Summary of attempts to replicate Anne Hyman's dissertation results

Following submission of a manuscript on the type specificity of the capsular polysaccharide of the swine pathogen *Haemophilus parasuis* to the journal PLoS One there was a request by reviewers for improved quality of photographs and other data, necessitating a postdoc in my lab and I to redo some experiments. First of all, new capsular polysaccharide was purified from a serotype 5 strain. There are 15 serotypes of *H. parasuis*, which we hypothesized were due to antigenic differences in the capsular polysaccharides of each serotype. Unfortunately, the experiments concerning the antiserum that Anne Hyman had generated were unable to be repeated, and were entirely out of line with the results described.

Anne immunized rabbits with what was supposed to be a purified polysaccharide of *H. parasuis.* She told me the rabbit was responding to the immunizations and making a response. Anne showed me ELISA data indicating that the titer of antiserum to the capsule was >1:10,000.

Anne's claims based on the antiserum she prepared:
1, the capsule is the serotype specific antigen of *H. parasuis*. This means that antibodies to the capsule react predominately, though not necessarily exclusively, with only that one serotype.

Anne's results presented to PLoS One:

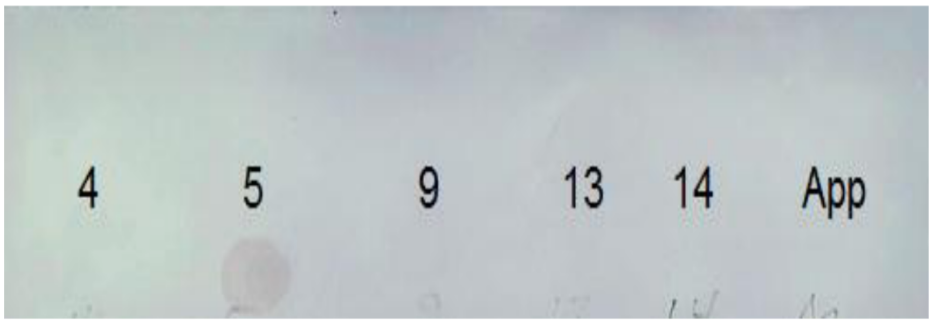


Fig. 3. Reactivity of *H. parasuis* serotypes with antiserum to *H. parasuis* serotype 5 CP. All serotypes were grown in broth, and 2 µl of each sample was dotted onto nitrocellulose and incubated with anti-CP 5 serum. App- *A. pleuropneumoniae.*

This figure shows that antiserum to the capsule of *H. parasuis* serotype 5 reacted with only the capsule of serotype 5, and not to the capsules of serotypes 4, 9, 13, 14 or *A. pleuropneumoniae* (App). Numbers refer to the *H. parasuis* serotype.

Our repeat of this experiment:

Our repeat shows that Anne's serum reacts with the capsule of all serotypes tested, as well as to whole cells of *A. pleuropneumoniae* and a very unrelated organism, *Francisella tularensis.*

2, the capsule is expressed by cells grown in broth, but not on agar plates.
Anne's results presented to PLoS One:

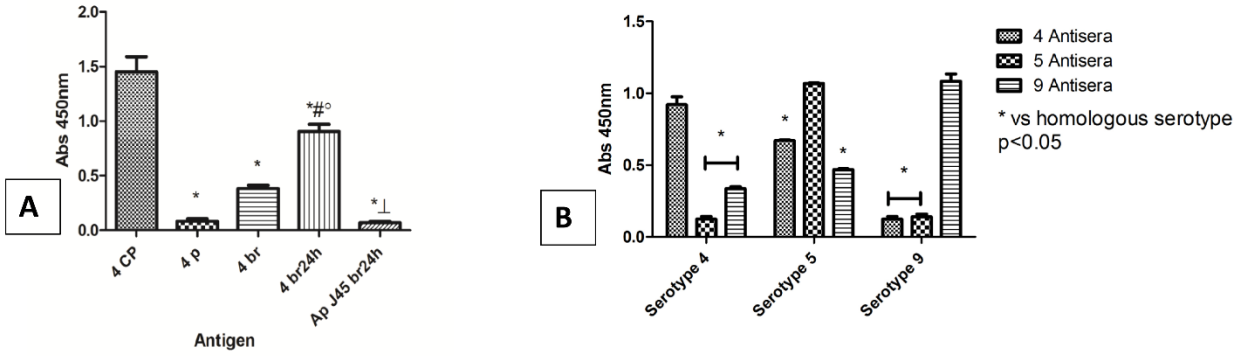
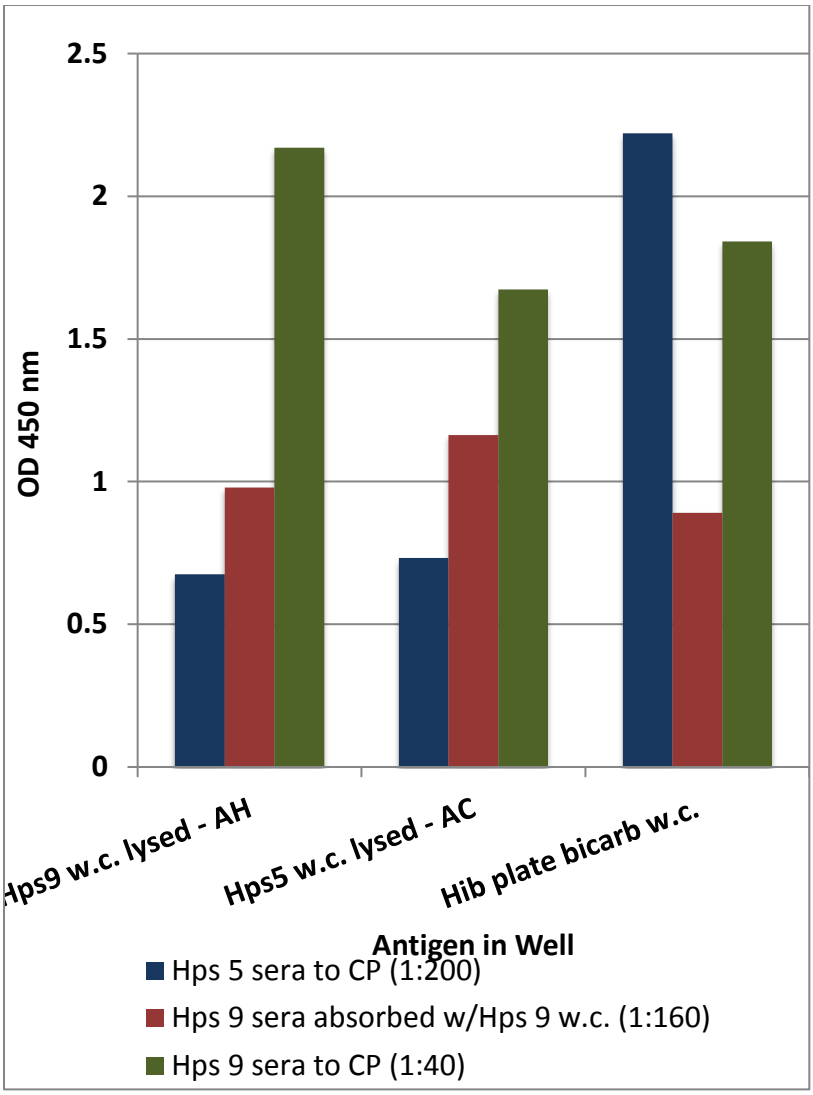


Fig. 4. Homologous and heterologous reactivity of antiserum to *H. parasuis* purified CP or whole cells by ELISA. Purified CP, or bacteria grown on agar (p), in broth to mid-log phase (br), or for 24 h (br24h) were used as antigens with homologous or heterologous antisera. A, reactivity of antiserum to serotype 4 CP with homologous CP, with agar-grown cells (p), with bacteria grown in broth for 12 hours (br) or for 24 h (br24h). *, indicates a significant difference compared to reactivity to serotype 4 CP ($p < 0.01$), #, indicates a significant difference compared to reactivity with serotype 4 cells (p) ($p < 0.01$), °, indicates a significant difference with reactivity to serotype 4 cells (br) ($p < 0.05$), ⊥, indicates a significant difference compared to reactivity with serotype 4 cells (br24h) ($p < 0.01$). B, reactivity of antisera to CPs 4, 5, or 9 with homologous and heterologous CPs. * indicates a significant difference in reactivity to heterologous antisera in comparison to reactivity with homologous antiserum (All significance = $p<0.05$).

This figure shows that antiserum to serotype 4 reacts with purified capsule, and to cells grown in broth, but not to cells grown on agar plates or to *A. pleuropneumoniae* grown in broth (A). B shows that antiserum to the same antigenic serotype reacts significantly greater with the capsule of that serotype than to capsules of other serotypes.

Our results:



These results do support Anne's results in that at least the antiserum to serotype 9 capsule reacts most strongly to serotype 9. However, antiserum to serotype 9 capsule also reacted more straongly with serotype 5 capsule than antiserum to serotype 5, and all the sera reacted strongly with *H. influenzae* type b, an unrelated species.

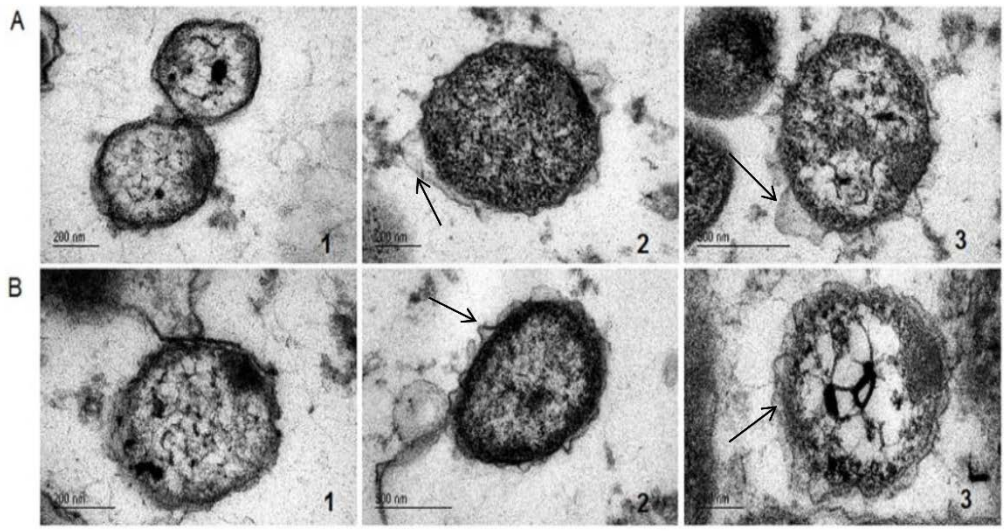
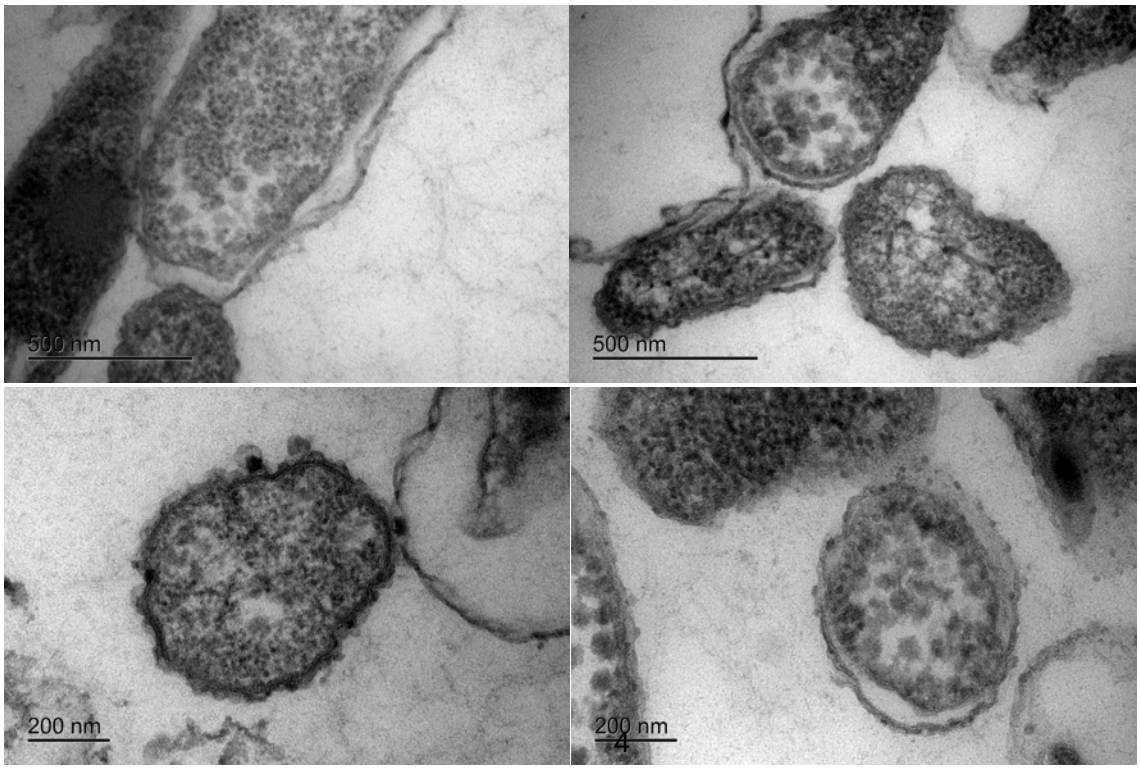
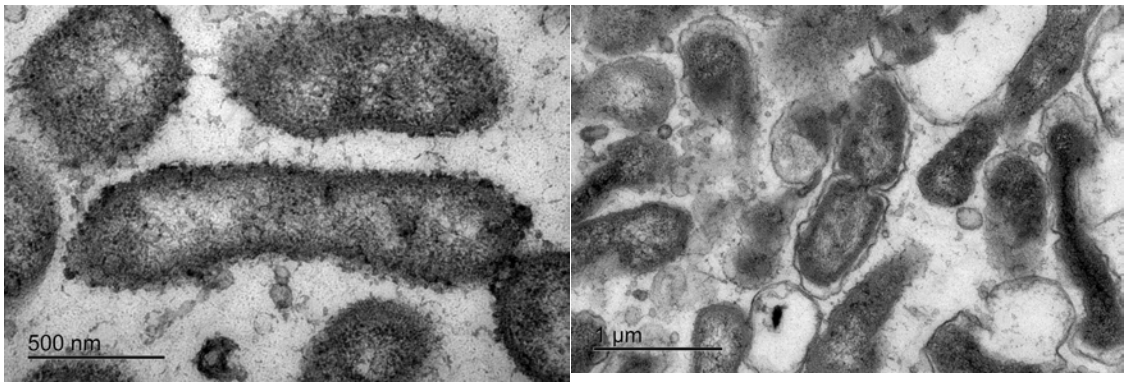


Fig. 6. Immuno-electron microscopy of *H. parasuis* for CP expression. *H. parasuis* serotypes 4 (A) or 5 (B) were grown on agar (1), or in broth to mid-log phase (2), or in broth for 24 h (3). The bacteria were washed and incubated with homologous antibody to CP. The bacteria were stained with Ruthenium red, fixed, dehydrated, stained with uranyl acetate, and examined by transmission electron microscopy. Bacteria grown on agar did not exhibit the ruffled, halo-like CP structure observed around broth-grown cells (arrows).

Another figure showing by electron microscopy that capsule is only expressed on the surface of cells grown in broth. The reviewers questions if the material pointed out is actually capsule.

Our results:





Top row- *H. influenzae* type b grown in broth
Middle row- *H. parasuis* type 5 grown in broth
Bottom row- *H. parasuis* type 5 grown on agar plates.

I see no clear evidence of a capsule on any of the bacteria tested. Because capsules consist of primarily water they are very difficult to see by electron microscopy, which is done in a vacuum.

3, bicarbonate increases capsule production on cells grown in broth and on agar plates

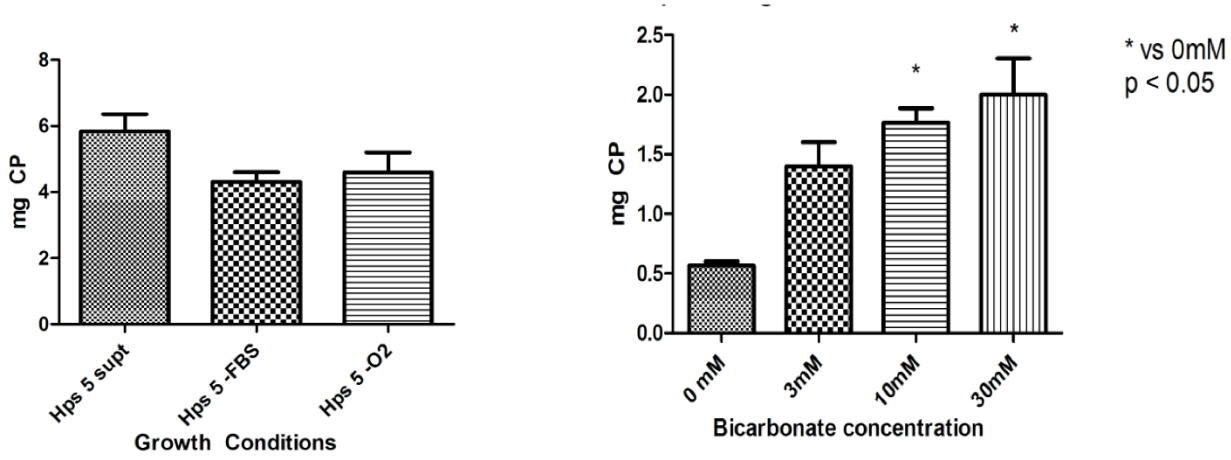
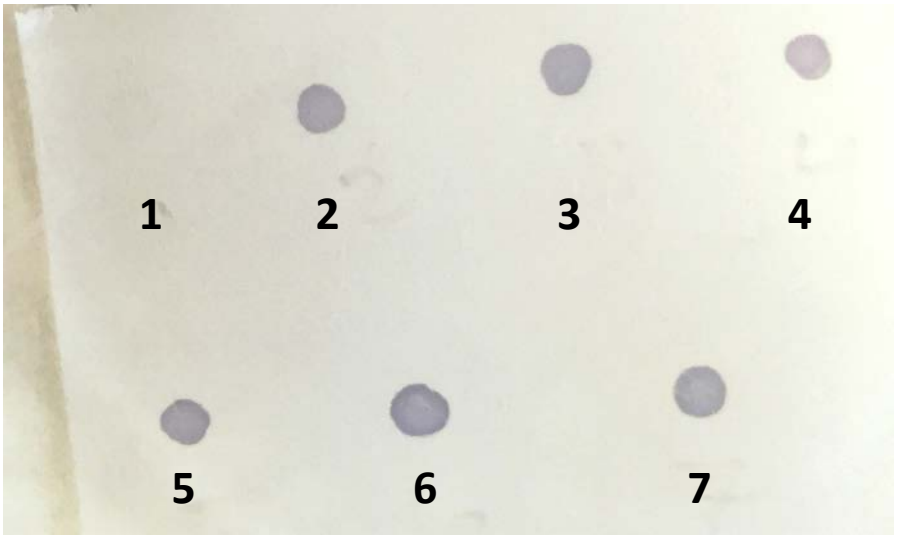


Fig. 9. Growth supplements affecting CP production. (A) *H. parasuis* (Hps) was grown in PPLO$^+$ broth containing 5% fetal bovine serum (FBS), NAD, and 1% glucose (supt); PPLO+ medium lacking FBS (-FBS); PPLO$^+$ medium supplemented with 10% Oxyrase to remove oxygen (-O2); or (B) PPLO$^+$ supplemented with 3-30 mM sodium bicarbonate. * indicates significantly more CP produced ($p < 0.05$ for 10 mM bicarbonate supplementation, $p<0.01$ for 30 mM bicarbonate supplementation) than the 0 mM bicarbonate control.

This figure shows that if the bacteria are grown in medium containing sodium bicarbonate in broth that capsule production is significantly increased.

Our results:



1. type 5 - Pure CP (capsular polysaccharide)
2. type 5 – Pl (cells grown on plates)
3. type 5 – Pl – bicarb (cells grown on plates with bicarbonate)
4. type 5 – Br (cells grown in broth medium)
5. type 5 – Br – bicarb (cells grown in broth medium with bicarbonate)
6. type 5 - Pl – lysed (cells that have been grown on plates and lysed)
7. type 5 – Pl – bicarb – lysed (cells that have been grown on plates with bicarbonate and lysed)

We see no evidence that bicarbonate enhances production or release of the capsule.

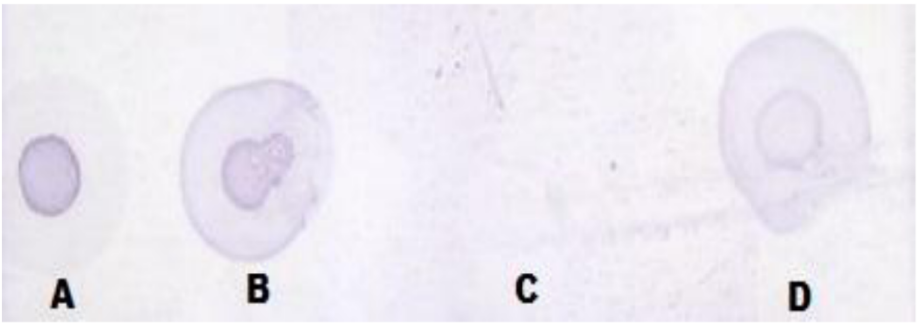


Fig 10. Immuno-blot of *H. parasuis* serotype 5 grown on agar with or without bicarbonate supplementation. A, purified serotype 5 CP; B, broth-grown serotype 5 cells; C, agar-grown serotype 5 cells on PPLO$^+$; D, agar-grown serotype 5 cells on PPLO$^+$ supplemented with 30 mM bicarbonate.

This figure also shows that if the bacteria are grown on agar plates with or without bicarbonate, that capsule can now be detected on cells grown with bicarbonate, but not without bicarbonate. Anne's blots were on nitrocellulose.

Our results:

Nitrocellulose. 1:250 anti-Hps5 CP sera

Hps5 w.c. plate Hps5 w.c. plat w/bicarb Hps5 w.c.–broth Hib w.c. broth Hps5

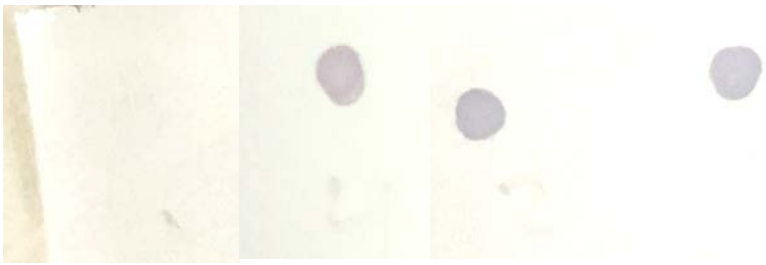
CP +Charged Nylon. 1: 250 anti-Hps5 CP sera

Hps5 w.c. plate Hps5 w.c. plat w/bicarb Hps5 w.c.–broth Hib w.c. broth Hps5 CP

Definitions: Hps5-*H. parasuis* serotype 5
w.c.-whole cells
plate-cells grown on agar plates
broth-cells grown in broth medium
CP-purified capsular polysaccharide
w/bicarb-sodium bicarbonate added to the growth medium

In our repeat experiment, new highly purified type 5 CP did not react with the antiserum on nitrocellulose, but did react on charged nylon paper. Cells of another encapsulated bacterium, *Haemophilus influenzae* type b, also reacted very strongly with the antiserum. *H. parasuis* cells grown on agar without bicarbonate reacted as well with the antiserum as cells grown with bicarbonate.

Also:



A **B** **C** **D**

An exact replica of Anne's blot on nitrocellulose showing that the Hp5 capsule does not appear, but all whole cells tested did, including cells off plates.

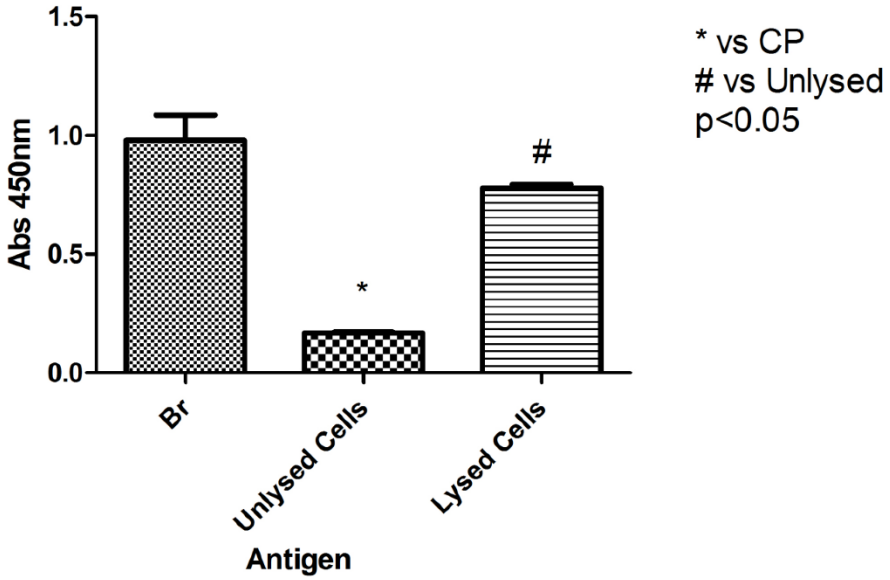
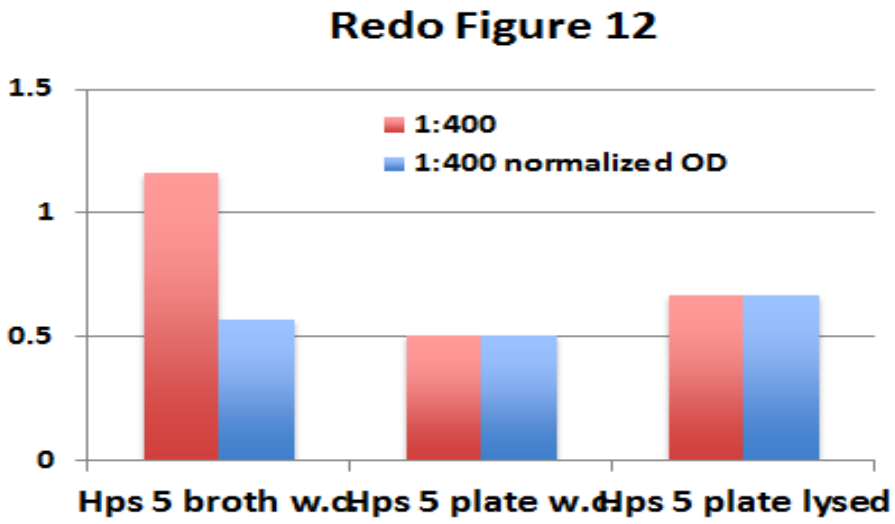


Fig. 12: Production of CP by agar-grown cells. The amount of CP from *H. parasuis* serotype 5 grown in broth (Br) was compared by ELISA to the material extracted from bacteria grown on agar that were lysed or left intact. There was significantly more CP produced by broth-grown bacteria than unlysed agar-grown bacteria (*, $p < 0.05$) and significantly more CP produced by lysed, agar-grown bacteria than unlysed agar-grown bacteria (#, $p < 0.05$). There was no significant difference between CP production by broth-grown bacteria or by lysed agar-grown bacteria ($p > 0.05$).

This figure shows that if the bacteria grown on agar plates are lysed, that capsule can be detected with the antiserum to the capsule by ELISA. This indicates that the capsule is made, but not exported when the bacteria are grown on agar plates, and that it is exported when grown in broth. This result would explain why the bacteria need to be lysed by autoclaving in order to be typed.

Our results:



There was slightly more capsule present from lysed cells, but this is not unusual. The difference in the amount of capsule from lysed cells and unlysed was not significant.

All of the data shown involved the use of the antiserum Anne made to “purified" *H. parasuis* capsule. Prior work by a commercial laboratory indicated that the capsule is not immunogenic in rabbits.

In summary, my conclusion after extensive investigation and many repeated experiments over the past year (many others are not shown here, but were supplied separately), is that Anne’s data could not be replicated as reported in the dissertation and that the amount of sialic acid on the capsule is greater than we thought and makes the capsule non-immunogenic.

In other experiments carried out by colleagues in pigs in Iowa, pigs immunized with the capsule conjugated to an immunogenic protein also did not make antibodies to the capsule.

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