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N. M. Clark

University of Nebraska - Lincoln

E. M. Berberov

University of Nebraska - Lincoln

M. Wang

University of Nebraska Medical Center, Omaha, NE

Rodney A. Moxley

University of Nebraska - Lincoln, rmoxley1@unl.edu

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Anti-capsular antibodies activate killing of *Escherichia coli* O8:K87 by the alternate complement pathway in porcine serum

N. M. Clark ^{1,*}, E. M. Berberov ^{1,†}, M. Wang ^{2,‡}, and R. A. Moxley ^{1,§}

¹ Department of Veterinary and Biomedical Sciences, University of Nebraska–Lincoln, Fair Street and East Campus Loop, Lincoln, NE 68583-0905, USA

² Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, Omaha, NE 68198-6025, USA

* Present address: Department of Pathology and Microbiology, 983135 Nebraska Medical Center, Omaha, NE 68198-3135, USA.

† Present address: Vaccine and Infectious Disease Organization, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Sask. S7N 5E3, Canada.

‡ Present address: Department of Applied Chemistry, College of Sciences West Campus, China Agricultural University, Beijing 100094, PR China.

§ Corresponding author. Tel.: +1 402 472 8460; fax: +1 402 472 9690.

Abstract: Enterotoxigenic *Escherichia coli* (ETEC) strains that produce K88 (F4)⁺ fimbria are important causes of diarrhea and post-diarrheal septicemia in swine. ETEC O8:K87, a serotype represented by a number of these strains, is typically serum resistant. Strain-specific antibodies are known to activate alternative C pathway-mediated killing of other serum-resistant *E. coli* [Hill, A.W., Shears, A.L., Hibbitt, K.G., 1978. The requirement of specific antibody for the killing of *E. coli* by the alternate complement pathway in bovine serum. *Immunology* 34, 131–136], but their antigenic targets have not been determined. We tested the hypothesis that anti-K87 antibodies activate alternative pathway-mediated killing of ETEC O8:K87. Pigs were immunized with ETEC O8:K87 strain 2534-86 cells or purified K87 polysaccharide. Post-, but not pre-immunization sera killed 2534-86 cells, and absorption with 2534-86 cells or by K87 affinity chromatography eliminated bactericidal activity. Complementation of absorbed serum with anti-K87 antibodies restored bactericidal activity, confirming the ability of these antibodies to activate C-mediated serum killing. Serum from age-matched, non-vaccinated control pigs also killed 2534-86. This activity was eliminated by absorption with 2534-86 cells, but not K87 affinity chromatography, indicating that specific non-capsular antibodies are also able to activate C-mediated killing. In all cases, Mg-EGTA-treated serum was as effective as non-treated serum in killing, suggesting that bactericidal activity was mediated predominantly if not exclusively via the alternative C pathway.

Keywords: *Escherichia coli*, Capsule, Polysaccharide, Alternative complement pathway, Enterotoxigenic *E. coli*, Pig, Porcine, Serum resistance, K87, Anti-capsular antibodies

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea and post-diarrheal septicemia in pre- and post-weaned pigs (Berberov et al., 2004). ETEC O8:K87, a serotype that includes some of these strains,

is typically serum resistant, but this phenotype is lost in spontaneous acapsular (K87[−]) mutants (Stawski et al., 1990). Strain-specific antibodies are known to activate alternative C pathway-mediated killing of serum-resistant *E. coli* O149:K91:H10:F4ac by bovine serum, but their antigenic targets have not been determined (Hill

et al., 1978). In the present study, we hypothesized that anti-K87 antibodies activate alternative C pathway-mediated killing of ETEC O8:K87 in porcine serum and removal of these specific antibodies mitigates this activity. We provide evidence herein supporting this hypothesis; we also found that these antibodies are highly prevalent in the swine population, and that specific non-capsular antibodies also activate alternative pathway-mediated killing.

2. Materials and methods

2.1. Bacterial strains

Strain 2534-86 (ETEC; O8:K87:NM:F4ac) was originally isolated from a piglet with diarrhea and septicemia, and causes these clinical manifestations in gnotobiotic piglets (Berberov et al., 2004). This strain was used to immunize animals against *E. coli* cells expressing K87 capsular polysaccharide, and was a source of the latter for purified antigen. *E. coli* O8:K⁻ strain F1321 (Meier and Mayer, 1985) and *E. coli* strain K12:F4ac (Berberov et al., 2004) were used to test the specificity of the indirect ELISA for K87 antibodies. *E. coli* strain DH5 α was used as a positive control to test for C-mediated bactericidal activity of serum.

2.2. Isolation and purification of K87 capsular polysaccharide antigen

Purified K87 capsular polysaccharide (PP) was isolated by phenol-water extraction (Jann, 1985) from strain 2534-86 cultures. Glucose concentration (Saha and Brewer, 1994) was 95.46% of the calculated yield per gram, suggesting that the extract was K87 polysaccharide. No evidence of protein, LPS or nucleic acid contamination was found; purity was confirmed by ¹H nuclear magnetic resonance spectroscopy (¹H NMR) of the extract conducted in D₂O. Chemical shifts and coupling patterns were identical to the K87 spectrum reported by Parolis et al. (1990).

2.3. ¹H NMR studies

¹H NMR spectroscopy was conducted on a Varian Unity 500 MHz spectrometer at 25 °C. A presaturation sequence was utilized to reduce the intensity of the H₂O peak which served as an internal standard at δ 4.773. ¹H NMR (δ , ppm, D₂O) 1.307 (Mc), 1.972, 1990, 2.083 (CH₃CO), 3.20–3.30, 3.42–3.58, 3.60–4.05, 4.10–4.20, 4.35–4.58, 4.62–4.75, 4.80–4.90, 5.15–5.21, 5.43–5.52.

2.4. Preparation of formalin-treated strain 2534-86 cells for immunization

Formalinized *E. coli* strain 2534-86 cells for immunization were prepared using the methods of Gross and Rowe (1985). Bacteria were washed twice by centrifugation and resuspension in 0.9% saline, and resuspended in 0.3% (v/v) solution of commercial 10% neutral-buffered formalin in 0.9% saline at a concentration equivalent to 1.0×10^9 CFU/ml.

2.5. Experimental animals

BALB/c mice (6–8-week-old adult females; $n = 16$), specific pathogen-free (SPF) New Zealand White rabbits (5–6 month-old adult females; $n = 2$), and cesarean-derived, colostrum-deprived, primary SPF purebred Yorkshire pigs ($n = 10$) were immunized against PP or 2534-86 cells. When pigs were 1 month old, seven were used for immunization, and three were non-vaccinated and kept in the same room as controls. Serum samples were obtained prior to the first immunization and at the conclusion of immunizations, at which time they were euthanatized and exsanguinated. Serum samples from 75 conventional pigs from 11 herds in five different counties in Nebraska were obtained to test for antibodies to K87; 40 pigs were 8 weeks old and 35 were 5–6 months old. Serum samples were stored at -70 °C until used in assays. Animal experiments were approved by the UNL Institutional Animal Care and Use Committee.

2.6. Immunization protocols

Immunization protocols for PP and 2534-86 cells were developed by modification of standard procedures. Mice ($n = 8$) were primed i.p. with 100 μ g PP and 100 μ l complete Freund's adjuvant (CFA), and boosted twice i.p. with 50 μ g PP and 50 μ l incomplete Freund's adjuvant (IFA), or ($n = 8$) primed i.p. with 1.0×10^8 CFU and 100 μ l CFA, and boosted twice i.p. with 1.0×10^8 CFU and 50 μ l IFA. Rabbits ($n = 1$) were primed s.c. with 150 μ g PP and 150 μ l CFA, and boosted twice s.c. with 75 μ g PP and 75 μ l IFA, or ($n = 1$) primed i.v. with 0.5×10^9 CFU, and boosted four times i.v. with 1.0 – 2.0×10^9 CFU. Pigs ($n = 4$) were primed s.c. with 200 μ g PP and 200 μ l CFA, and boosted twice s.c. with 100 μ g PP and 100 μ l IFA, or ($n = 3$) primed i.v. with 0.5×10^9 CFU, and boosted twice i.v. with 0.5×10^9 CFU. Cells used for the first dose given to the pigs were inadvertently boiled, although the remaining doses were not.

2.7. Indirect enzyme-linked immunosorbent assay (ELISA) to detect anti-K87 antibodies

An indirect ELISA was developed using standard procedures to measure the titers of anti-K87 antibodies in sera. Polystyrene 96-well plates were coated with antigen, each well with 50 μ l of a PBS solution containing 10 ng PP. Sera were diluted from 1:100 to 1:204,800 in wells of the plates. Goat anti-species IgG labeled with horseradish peroxidase was used as the secondary anti-

body, with peroxide and *o*-phenylenediamine for color development. Absorption was measured at 490 nm. The antibody titer was the highest dilution having an absorbance (mean $\pm$ 1 S.E.M.) greater than the negative control (mean $\pm$ 1 S.E.M.).

2.8. Absorption of specific antibodies from serum

Antibodies to K87 and other surface antigens on *E. coli* strains were absorbed from sera with bacteria us-

Figure 1. (A) Indirect ELISA for detection of anti-K87 antibodies. Serum from rabbit immunized with formalin-treated *E. coli* O8:K87 strain 2534-86 cells. Purified K87 polysaccharide (PP) was coated onto polyvinyl chloride microtiter plate wells, and serum dilutions from immunized animals were added. Binding of antibodies to the immobilized polysaccharide was detected by goat anti-species IgG labeled with horseradish peroxidase, and *o*-phenylenediamine substrate, and reactions were read at 490 nm. The specificity of the assay for anti-K87 antibodies was validated by absorption with cells expressing F4ac fimbria, O8 antigen and other surface antigens commonly found on *E. coli* O8 bacteria. (B) Serum from primary SPF pigs immunized with formalinized 2534-86 cells and absorbed with 2534-86 or *E. coli* O8:K⁻ strain F1321 cells. (C) Serum from primary SPF pigs immunized with PP and absorbed with 2534-86 or F1321 cells.

ing standard procedures (Gross and Rowe, 1985), or by affinity chromatography using epoxy-activated Sepharose 6B beads (Amersham Pharmacia Biotech) coupled to PP. PP was added at a concentration of 400 µg/ml and using a gel:buffer ratio of 1:2. Absorption control sera were prepared with a column containing Sepharose 6B beads not coupled to PP. Sera were passed through the column and absorbed antibodies were eluted with 0.1 M sodium citrate (pH 3.0). Two hundred microliters eluate fractions were collected and the pH adjusted to 7.5 with 1 M Tris-HCL (pH 9.0). Fractions containing protein (bicinchoninic assay) were added to absorbed sera. All sera to which eluate was not added were diluted with an equal volume of buffer, and adjusted to pH 7.3 in order to standardize test and control samples.

2.9. Serum bactericidal assay

Serum bactericidal assays were performed with pre- and post-immunization sera of primary SPF pigs immunized with PP or strain 2534-86 cells, and control pigs. Assays were performed with serum that was untreated, treated with $MgCl_2$ and ethylene glycol tetraacetic acid (Mg-EGTA), and heat-inactivated (56 °C for 30 min; Burns and Hull, 1998). Buffered saline gelatin (BSG) with

Mg^{2+} (10 or 20 mM $MgCl_2$) and EGTA (10 or 20 mM) was added to serum.

3. Results and discussion

The indirect ELISA to detect anti-K87 antibodies was validated by absorption of serum from a rabbit immunized with 2534-86 cells using *E. coli* cells exposing O8, F4ac fimbria, and other surface antigens (Figure 1A). No reduction in titer occurred following absorption of post-immunization serum with formalinized strain F1321 (O8:K⁻) cells exposing O8 and other *E. coli* surface antigens or boiled cells exposing O8 antigen, or following absorption with formalinized K12:F4ac cells exposing F4ac fimbriae. However, absorption with formalinized 2534-86 cells exposing K87 antigen eliminated the titers. Immunization of mice with 2534-86 cells or PP caused seroconversion (data not shown). All conventional pigs tested were positive for antibodies to K87, with titers ranging from 1:6400 to 1:204,800. Immunization of colostrum-deprived, primary SPF pigs with 2534-86 cells and PP caused seroconversion to K87 antigen (Figure 1B and C, respectively), whereas non-vaccinated age-matched (68–72-day-old) control pigs were seronegative (data not shown). Absorption of post-im-

Figure 2. Indirect ELISA for detection of anti-K87 antibodies. (A) Serum from primary SPF pig immunized with formalin-treated strain 2534-86 cells. Serum was subjected to 2–3 cycles of affinity chromatography with Sepharose 6B beads coupled to K87 polysaccharide (PP), or Sepharose beads not coupled to PP. (B) Serum from primary SPF pig immunized with PP. Serum was subjected to 2–3 cycles of affinity chromatography with beads coupled or not to PP.

munization pig sera with formalinized 2534-86 cells but not F1321 (O8:K⁻) cells eliminated the anti-K87 titers (data not shown). K87 affinity chromatography caused complete removal of detectable anti-K87 antibodies in sera from primary SPF pigs immunized with 2534-86 cells (Figure 2A) and PP (Figure 2B), whereas absorption control sera retained these antibodies.

To address whether anti-K87 antibodies specifically activate C-mediated bactericidal activity, sera from primary SPF pigs immunized with formalinized 2534-86 cells or PP were tested in bactericidal assays against 2534-86 cells. Pre-immunization sera had no bactericidal activity after 3 h incubation, whereas sera from pigs immunized with formalinized 2534-86 cells or PP elicited

Figure 3. Bactericidal assay with antiserum from primary SPF pigs immunized with formalinized *E. coli* O8:K87 strain 2534-86 cells ($n = 3$) or purified K87 polysaccharide (K87, $n = 4$). (A) Untreated serum; (B) Mg-EGTA-treated serum; (C) heat-inactivated serum. Pre-immunization: mean values of all pigs combined prior to immunization, anti-2534: serum from pigs immunized with 2534-86 cells, anti-K87: serum from pigs immunized with K87, abs-2534: serum from pigs immunized with 2534-86 cells and absorbed with 2534-86 cells, abs-K87: serum from pigs immunized with K87 and absorbed with 2534-86 cells, affin-2534: serum from pigs immunized with 2534-86 cells and absorbed by affinity chromatography with beads coupled to K87, affin-K87: serum from pigs immunized with K87 and absorbed by affinity chromatography with beads coupled to K87, affin-2534AB: serum from pigs immunized with 2534-86 cells, absorbed by affinity chromatography with beads coupled to K87, and eluted anti-K87 antibodies from column added back to serum and affin-K87AB: serum from pigs immunized with K87, absorbed by affinity chromatography with beads coupled to K87, and eluted anti-K87 antibodies from column added back to serum.

>99% killing after 2 h incubation (Figure 3). The bactericidal activity of Mg-EGTA-treated sera in both groups was equivalent to that of untreated serum. These results suggested that killing activity occurred predominantly, if not exclusively by the alternative C pathway since EGTA chelates Ca^{2+} , inactivating the classical C pathway, but has a lower affinity for Mg^{2+} ; the addition of Mg^{2+} allows the alternative pathway to proceed (Burns and Hull, 1998). Absorption of sera with 2534-86 cells completely removed bactericidal activity (Figure 3), whereas absorption of the same sera with formalinized strain F1321 (*E. coli* O8:K⁻) cells had no apparent effect (data not shown). Hence, specific antibodies are needed for bactericidal activity against *E. coli* O8:K87. Sera from primary SPF pigs immunized with purified PP retained bactericidal activity against *E. coli* DH5 α following absorption with formalinized 2534-86 cells (data not shown), which confirmed that the absorption process had not eliminated the C-mediated bactericidal activity against serum-sensitive bacteria. Absorption by K87 affinity chromatography eliminated bactericidal activity, whereas absorption control sera from these same pigs had retained activity (Figure 3). Untreated and Mg-EGTA-treated absorbed sera from pigs immunized with either PP or 2534-86 cells complemented with eluted anti-K87 antibodies had restoration of killing activity (Figure 3). The anti-K87 titers of pre-absorbed serum and complemented serum were comparable (data not shown). Restoration of bactericidal activity in complemented serum confirmed the ability of anti-K87 antibodies to activate C-mediated killing.

To determine whether antibodies other than anti-K87 could activate killing of 2534-86, bactericidal assays were conducted with serum from primary SPF age-matched (68–72-day-old) control pigs lacking anti-K87 antibodies. This serum effected >99% killing by 3 h incubation, and bactericidal activity of Mg-EGTA-treated serum was equivalent to that of untreated serum. The killing activity of age-matched control pig serum was eliminated by absorption with 2534-86 cells; however, it was not eliminated by K87 affinity chromatography (data not shown). These results indicated that specific antibodies other than anti-K87 can activate killing, although the antigen against which these non-capsular antibodies were directed was not addressed.

Since Mg^{2+} and Ca^{2+} are needed for the alternative and classical C pathways, respectively, to proceed, their concentrations in absorbed and non-absorbed sera from two primary SPF pigs tested in the bactericidal

assays were determined. The Mg^{2+} and Ca^{2+} concentrations of non-absorbed sera of both pigs were within the normal range for porcine serum; however, concentrations of both cations in one of these sera after affinity chromatography were below the normal range. Addition of MgCl_2 restored its bactericidal activity when complemented with eluted antibodies. By contrast, the Mg^{2+} and Ca^{2+} concentrations of the serum of the second pig, absorbed at a higher flow rate (4 ml/min), were within the normal range, indicating that the loss of bactericidal activity in affinity chromatography-absorbed serum was not due to an alteration in the concentration of these cations.

The ability of anti-capsular antibodies to activate alternative pathway killing of *E. coli* O8:K87 extends the observations reported by Hill et al. (1978). In that study, strain-specific antibodies activated alternative pathway killing of *E. coli*; however, bovine serum and a different serotype of *E. coli* were used, and the antigenic target was not identified. Interestingly, strains of the serotype used in that study (O149:K91) are typically enterotoxigenic, express F4 fimbria and are only known to cause natural disease in swine (Morris and Sojka, 1985). Similarly, anti-capsular antibodies, specifically of the IgG class, kill *Haemophilus influenzae* type B (Hib), in a process that occurs exclusively by the alternative pathway (Tarr et al., 1982); however, non-capsular antibodies are capable of C activation and killing of Hib, but this occurs exclusively by the classical pathway (Steele et al., 1984). Collectively, these studies suggest that alternative C pathway activation by anti-capsular antibodies may represent a common defense mechanism in mammalian species against encapsulated, serum-resistant Gram-negative bacteria. In the case of swine, anti-K87 antibodies appear to be highly prevalent, and we deduce that they may play an important role in host defense against ETEC-mediated post-diarrheal septicemia.

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References

- Berberov et al., 2004:** E.M. Berberov, Y. Zhou, D.H. Francis, M.A. Scott, S.D. Kachman and R.A. Moxley, Relative importance of heat-labile enterotoxin in the causation of severe diarrheal disease in the gnotobiotic piglet model by a strain of enterotoxigenic *Escherichia coli* that produces multiple enterotoxins, *Infect. Immun.* **72** (2004), pp. 3914–3924.
- Burns and Hull, 1998:** S.M. Burns and S.I. Hull, Comparison of loss of serum resistance by defined lipopolysaccharide mutants and an acapsular mutant of uropathogenic *Escherichia coli* O75:K5, *Infect. Immun.* **66** (1998), pp. 4244–4253.
- Gross and Rowe, 1985:** R.J. Gross and B. Rowe, Serotyping of *Escherichia coli*. The virulence of *Escherichia coli*: review and methods. In: M. Sussman, Editor, *Society for General Microbiology*, Academic Press, Orlando, FL (1985), pp. 345–363.
- Hill et al., 1978:** A.W. Hill, A.L. Shears and K.G. Hibbitt, The requirement of specific antibody for the killing of *E. coli* by the alternate complement pathway in bovine serum, *Immunology* **34** (1978), pp. 131–136.
- Jann, 1985:** K. Jann, Isolation and characterization of capsular polysaccharides (K antigens) from *Escherichia coli*. The virulence of *Escherichia coli*: review and methods. In: M. Sussman, Editor, *Society for General Microbiology*, Academic Press, Orlando, FL (1985), pp. 375–379.
- Meier and Mayer, 1985:** U. Meier and H. Mayer, Genetic location of genes encoding enterobacterial common antigen, *J. Bacteriol.* **163** (1985), pp. 756–762.
- Morris and Sojka, 1985:** J.A. Morris and W.J. Sojka, *Escherichia coli* as a pathogen in animals. The virulence of *Escherichia coli*: review and methods. In: M. Sussman, Editor, *Society for General Microbiology*, Academic Press, Orlando FL (1985), pp. 47–77.
- Parolis et al., 1990:** H. Parolis, L.A.S. Parolis and S.M.R. Stanley, The structure of the capsular antigen from *Escherichia coli* O8:K87:H19, *Carbohydr. Res.* **205** (1990), pp. 361–370.
- Saha and Brewer, 1994:** S.K. Saha and C.F. Brewer, Determination of the concentration of oligosaccharides, complex type carbohydrates, and glycoproteins using the phenol–sulfuric acid method, *Carbohydr. Res.* **254** (1994), pp. 157–167.
- Stawski et al., 1990:** G. Stawski, L. Nielsen, F. Orskov and I. Orskov, Serum sensitivity of a diversity of *Escherichia coli* antigenic reference strains, *Acta Pathol. Microbiol. Immunol. Scand.* **98** (1990), pp. 828–838.
- Steele et al., 1984:** N.P. Steele, R.S. Munson Jr., D.M. Granoff, J.E. Cummins and R.P. Levine, Antibody-dependent alternative pathway killing of *Haemophilus influenzae* type b, *Infect. Immun.* **44** (1984), pp. 452–458.
- Tarr et al., 1982:** P.I. Tarr, S.W. Hosea, E.J. Brown, R. Schneerson, A. Sutton and M.M. Frank, The requirement of specific anticapsular IgG for killing of *Haemophilus influenzae* by the alternative pathway of complement activation, *J. Immunol.* **128** (1982), pp. 1772–1775.