was not possible to compare XC10 and Tepc15 directly, the ELISA and Western blot data showed that the affinity and specificity for PC were the same. Both antibodies bound to bacteria expressing PC on its surface. Since the concentration of XC10 in the mouse serum sample could not be determined, the antibodies could only be compared indirectly. The reason for the failure in determining the XC10 concentration was the insufficient removal of albumin from the sample. As discussed by Chen *et al* (2005) highly abundant proteins such as albumin can efficiently reduce the chance of detecting biomarkers, such as the XC10 antibody. Albumin is usually present in concentrations of mg/ml whereas biomarkers are present in nm-pm/ml (Chen *et al* 2005). The separation methods used in these experiments did not give adequate separation between the antibody XC10 and albumin. Albumin is present in all sera and has the same size as the light chain of XC10. Hence it is necessary to remove it in order to be able to detect the antibody. Albumin will also surpass the antibody when it comes to binding to the affinity column. Using an affinity column is a widely used method to remove albumin but a disadvantage is its lack of specificity which means that a lot of other proteins will bind to the column instead, possibly even the biomarker of interest (Chen *et al* 2005). This will make the separation process even more difficult, especially since albumin is present in a much higher concentration than the antibody. The purification in the experiments described here used various albumin depleting kits with filters containing cibracron blue and centrifugation methods based on size. The best explanation to the predicament of the difficulties in getting the XC10 antibody to bind to the affigel column was that XC10 was outcompeted by the albumin.

A small fraction of the antibody did bind to the affigel column however, and could be detected on an SDS polyacrylamide gel, even though this was not enough to purify the sample. Nonetheless, with more time, it should be possible to separate the two proteins. With increasing interest in proteomics, the methods for reducing abundant proteins from sera are constantly refined and other techniques can be tried in order to remove albumin. These methods could be adjusted to suit these specific proteins better (Lee *et al* 2008**,** Chen *et al* 2005). Lack of time was the main reason for the failure in this separation process.

### Elucidating the importance of the position of phosphocholine

Initially it seemed that XC10 was the only one of the two antibodies that could distinguish between one or two PC attached to LPS. But these initial results could not be confirmed and no conclusion could be drawn from the first experiment. Whether the two antibodies can distinguish between one or more PC attached, and if the site of attachment is important for their recognition, should be determined by further studies. In order to do this successfully, isogenic strains of *H. influenzae* that only differ in the number and location of the PC attached, are needed. Repeating these experiments with all four mutants of *H. influenzae* (Fig 10) would possibly give answers regarding PC's role for antibody recognition. It is widely established that Tepc15 recognises PC but to what extent and if it can distinguish between different positions and different magnitudes of PC expression has yet to be determined (Fox *et al* 2008). It would be of great interest to verify if one of the sites for PC attachment is more important than the others, and whether steric hindrance will keep the antibody from distinguishing

between attachments of one or two sites etc. The isogenic strains could also be used in more general studies of PC's role in attachment and colonization of the host. Knowing more of how *H. influenzae* colonizes its host will greatly benefit the discovery and studies of new drugs, and also the development of new screening method for this very serious human pathogen (Fox *et al* 2008), for which the antibody XC10 could be used.

## Materials and Methods

### Bacterial strains and culture conditions

Strains from both *H. influenzae* and *N. meningitidis* were used in these experiments (Table 1). They were grown on blood heart infusion (BHI) agar (5.2 % BHI agar, 1 % (w/w) proteose peptone, 0.09 M NaCl, 0.02 M $Na_2HPO_4$, 0.02 % (w/w) dextrose and 10 % (w/w) Levinthal base) at 37 ˚C over night. Colonies were scraped and resuspended in 1 ml PBS (0.14 M NaCl, 2.7 mM KCl, 10.1 mM $Na_2HPO_4$ 1.8 mM $KH_2PO_4$, pH adjusted to 7.4). Absorbance was measured at 600 nm ($A_{600}$).

Table 1. Bacterial strains

| Organism | Strain | Description | Reference |
|---|---|---|---|
| *H. influenzae* | 1159 (wt) | A non-typable *H. influenzae* isolate containing a duplication of the *lic1* locus | Cody *et al*. 2003 |
| | 1158*lic1D* | A chloramphenicol cassette inserted within the second *lic1D* gene | K. Fox *et al.* unpublished |
| | 1159*lic1* | A deletion in the first copy of the *lic1* locus and a tetracycline cassette inserted | K. Fox *et al.* unpublished |
| | 375 | A non-typeable *H. influenzae* isolate with PC expressed on extensions from HepI | Cody *et al*. 2003 |
| | 486 | A non-typeable *H. influenzae* isolate with PC expressed on extensions from HepII | Cody *et al*. 2003 |
| | Eagan | A non-typeable *H. influenzae* isolate with PC expressed on extensions from HepIII | Cody *et al*. 2003 |
| *N. meningitidis* | C311 (wt) | *N. meningitidis* serotype B strain | Freda Jen, unpublished |
| | C311*pptA* | *pptA* gene disrupted by a insertion of a kanamycin cassette. | Freda Jen, unpublished |
| *E.coli* | DH5α | | |

strains 1158 and 1159 were taken from the same patient on the same day and are essentially the same strain (Cody *et al* 2003)

### Antibodies

The XC10 antibody was used with mouse IgG as the secondary antibody and Tepc15 was used with mouse IgA as the secondary antibody. The antibodies were diluted in 3 % BSA-TBST (0.15 M NaCl, 2.7 mM KCl, 0.3 % (w/w) Tris base, $5x10^{-9}$ mM Tween-20 and 3 % (w/w) BSA) at the concentrations found in Table 2.

Table 2. Antibodies

| Primary antibody | Dilution Western Blot (in BSA-TBST) | Dilution ELISA assay (in 3 % skim milk block) | Secondary antibody | Dilution Western Blot (in BSA-TBST) | Dilution ELISA assay (in 3 % skim milk block) |
|---|---|---|---|---|---|
| XC10 (M. Jennings, unpublished) | 1:10 | 1:10 | IgG, mouse (Sigma) | 1:10000 | 1:1000 |
| Tepc15 (Sigma) | 1:4000 | 1:100 | IgA, mouse (Sigma) | 1:7000 | 1:1000 |

**Cloning**

A pGEM-Teasy (Promega) plasmid was used as the cloning vector in which the polylinker was located in the *lacZ* gene.

Ligation reactions consisted of 1 µl vector, 7.5 µl quick ligase 2x buffer, 1 µl quick ligase and an appropriate volume of insert such that the vector:insert molar ratio was approximately 1:3. Reactions were made in a final volume of 15 µl with sterile $dH_2O$. The reaction was incubated at room temperature over night. The ligation products were ethanol precipitated prior to transformation (1 µl tRNA, 2 µl sodium acetate, 50 µl 100 % ethanol). DNA was incubated on ice for 15 min prior to centrifugation at 16000 g for 20 min. The supernatant was removed and the pellet was washed with 70 % ethanol, centrifuged again at 16000 g for 10 min. The pellet was air dried and resuspended in 20 µl sterile water.

Colonies of *E. coli* DH5α were used to inoculate 5 ml LB (1 % (w/w) Bacto-tryptone, 0.5 % (w/w) yeast extract and 0.2 M NaCl) which was incubated at 37 ˚C until an optical density of 0.8, measured at 600 nm ($A_{600}$), was reached. The cells were pelleted, the supernatant removed, and the pellet was suspended in a series of washes with ice-cold sterilised water. First the cell suspension was centrifuged at 16000 g for 1 min, the pellet was washed with 1 ml ice-cold water and the same procedure was repeated. Thereafter, the cells were resuspended in 0.5 ml of water, centrifuged and the pellet resuspended in 0.2 ml of water and centrifuged. The supernatant was removed and the cells resuspended in 0.1 ml of water. The ligation reaction was added to the competent cells. The mix was transferred to a pre-chilled 0.1 cm electroporation cuvette. Cells were electroporated using a Bio-Rad Gene Pulser™ (1.8 kV, 2.5 µF and 200 Ω). Immediately after electroporation the cells were moved to pre-warmed LB and the cell culture was allowed a 1.5 h recovery at 37˚C before being plated on LA plates containing ampicillin (50 µg/ml), Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM ) and 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-gal) (60 µg/ml). The plates were incubated over night at 37 ˚C. Transformants containing the insert was identified based on blue/white screening. The transformants giving white colonies were regrown on LA containing ampicillin, IPTG and X-gal.

**Polymerase chain reaction**
Amplification was carried out in a 30 µl reaction consisting of 6 µl 10x reaction buffer, 3 µl $MgCl_2$, 0.24 µl deoxynucleaside triphosphates (dNTPs), 1 µl of Taq DNA polymerase, 0.6 µl of each forward and reverse primer, 1 µl of template DNA and 22 µl sterilised and deionised water. The polymerase chain reaction (PCR) was carried out using an Eppendorf thermocycler. PCR purification was performed using a QIAGEN QIAquick gel extraction kit.

**Agarose gel electrophoresis**
A 1 kb ladder (Invitrogen) and the DNA samples were run in a 1 % agarose gel containing 0.5 µg/ml ethidium bromide. The gel was run at 120 V for 20 min in 1x TBE (0.06 M Tris base, 0.18 M Boric acid, 4.5 mM EDTA) prior to being visualized with UV illumination. DNA was extracted from the agarose gel was performed using a QIAGEN gel extraction kit.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis**
Cell lysates were prepared by resuspending plate-grown bacteria in Tris buffer. When studying LPS, 2.5 µl protein kinase and 15 µl 10 % sodium dodecyl sulfate (SDS) was added to 250 µl of the cell suspension which was incubated at 56˚C over night.

The LPS of *H. influenzae* was separated from other proteins on a 16% tricine gel (Invitrogen) and the pili of *N. meningitidis* were separated by a 4-12 % Bis-Tris gel (Invitrogen). The samples to be loaded on the gel were diluted 1:10 in Tris buffer, dye was added and the samples were boiled for 5 min prior to loading on the gel. Electrophoresis was carried out according to the manufacturer's description and running buffer made up accordingly. Two gels were used when running gels for Western blotting, one for silver stain and one for Western blotting.

**Silver stain**
The gel was incubated in fix solution (8.7 M ethanol, 0.8 M acetic acid) for at least 1 h. The gel was immersed in periodic acid solution (0.03 M periodic acid in fix solution) for 10 minutes and then washed thoroughly 3x 10 minutes with milliQ water. Staining solution (0.186 M NaOH, 0.4 M $NH_4OH$, 0.04 M $AgNO_3$) was added to the tray and left for 10 minutes. The gel was washed 3x 10 minutes with milliQ water and immersed in developer (2.8 mM citric acid, 0.34 mM formaldehyde) and left to develop in the dark. Developing was stopped with stop solution (0.16 M acetic acid). The gel was kept on agitation throughout all steps.

**Western Blotting**
The SDS polyacrylamide gel was blotted onto polyvinylidene difluoride (PVDF) membranes (GeneScreen). Transfer buffer was made up according to the manufacturer's instructions (GeneScreen). The blot was run at 30 V for 75 min. Non-specific sites were blocked with 3 % BSA in TBST blocking solution for at least 1h. The membrane was washed 3x 3min with TBST and then incubated in primary antibody solution (Table 2) over night in the presence of 23 µM sodium azide. The membrane was washed 3x 3 min with TBST and then immersed in secondary antibody solution for 1h. Before adding the substrate the membrane was washed thoroughly with TBST. The bound antibodies were visualized by adding NBT/BCIP (Roche) tablets dissolved in 10 ml $dH_2O$ and left to develop in the dark. All steps were completed with agitation.

**Enzyme-linked immunosorbent assay**
Enzyme-linked immunosorbent assay (ELISA) plates were coated with cell suspension (OD $A_{600}$ of 0.2), strains 1159 (wt) and 1159*lic1* was used. The antigen was dried onto the plate over night. Unspecific binding was blocked with a 3 % skim milk solution for 1h. The plate was washed with PBST (0.14 M NaCl, 2.7 mM KCl, 10.1 mM $Na_2HPO_4$, 0.001M $KH_2PO_4$, 0.2 % Tween-20). A primary antibody solution (Table 2) was added (100 µl per well) and incubated for 1h. The plate was washed with PBST and the secondary antibody solution (100 µl per well) was added and left for 1h. Substrate was added to the wells (10 ml substrate with 38 mM 2.2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 0.58 µM $H_2O_2$).The colour intensity was measured after no less than 40 min.

**Albumin removal**
Two methods were attempted, albumin depleting discs (Pall) and size exclusion centrifugation tubes (Pall).

One albumin depleting disc from the albumin depleting kit (Pall) was put into each of four tubes used. The disc was resuspended in and samples centrifuged at 14000 g according to the manufacturer's instructions. The flow through was sampled.

In an attempt to remove more albumin, 15 albumin depleting discs were pooled into one column. The XC10 sample was loaded on to the column and the flow through was collected. The albumin was eluted from the column by running 2 M guanidine hydrochloride through the column.

Two types of size exclusion centrifugation tubes were used, a 30 K red microseparation centrifugal tube (Pall) and a 100 K Centricon centrifugal tube (Millipore). The XC10 sample was diluted 1:2 with PBS and centrifuged according to the manufacturer's instructions. The 30 K red microseparation centrifugal tube was centrifuged at 67000 g and the flow through was collected; the 100 K Centricon centrifugal tube at 11700 g and the top layer was sampled.

All albumin depleted samples were given a concentration of 0.8 M $(NH_4)_2SO_4$ before being added to the Affigel column.

**Affinity column**
An Affigel column (Bio-Rad) was equilibrated with 0.8 M sodium sulphate phosphate buffer (0.8 M $(NH_4)_2SO_4$ in $Na_2PO_4$ buffer, pH 7.5). The sample was loaded on to the column and washed with 10 ml with sodium sulphate phosphate buffer. The eluate was recovered by running phosphate buffer through the column and collecting fractions.

The Affigel column was rinsed with 3 % isopropanol solution and re-equilibrated with sodium sulphate phosphate buffer between the experiments.

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