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VACCINES

Development and Qualification of an Opsonophagocytic Killing Assay To Assess Immunogenicity of a Bioconjugated *Escherichia coli* Vaccine

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ABSTRACT The global burden of disease caused by extraintestinal pathogenic *Escherichia coli* (ExPEC) is increasing as the prevalence of multidrug-resistant strains rises. A multivalent ExPEC O-antigen bioconjugate vaccine could have a substantial impact in preventing bacteremia and urinary tract infections. Development of an ExPEC vaccine requires a readout to assess the functionality of antibodies. We developed an opsonophagocytic killing assay (OPA) for four ExPEC serotypes (serotypes O1A, O2, O6A, and O25B) based on methods established for pneumococcal conjugate vaccines. The performance of the assay was assessed with human serum by computing the precision, linearity, trueness, total error, working range, and specificity. Serotypes O1A and O6A met the acceptance criteria for precision (coefficient of variation for repeatability and intermediate precision, ≤50%), linearity (90% confidence interval of the slope of each strain, 0.80, 1.25), trueness (relative bias range, −30% to 30%), and total error (total error range, −65% to 183%) at five serum concentrations and serotypes O2 and O25B met the acceptance criteria at four concentrations (the lowest concentration for serotypes O2 and O25B did not meet the system suitability test of maximum killing of ≥85% of *E. coli* cells). All serotypes met the acceptance criteria for specificity (opsonization index value reductions of ≤20% for heterologous serum preadsorption and ≥70% for homologous serum preadsorption). The assay working range was defined on the basis of the lowest and highest concentrations at which the assay jointly fulfilled the target acceptance criteria for linearity, precision, and accuracy. An OPA suitable for multiple *E. coli* serotypes has been developed, qualified, and used to assess the immunogenicity of a 4-valent *E. coli* bioconjugate vaccine (ExPEC4V) administered to humans.

KEYWORDS assay qualification, bacteremia, conjugate vaccine, *Escherichia coli*, ExPEC, invasive disease, opsonophagocytic killing assay

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Bacteremia and septicemia, forms of invasive disease resulting from localized infections, are caused by various bacteria and bacterial toxins in the bloodstream (1) and are a significant cause of clinical morbidity and mortality. Septicemia is the 6th most frequent cause of hospitalization (1) and is the 10th leading cause of death in the United States (2). Associated economic costs are significant, and septicemia is the most expensive condition treated in U.S. hospitals, with 2009 aggregate expenditures approaching $15.4 billion, or approximately 4.3% of all hospital costs (1, 3). Extraintestinal pathogenic *Escherichia coli* (ExPEC) is the organism most frequently associated with sepsis in patients with a principal septicemia diagnosis (1), with *E. coli* septicemia case fatality rates ranging from 5% to 30% (4). Furthermore, ExPEC strains are the most

frequent cause of urinary tract infections (UTIs) and are implicated in other localized infections, including neonatal meningitis, intra-abdominal infections, surgical site infections, osteomyelitis, soft tissue infections, pneumonia, and infections of intravascular devices (5). Increasing rates of drug resistance among ExPEC strains have led to rising rates of hospitalization, increased numbers of treatment failures, and increased treatment costs (1, 3). The rapid expansion of the multidrug-resistant O25 sequence type 131 (ST131) clone has been a significant factor in the worldwide increase in the incidence of antimicrobial-resistant ExPEC strains (6). The prevention of infection with an effective vaccine targeting the most commonly circulating ExPEC serotypes, including highly resistant strains, such as ST131 strains, could have a substantial impact in reducing the incidence of ExPEC disease, including drug-resistant infections (7).

Although there are more than 180 O serotypes of *E. coli*, most cases of human ExPEC disease are caused by between 10 and 12 O serotypes (8). Bioconjugation is a novel technology whereby polysaccharide and carrier protein are biosynthesized within *E. coli* cells, with *in vivo* coupling being achieved using an oligosaccharyltransferase (9). Bioconjugation can result in the production of multiple specific O polysaccharides with a homogeneous structure conjugated to any protein carrier and removes the requirement for chemical detoxification. A phase 1 study of a 4-valent *E. coli* bioconjugate vaccine (ExPEC4V) conducted in women with recurrent UTI was recently completed (10). ExPEC4V is formulated with O antigens from *E. coli* serotypes O1A, O2, O6A, and O25B coupled to a detoxified variant of the protein exotoxin A from *Pseudomonas aeruginosa* (EPA).

Development of a new vaccine requires measurement of its immunogenicity, ideally against a known correlate of protection. While the measurement of antibody levels in serum using enzyme-linked immunosorbent assay (ELISA) methods can demonstrate the immunogenic potential of a vaccine, antibody levels do not always directly correlate with protection (11). This is the case for pneumococcal conjugate vaccines (PCVs), where measurement of functional antibody responses in opsonophagocytic killing assays (OPA) may correlate with clinical protection more closely than ELISA antibody levels (12–14). OPAs measure the ability of antibodies to mediate phagocytic killing, usually in the presence of complement. Immunity to *E. coli* is via complement-mediated lysis and opsonophagocytic killing (15, 16). Cross et al. used an OPA with human neutrophils to assess the functional activity of antibodies raised by vaccination with a polyvalent O-antigen conjugate *E. coli* vaccine (17). Because the development of a multivalent ExPEC conjugate vaccine shares some of the same challenges addressed during the development of PCVs, we built on the large experience with OPAs for assessment of PCVs to establish an assay to measure functional antibody responses to an ExPEC bioconjugate vaccine (18). Here we describe the development and qualification of four singleplex OPAs for *E. coli* serotypes O1A, O2, O6A, and O25B using HL60 cells as effector cells. The OPAs described in this report are based on those described in previous studies (14, 19) with some additional modifications, such as the use of human complement rather than baby rabbit complement (BRC) and the addition of a complement preadsorption step to decrease the high levels of nonspecific killing in human serum samples.

RESULTS

OPA optimization. In order to adapt the previously described pneumococcal OPA protocols for use with *E. coli* strains (14, 19), the following modification were introduced into the assay: (i) an increased ratio of HL60 cells to *E. coli* cells of at least 600:1 was found to be optimal for assay performance (data not shown), (ii) human complement instead of baby rabbit complement was used, (iii) the percentage of human complement used was changed and a preadsorption step was added, (iv) fetal bovine serum was removed from the opsonization buffer (Hanks' balanced salt solution [HBSS] with calcium, magnesium, and 0.1% gelatin comprised the assay buffer), and (v) the composition of agar used for bacterial enumeration was changed.

TABLE 1 Comparison of baby rabbit complement to human complement using *E. coli* OC24452 (O1A)[h]

Serum sample	OI[a] with:			
	2% BRC[b]	5% BRC	10% BRC	2% HC[c]
HS[d] (Sigma)	275	440	807	49
HS37[e]	261	221	273	40
HS38[e]	30	124	116	19
HS39[e]	34	143	270	33
HS40[e]	0	29	39	0
Rat pre-278[f]	0	0	0	0
Rat post-278[g]	0	0	0	96
Rat pre-378[f]	0	0	0	0
Rat post-378[g]	0	0	0	90

[a]OI, opsonization index, which is defined as the serum dilution that kills 50% of bacteria.
[b]BRC, baby rabbit complement.
[c]HC, human complement.
[d]HS, human serum.
[e]HS37, HS38, HS39, and HS40, human serum from 4 different healthy unvaccinated subjects.
[f]Serum from rats 278 and 378 prior to vaccination with ExPEC4V vaccine.
[g]Serum from rats 278 and 378 after vaccination with ExPEC4V vaccine.
[h]The nonspecific killing obtained with 2%, 5%, or 10% BRC or 2% HC was 9%, 23%, 57%, or 25%, respectively. The nonspecific killing obtained with 16.5% BRC was 98%, so OI titers could not be determined.

Initially, the *E. coli* OPA was performed using BRC. However, BRC was unable to discriminate a postvaccination response from the prevaccination condition in rat serum (Table 1). The use of human complement produced lower opsonization index (OI) titers with serum from healthy nonvaccinated subjects than the use of BRC. Only human complement yielded an increased OI titer for postvaccination serum compared with that for prevaccination serum from rats.

Additional improvement of the OPA involved adjustment of the human complement concentration and the preadsorption of complement with bacteria. A pool of human complement from three donors at 4% or 7% yielded high levels of nonspecific killing (NSK) ($\geq$85%) for all four OPA strains (Table 2). Preadsorption with glutaraldehyde-treated *E. coli* at a ratio of 2×10^9 CFU of each strain (strains OC24452, OC24453, OC24454, and OC24176) per 1 ml of complement reduced NSK levels to $<$30% for a complement concentration of 4%. NSK levels where higher for strains OC24454 (76%) and OC24176 (35%) for a complement concentration of 7%. Increasing the amount of the OC24454 and OC24176 strains used in the preadsorption step to 7×10^9 CFU and 4×10^9 CFU, respectively, decreased the rate of NSK for these two strains to $\leq$42%.

In the pneumococcal OPA (20), enumeration of the bacterial colonies is done by plating the samples onto Todd-Hewitt yeast extract agar followed by placement of an overlay of Todd-Hewitt yeast extract agar containing 2,3,5,-triphenyltetrazolium chloride (TTC) and incubation overnight at 37°C. These procedures resulted in the growth of *E. coli* colonies on top of as well as within the overlay, making counting of the colonies difficult. The switch to glycerol yeast extract agar (GYA) medium without an agar overlay and with a decreased incubation temperature (33°C) led to the formation of discrete colonies that could be accurately enumerated (data not shown).

To evaluate whether *E. coli* killing was mediated by an opsonophagocytic process or by the direct lytic activity of complement, OPA was performed with the removal of each of the key assay components. The results showed that in the absence of phagocytes (HL60 cells), bacterial killing was abrogated (Fig. 1). Similarly, if the complement source and serum antibodies were absent, bacterial killing was also reduced. Importantly, no direct complement lytic activity was observed when *E. coli* was incubated only with complement (Fig. 1). A statistically significant increase in the area under the curve (AUC) under all four conditions (in which key components of the assay were omitted) compared to that under the reference condition (with serum, bacteria, complement, and HL60 cells) was observed ($P < 0.0001$ under all four

TABLE 2 Effect of human complement concentration and preadsorption on nonspecific killing and OI

Complement	Complement concn (%)	Strain (serotype)	Result for human serum sample 58		
			NSK[a] (%)	OI[b]	Maximum killing (%)
Pooled human, nonadsorbed	4	OC24452 (O1A)	91	—[c]	—
	4	OC24453 (O2)	90	—	—
	4	OC24454 (O6A)	85	—	—
	4	OC24176 (O25B)	90		
	7	OC24452 (O1A)	98	—	—
	7	OC24453 (O2)	96	—	—
	7	OC24454 (O6A)	95	—	—
	7	OC24176 (O25B)	99	—	—
Pooled human, preadsorbed, low[d]	4	OC24452 (O1A)	9	189	99
	4	OC24453 (O2)	10	205	94
	4	OC24454 (O6A)	28	602	96
	4	OC24176 (O25B)	18	460	100
	7	OC24452 (O1A)	3	549	100
	7	OC24453 (O2)	8	462	99
	7	OC24454 (O6A)	76	3,053	95
	7	OC24176 (O25B)	35	605	100
Pooled human, preadsorbed, high[e]	7	OC24452 (O1A)	12	193	99
	7	OC24453 (O2)	16	285	99
	7	OC24454 (O6A)	42	764	100
	7	OC24176 (O25B)	14	647	100

[a]NSK, nonspecific killing.
[b]OI, opsonization index.
[c]—, not determined because NSK was ≥85%.
[d]Complement preadsorbed with glutaraldehyde-treated *E. coli* at a ratio of 2×10^9 CFU of each strain (OC24452, OC24453, OC24454, OC24176) per 1 ml complement.
[e]Complement preadsorbed with glutaraldehyde-treated *E. coli* at a ratio of 2×10^9 CFU of strain OC24452 or OC24453, 7×10^9 CFU of strain OC24454, and 4×10^9 CFU of strain OC24176 per 1 ml complement.

conditions). To further investigate the role of phagocytes in mediating bacterial killing, OPA was performed in the presence of the actin polymerization inhibitor cytochalasin B. The results show that 10 μM cytochalasin B in the presence of HL60 cells could significantly inhibit the killing of *E. coli* serotypes O1A, O2, and O25B (Fig. 2). The overall decrease in percent killing was estimated to be −71.6% across serotypes ($P = 0.0055$; 95% confidence interval [CI], −100%, −40%). Notably, the process of phagocytic killing of *E. coli* serotype O6A was completely abrogated by 10 μM cytochalasin B (Fig. 2).

OPA qualification. The OPA qualification was performed using an HL60 cell-to-bacterial cell ratio of at least 600:1 and a final human complement concentration of 7%.

The performance of the assay was assessed for precision, linearity, trueness, accuracy, assay range, and specificity with pooled serum samples from subjects that had been vaccinated with ExPEC4V (21, 22).

The ranges for linearity (90% CI of the slope, 0.80 to 1.25), precision (coefficient of variation [CV] for repeatability and intermediate precision, ≤50%), accuracy (total error, −65% to 183%), and trueness (relative bias, −30% to 30%) were defined as the target acceptance criteria prior to the qualification and were based on previous experience with cell-based assays. The assay working range was defined as the lowest and highest concentration at which the assay jointly fulfilled the target acceptance criteria for linearity, precision, trueness, and accuracy. The qualification criteria were evaluated with different concentrations using data that passed the system suitability test of maximum killing of ≥85%. Of five serum concentrations assessed, the lowest concentration for serotypes O25B and O2 did not meet the system suitability criteria. Therefore, five concentrations for serotypes O1A and O6A and four concentrations for serotypes O25B and O2 were evaluated. Repeatability (intra-assay) variances and between-run (interassay) variances were estimated by *E. coli* serotype and by concen-

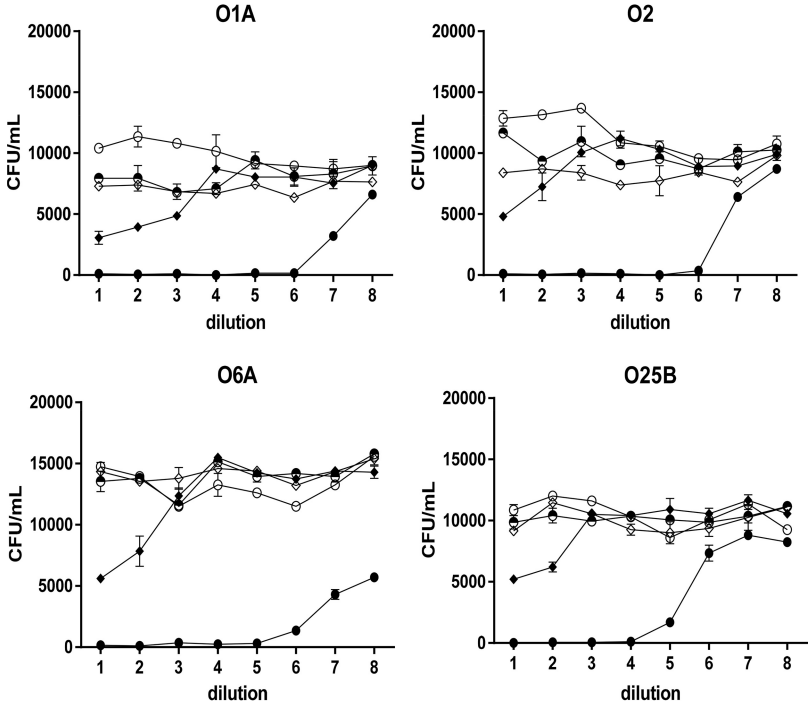

FIG 1 Evaluation of the contribution of key components of OPA. OPA was performed in the presence of all components (standard condition), including HL60 cells (phagocytes), *E. coli* bacterial cells, complement, and human serum antibodies (filled circles) or with the removal of phagocytes (open circles), complement (filled diamonds), serum antibodies and complement (open diamond), or serum antibodies and phagocytes (half-filled circles). The results are expressed as the mean number of CFU per milliliter ± SEM determined for duplicates of a 3-fold dilution curve. The assay was performed with human complement (7%) from a single donor. The area under the curve (AUC) for each condition and serotype was calculated, and pairwise comparisons were performed between conditions, omitting key assay components and the reference condition (serum, bacteria, complement, HL60 cells) while correcting for serotype. Dunnett's method was applied to correct for multiple comparisons. *P* was <0.0001 for all four conditions tested.

tration using a linear mixed model fitted on the $\log_{10}$ titers, including assay run (a combination of runs, days, and operators) as a single random factor (series). To obtain the variance components by *E. coli* serotype across concentrations, a second model was fitted by including series as the random factor and concentration as the fixed factor. Repeatability variances and between-run variances were determined for each model. Intermediate precision variance is the sum of the repeatability variance and between-run variance. Repeatability standard deviations and coefficients of variation (CVs) and intermediate precision standard deviations (SDs) and CVs were estimated. A CV for the repeatability and intermediate precision of the assay per concentration and overall of $\leq$50% was considered acceptable.

The results show that for serotype O1A the main source of variability was from assay run to assay run (CV, 9% to 31%), whereas for serotypes O2 and O6A, the main source of variability was repeatability (CV, 8% to 18%) (Table 3). For serotype O25B, both sources of variability (repeatability and run to run) contributed equally to the total variation (Table 3).

For each serotype and concentration, the CVs for intermediate precision reached a maximum of 33% for serotype O1A, 31% for serotype O2, 17% for serotype O6A, and 22% for serotype O25B (Table 3). Estimates of repeatability and intermediate precision were computed for each serotype across the whole range of concentrations tested (Table 3). The CVs for intermediate precision were $\leq$23% for all serotypes.

Linearity was assessed by plotting the $\log_{10}$-transformed titers against the $\log_{10}$ theoretical titer expected from serial dilution of the titer observed with the first concentration tested for each serotype. A linear regression model was fitted to the

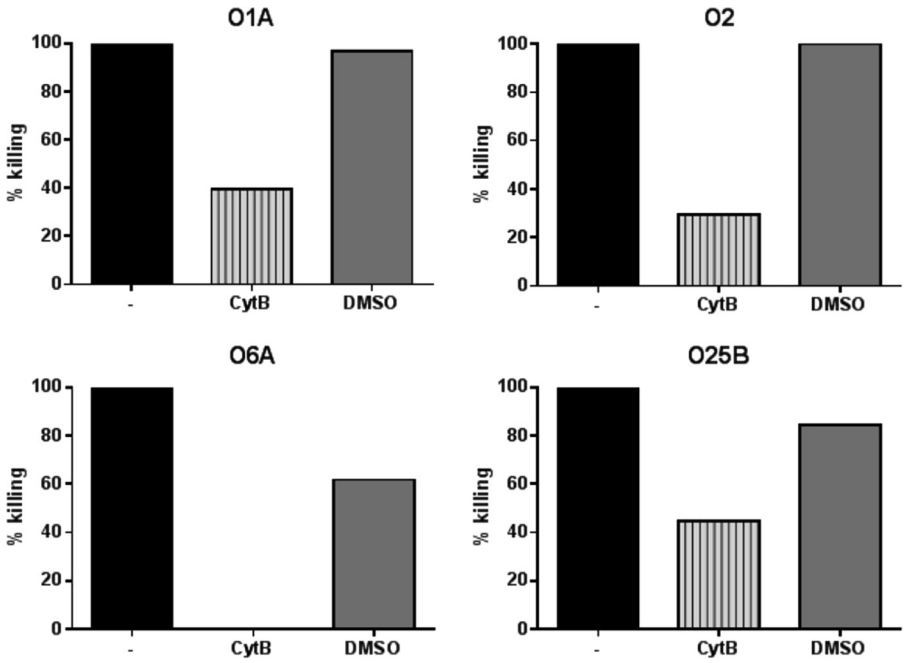

FIG 2 Phagocytosis is required for *E. coli* killing in the OPA. HL60 cells were incubated for 30 min with cytochalasin B (CytB; 10 μM) or dimethyl sulfoxide (DMSO; vehicle). The opsonization index (OI) obtained for the standard condition (−, black bars) was set as 100% killing, and the tested conditions were calculated as follows: percent killing = (OI for the test condition/OI for the standard condition) × 100. A paired *t* test was used to compare the percent killing between cytochalasin B treatment and the reference condition (no treatment) across serotypes ($P = 0.0055$; lower and upper 95% confidence limits [CL], −100% and −40%, respectively).

$\log_{10}$ titers as a function of the $\log_{10}$ theoretical titers for each serotype. Linearity was considered acceptable if the 90% confidence interval (CI) of the slope of each strain was within the range of 0.80 to 1.25.

Regression modeling indicated the linearity of the assay (Fig. 3), with the 90% CI of the slopes of all serotypes being fully included in the predefined acceptance limits of 0.80 to 1.25 (Table 4).

Trueness was assessed by means of relative bias since the true concentration of the samples was unknown. The relative bias was calculated as the difference between the geometric mean of the observed titers and the theoretical titer divided by the geometric mean of the theoretical titer on the original scale, expressed as a percentage. The geometric means of the observed titers were obtained from the linear mixed model by concentration and serotype after back calculation of the mean $\log_{10}$ titers to the original scale. Trueness was considered to be within acceptable levels if the relative bias at each concentration was within the range of −30% to 30%. The accuracy or total error of the results generated by the OPA was estimated by calculating the 90% beta-expectation tolerance interval on the $\log_{10}$ titers by including series as a random effect and using the linear mixed model described above for each concentration. The 90% beta-expectation tolerance interval expressed as relative error at each theoretical titer was acceptable if the values fell within the range of −65% to 183%.

Data analysis showed that the relative bias was always less than approximately 30% for all serotypes and concentrations evaluated (data not shown). The 90% beta-expectation tolerance intervals fell completely within the acceptance criterion range of −65% to 183% for all serotypes and concentrations evaluated (Fig. 4).

The assay range for each serotype was determined to be the lowest and highest concentrations evaluated during the qualification in which the assay showed acceptable linearity, precision, trueness, and total error. The lower and upper limits of the assay range were set at the theoretical titers. For serotypes O1A and O6A, five

TABLE 3 Precision by concentration and serotype[a]

Serotype	Concn	Log_{10} titer[b]	Titer[b]	SD			% CV[c]		
				Repeatability variance	Between-run variance	Intermediate precision	Repeatability variance	Between-run variance	Intermediate precision
O1A	1	3.584	3,841	0.040	0.055	0.068	9.23	12.67	15.72
O1A	2	2.982	960	0.071	0.095	0.119	16.50	22.08	27.80
O1A	3	2.380	240	0.039	0.039	0.055	9.05	8.93	12.74
O1A	4	1.778	60	0.049	0.092	0.104	11.23	21.52	24.39
O1A	5	1.176	15	0.046	0.132	0.140	10.67	31.17	33.11
O1A	Overall			0.058	0.078	0.097	13.40	18.01	22.58
O2	1	3.717	5,208	0.071	0.000	0.071	16.38	0.000	16.38
O2	2	3.018	1,042	0.066	0.080	0.104	15.34	18.63	24.30
O2	3	2.319	208	0.076	0.108	0.132	17.67	25.22	31.12
O2	4	1.620	42	0.051	0.000	0.051	11.78	0.000	11.78
O2	Overall			0.082	0.051	0.096	18.96	11.80	22.44
O6A	1	3.164	1,458	0.055	0.007	0.056	12.75	1.60	12.85
O6A	2	2.687	486	0.053	0.052	0.074	12.27	11.97	17.21
O6A	3	2.209	162	0.046	0.015	0.048	10.52	3.54	11.11
O6A	4	1.732	54	0.035	0.037	0.051	8.09	8.62	11.84
O6A	5	1.255	18	0.049	0.027	0.056	11.33	6.14	12.91
O6A	Overall			0.048	0.033	0.058	11.01	7.52	13.36
O25B	1	2.502	317	0.058	0.056	0.081	13.52	12.87	18.75
O25B	2	2.024	106	0.064	0.033	0.072	14.86	7.71	16.78
O25B	3	1.547	35	0.056	0.060	0.082	12.94	13.93	19.10
O25B	4	1.070	12	0.053	0.076	0.093	12.33	17.75	21.72
O25B	Overall			0.056	0.060	0.083	13.05	13.96	19.19

[a]Precision experiments used human serum. Precision was evaluated on the basis of the results of 4 runs performed by 2 operators using a pool of high-potency serum samples that were diluted to 4 or 5 concentrations, as indicated.
[b]Theoretical titers at the original and log_{10} scale.
[c]CV, coefficient of variation.

concentrations fell within the assay working range, and for serotypes O2 and O25B, four concentrations fell within the assay working range (Table 5).

The specificity of ExPEC4V-induced serum antibodies for each of the four *E. coli* serotypes was demonstrated by preadsorbing serum with *E. coli* cells of each serotype: specificity was shown by the selective decrease in OI titers to the homologous serotype compared to the titers to the remaining three heterologous serotypes. Nonspecific activity and specific activity were defined as the percent reduction in the OI value of heterologous and homologous serum preadsorptions, respectively. Vaccine-induced antibodies were determined to be specific to each of the serotypes if the reduction of the OI value was $\leq$20% for heterologous serum preadsorption OPA combinations and $\geq$70% for homologous serum preadsorption OPA combinations.

Preadsorption of serum pools with homologous O-serotype *E. coli* cells yielded OPA titer reductions of $\geq$99.6%, while preadsorption of serum pools with heterologous O-serotype *E. coli* cells yielded OPA titer reductions of $\leq$20.6% (Table 6).

Evaluation of ExPEC4V immunogenicity using the qualified OPA. The qualified OPA was used to assess the immunogenicity of a 4-valent *E. coli* bioconjugate vaccine (ExPEC4V) administered to humans in a phase 1 study of women with recurrent UTIs (10). Representative OI data for the sera from 10 subjects vaccinated with ExPEC4V and 2 subjects vaccinated with a placebo are shown in Table 7. For all four O serotypes, vaccine-mediated increases in titers (1.2- to 241.6-fold) were seen when the titers from day 1 (prevaccination) were compared to those from day 30 for the vaccinated subjects, while the titers for the placebo-vaccinated subjects showed little or no change when those on day 1 were compared to those on day 30.

DISCUSSION

The World Health Organization recognizes the demonstration of the OPA titer to be a key secondary readout for the clinical efficacy of PCVs and requires measurement of

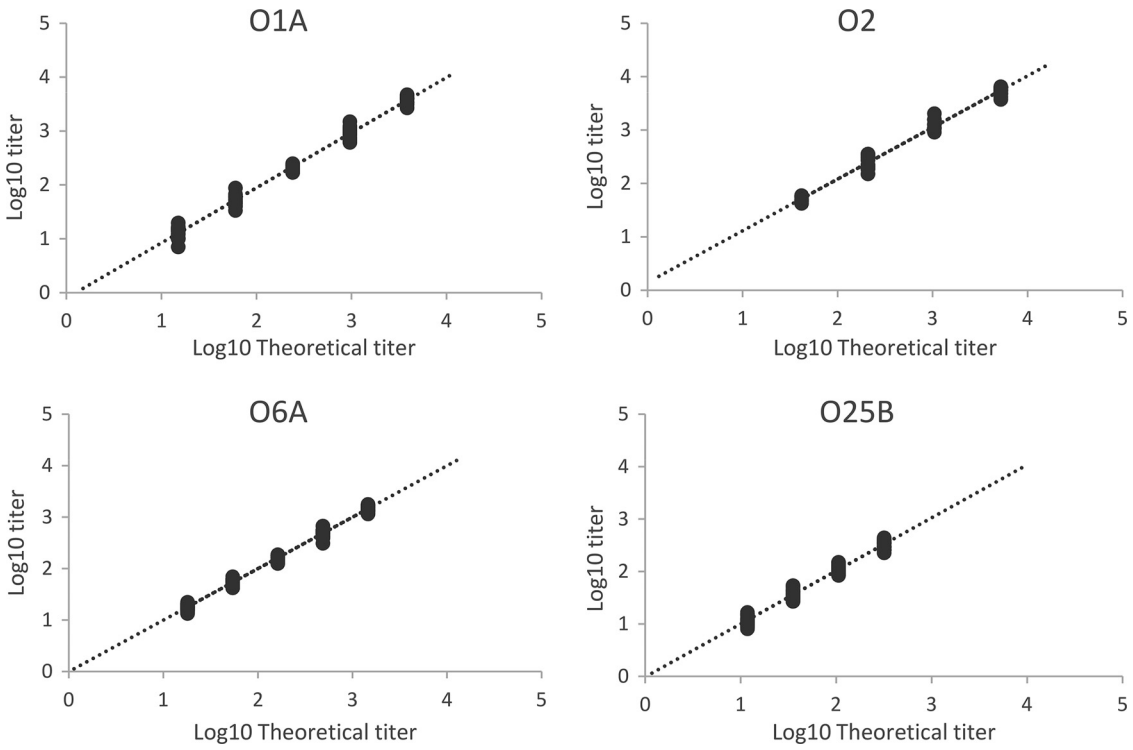

FIG 3 Assay linearity evaluation. Plots of the $\log_{10}$ titers versus $\log_{10}$ theoretical titers per serotype are shown. Dashed line, the unit line between the $\log_{10}$ titers and the $\log_{10}$ theoretical titers.

the OPA titer in a subset of samples to support licensure of new vaccines (23). We drew on parallels between the production and mechanisms of immune protection between ExPEC vaccines and PCVs to develop an OPA suitable for the evaluation of human serum in clinical trials of a bioconjugate vaccine (ExPEC4V). To date, guidelines for the development of ExPEC vaccines and requirements for the demonstration of immunogenicity are not available. However, OPA is expected to be an important readout to demonstrate immunogenicity to support the licensure of ExPEC vaccines.

Measurement of antibodies using ELISA is a well-established method of evaluating the immunogenicity of vaccines. However, the OPA more accurately reflects the *in vivo* protective capacity of these antibodies; the OPA result correlates more closely with clinical protection than the ELISA result in infants vaccinated with a PCV (12–14) and is likely to be a surrogate marker of protection for other bacteria, such as *E. coli*, for which opsonophagocytosis is an important clearance mechanism in the human host (24–26).

The information in the literature regarding *E. coli* OPAs is minimal in comparison to that regarding other bacteria, such as pneumococci, and typically comes from studies involving the use of neutrophils as effector cells, as described by Cross et al. (17). The use of HL60 cells in an *E. coli* killing assay is rarely reported (27), and there are no reports, to our knowledge, of a qualified *E. coli* OPA using HL60 cells. In order to avoid

TABLE 4 Linearity[a] results

Serotype	Slope	90% CI[b]	
		Lower	Upper
O1A	1.02	1.01	1.04
O2	0.97	0.94	1.00
O6A	1.00	0.99	1.01
O25B	1.01	0.98	1.04

[a]Linearity experiments used human serum.
[b]CI, confidence interval.

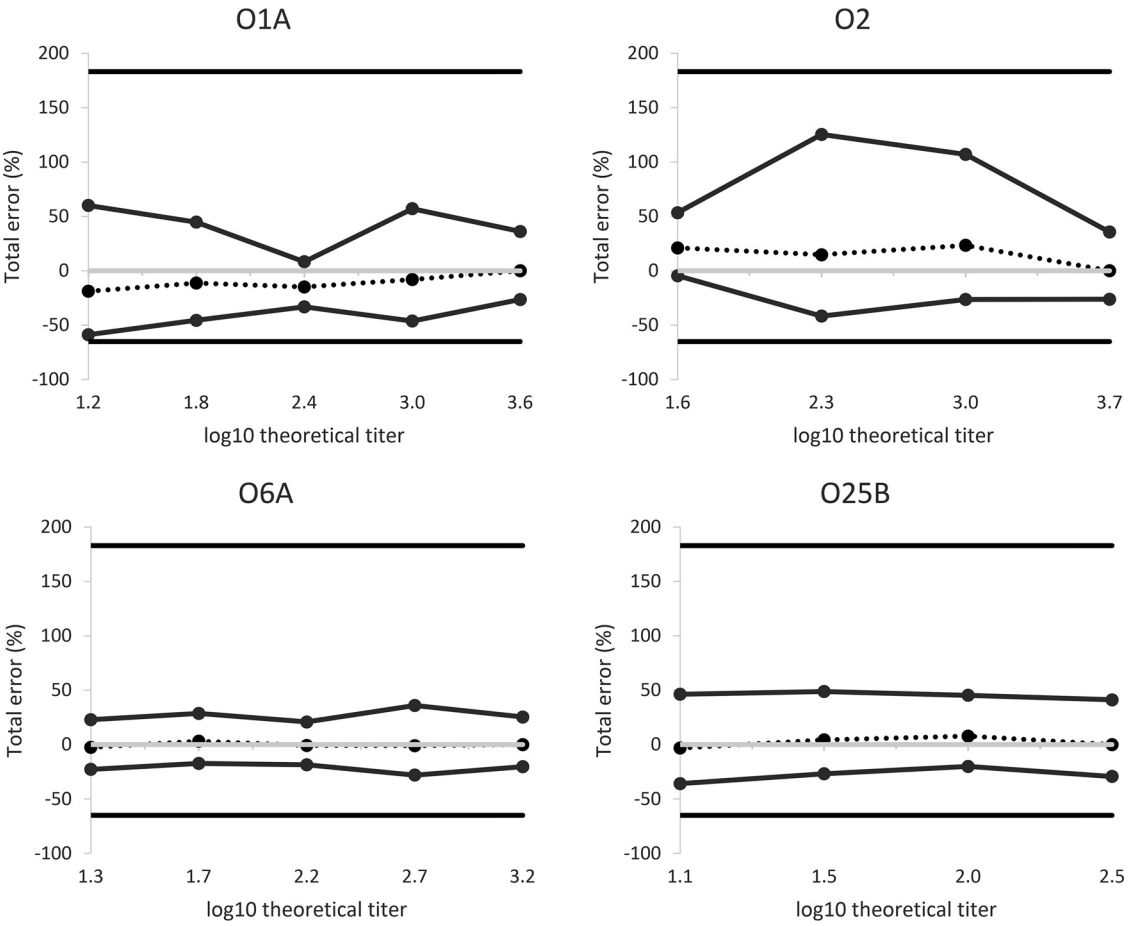

FIG 4 Total error profiles for the different concentrations per serotype. The dark gray solid lines connect the lower and the upper 90% beta-expectation tolerance limits, the black dotted line connects the percent relative bias, the outer black solid lines refer to the lower and upper acceptance criteria, and the light gray solid line refers to relative bias equal to 0%.

the technical challenges and variability associated with the isolation and use of human neutrophils and to better align with other OPAs already recognized by regulatory authorities (28), the previously described pneumococcal OPAs (14, 19) that use HL60 cells were adapted for use with *E. coli*. The original assay using BRC proved insensitive to differences between pre- and postvaccination serum samples. Human complement improved the ability of the assay to differentiate between pre- and postvaccination samples but required the addition of a preadsorption step to reduce the high levels of NSK activity against *E. coli* strains, likely due to the presence of preexisting antibodies (17). The ratios of HL60 cells to bacteria were optimized, and growth conditions for bacterial enumeration were modified to suit the characteristics of *E. coli*.

Qualification steps showed that for serotypes O1A, O2, O25B, and O6A, the accep-

TABLE 5 Assay working range[a]

	Log_{10} scale		Original scale	
Serotype	LLOQ[b]	ULOQ[c]	LLOQ	ULOQ
O1A	1.176	3.584	15	3,841
O2	1.620	3.717	42	5,208
O6A	1.255	3.164	18	1,458
O25B	1.070	2.502	12	317

[a]Assay working range experiments used human serum.
[b]LLOQ, lower limit of quantification.
[c]ULOQ, upper limit of quantification.

TABLE 6 OPA specificity expressed as a percentage of OI[a] reduction

	Specificity[b] (% reduction in OI)			
Serotype	O1	O2	O6	O25
O1A	99.6	17.2	12.3	20.6
O2	6.5	98.9	14.2	3.3
O6A	0.5	−3.3	100	−11.3
O25B	8.8	−0.2	−32.0	100

[a]OI, opsonization index.
[b]Specificity experiments used human serum.

tance criteria for precision, trueness, linearity, and total error were fulfilled within the working range of the assay. The assay was shown to be specific for all serotypes: OPA titers were reduced by ≥99.6% when serum pools were preincubated with *E. coli* cells of the homologous serotype, while OPA titers were reduced by ≤20.6% when serum pools were preadsorbed with *E. coli* cells of heterologous serotypes. These results are consistent with the predetermined targets and therefore qualified the OPA for its purpose and support the serotype specificity of the antibodies generated by ExPEC4V.

Further work to be done includes investigation of a potential protective OPA titer threshold for ExPEC infections. It is unlikely that the same threshold will apply to all serotypes or to different clinical syndromes, such as UTIs in healthy women versus bacteremia in elderly individuals. Future studies to evaluate the levels of anti-*E. coli* functional antibodies present in the serum of subjects with a history of recurrent urinary tract infection or bacteremia in comparison with those in the serum of healthy controls could support the establishment of a correlate of protection for ExPEC infections.

Standardization of OPAs between laboratories has been a significant challenge in the use of OPAs for PCVs (14, 29) but was fundamental for PCV clinical development. Therefore, interlaboratory standardization of the *E. coli* OPA will become important not only for vaccine studies but also for natural immunity studies and will support the further development of an ExPEC vaccine. Progress on this front and existing collaborations between manufacturers, regulatory agencies, and WHO can be applied to new *E. coli* OPAs.

The qualified *E. coli* OPA was used to evaluate human serum from a phase 1 clinical trial and successfully demonstrated a vaccine-mediated increase in OI titers at 30 days postvaccination (10); this increase in titers was similar to that reported for other conjugate vaccines, such as the pneumococcal 7-valent conjugate vaccine Prevnar7 (30, 31). In conclusion, we have developed an OPA based on the pneumococcal OPA

TABLE 7 OI values determined using the qualified OPA with human serum from a phase 1 study evaluating ExPEC4V

		OI[a]							
		Serotype O1A		Serotype O2		Serotype O6A		Serotype O25B	
Subject no.	Vaccine	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
1	ExPEC4V	249	1,451	340	12,922	491	7,626	77	226
2	ExPEC4V	149	1,151	228	8,897	497	1,292	338	606
3	ExPEC4V	42	3,578	167	23,210	406	3,784	210	3,423
4	ExPEC4V	122	9,641	560	9,824	541	4,559	6	1,450
5	ExPEC4V	126	15,207	674	26,244	1,292	3,390	476	1,265
6	ExPEC4V	177	1,121	484	15,609	812	2,140	86	184
7	ExPEC4V	147	5,889	128	10,371	509	2,492	23	87
8	ExPEC4V	361	510	5,385	10,318	508	13,407	212	1,133
9	ExPEC4V	15	477	21	6,135	2,091	9,655	4,609	5,360
10	ExPEC4V	173	379	187	7,284	999	2,759	6	81
11	Placebo	229	197	374	304	353	392	265	208
12	Placebo	197	197	230	210	404	457	320	249

[a]OI, opsonization index. OI titers at or below the lower limit of quantification were replaced with the respective values of one-half the lower limit of quantification. Day 1 is prevaccination.

suitable for multiple *E. coli* serotypes and have demonstrated the ability of this assay to measure a functional antibody response in human serum from a phase 1 clinical trial of ExPEC4V (10).

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains OC24452 (O serotype O1A), OC24453 (O serotype O2), OC24454 (O serotype O6A), and OC24176 (O serotype O25B) were used in the OPA. These strains are clinical isolates that were selected on the basis of their subtype specificity and their sensitivity in discriminating pre- from postvaccination serum responses.

The O serotype of each isolate was confirmed by agglutination using a single *E. coli* O-serotype-specific antiserum (Statens Serum Institut, Denmark) and PCR using O-serotype-specific primers described by Fratamico et al. (32) and Li et al. (33). Assay working stocks were created on the basis of the method described by Burton and Nahm (34). In brief, bacteria from master stocks were streaked onto blood agar plates and incubated overnight at 37°C in ambient air. Sterile Todd-Hewitt broth (Becton Dickinson, Sparks, MD) with 0.5% yeast extract was inoculated with multiple colonies, which were incubated at 37°C in ambient air with shaking at 110 rpm until the optical density at 600 nm was 0.6 to 0.9. Working assay stocks were stored until needed by cryopreservation with 15% glycerol at −80°C.

In the OPA, the enumeration of bacterial colonies was performed by spotting bacteria on glycerol yeast extract agar (GYA) containing 2,3,5,-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich). The GYA medium comprised M9 minimal salts (Becton Dickinson), $MgSO_4$ (Sigma-Aldrich), yeast extract (Becton Dickinson), glycerol (Sigma-Aldrich), and Bacto agar (Becton Dickinson).

Phagocytes. HL60 cells were obtained, propagated, and differentiated as described previously (19), except that the differentiation with dimethyl formamide was done for 3 days. Cells were assessed for their suitability for use in the OPA by flow cytometry, as described previously (19). These cells were used as the source of phagocytes for the OPA.

Serum samples. Human serum (HS) from healthy subjects was obtained from Sigma-Aldrich (St. Louis, MO; sample HS) or Biological Specialty Corporation (Colmar, PA; samples HS37, HS38, HS39, HS40, and HS58). Rat serum samples were obtained from preclinical studies in which animals were vaccinated with ExPEC4V (35). Human serum was also obtained before and after vaccination (12 paired samples, $n = 24$) from subjects participating in a phase 1 clinical trial assessing the safety and immunogenicity of a bioconjugate *E. coli* vaccine (ClinicalTrials.gov registration number NCT02289794) (10). For OPA qualification, human serum samples obtained from 4 subjects after vaccination with ExPEC4V were pooled. Rats were maintained, immunized, and bled at the animal facility of Eurogentec SA, and studies were conducted according to institutional guidelines. The phase 1 study and all protocol amendments were reviewed and approved by the independent ethics committees of each site and by the Swiss Agency for Therapeutic Products (Swissmedic).

Complement source. The complement initially used for OPA development was from 3- to 4-week-old rabbits (Pel-Freez, Rogers, AR, or Cedarlane, Burlington, NC). OPA qualification used pooled human complement (Cedarlane) from three different donors. To reduce nonspecific killing (NSK), human complement was preadsorbed with glutaraldehyde-treated *E. coli* bacteria at a ratio of 1 ml complement per 2×10^9 CFU of OC24452 (O1A), 2×10^9 CFU of OC24453 (O2), 7×10^9 CFU of OC24454 (O6A), and 4×10^9 CFU of OC24176 (O25B). Glutaraldehyde-treated *E. coli* bacteria and human complement were incubated for 60 min at 4°C with gentle rocking. After incubation, the glutaraldehyde-treated *E. coli* bacteria were removed by centrifugation (16,748 × *g*, 5 min, 4°C) and the complement was either used immediately or aliquoted and stored at −80°C.

OPA procedure. The assay was based on the OPA described by Romero-Steiner et al. (14) and Burton and Nahm (19) for the evaluation of pneumococcal antibodies. Assays were carried out using round-bottom 96-well plates (Costar). Serum samples were heat inactivated by incubation at 56°C for 30 min and added to the assay plate (20 μl/well) at a starting dilution of 1:12, followed by 7 additional serial dilutions in 3-fold steps. All serum samples were tested in duplicate. Frozen stocks of *E. coli* were thawed, washed with opsonization buffer B (OBB; Hanks' balanced salt solution [HBSS] with magnesium, calcium, and 0.1% gelatin) by centrifugation (12,000 rpm, 2 min), diluted to ~10^5 CFU/ml, and dispensed at 10 μl/well. After 30 min of incubation at room temperature, 5.6 μl of complement, 36.8 μl of HL60 cells, and 7.6 μl of OBB were added to each well. Before use, HL60 cells were washed twice with HBSS by centrifugation (350 × *g*, 5 min) and adjusted in OBB to 1.6×10^7 cells/ml. The plates were incubated for 60 min at 37°C in 5% CO_2 with shaking (Bellco Biotechnology, Vineland, NJ) at 700 rpm. Afterwards, the plates were placed on ice for 20 min and 10 μl of the final reaction mixture was spotted onto GYA plates containing TTC. The GYA plates were incubated for 15 to 16 h at 33°C.

The number of bacterial colonies on the agar plates was enumerated by digitally photographing the plates (Nikon D3200) and uploading the images to a computer with colony-counting software (NICE, U.S. National Institute of Standards and Technology [http://www.vaccine.uab.edu/uploads/mdocs/NICE.pdf]). Opsonization index (OI) values (referred to here as titers) were defined as the serum dilution that killed 50% of the bacteria and were determined using the Opsotiter3 Excel-based program developed at the University of Alabama at Birmingham, Birmingham, AL, USA.

OPA qualification. The performance of the assay was assessed for repeatability, intermediate precision, linearity, trueness, total error, assay range, and specificity with the pooled human serum samples from subjects vaccinated with ExPEC4V (21, 22).

The assay target acceptance criteria were defined as follows: linearity, 90% CI of the slope of 0.80 to 1.25; precision, CV for repeatability and intermediate precision of ≤50%; accuracy, total error of −65%

to 183%; and trueness, relative bias of −30% to 30%). The assay working range was defined as the lowest and the highest concentrations where the assay jointly fulfilled the target acceptance criteria for linearity, precision, trueness, and accuracy. The assay was considered specific if the OI reduction with was ≤20% for heterologous serum preadsorption and ≥70% for homologous serum preadsorption.

Statistical analysis. Data for the OPA qualification were analyzed using SAS (version 9.4) software (SAS, Cary, NC). To evaluate the contribution of key components of the opsonophagocytosis killing assay to bacterial killing, the area under the curve (AUC) for each dilution series per treatment and per serotype was calculated. In addition, a model was fitted to the $\log_{10}$ AUC by including serotype and treatment as fixed effects. Pairwise comparisons between treatment conditions were performed by omitting key components of the assay and the reference condition (serum, bacteria, complement, and HL60 cells). Dunnett's method was applied to correct for multiple comparisons. A paired *t* test was used to evaluate the effect of cytochalasin B treatment on the percent *E. coli* killing compared to the amount of *E. coli* under the reference condition (no treatment) across serotypes. *P* values of ≤0.05 were considered significant.

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REFERENCES

1. Elixhauser AFB, Stranges E. 2011. Septicemia in U.S. hospitals, 2009: statistical brief no. 122. Healthcare Cost and Utilization Project (HCUP) statistical briefs. Agency for Health Care Policy and Research, Rockville, MD.
2. Kung HC HD, Xu J, Murphy SL. 2008. Deaths: final data for 2005. *In* National vital statistics reports, vol 56, no. 10. National Center for Health Statistics, Hyattsville, MD.
3. de Kraker ME, Jarlier V, Monen JC, Heuer OE, van de Sande N, Grundmann H. 2013. The changing epidemiology of bacteraemias in Europe: trends from the European Antimicrobial Resistance Surveillance System. Clin Microbiol Infect 19:860–868. https://doi.org/10.1111/1469-0691.12028.
4. Laupland KB, Gregson DB, Church DL, Ross T, Pitout JD. 2008. Incidence, risk factors and outcomes of Escherichia coli bloodstream infections in a large Canadian region. Clin Microbiol Infect 14:1041–1047. https://doi.org/10.1111/j.1469-0691.2008.02089.x.
5. Russo TA, Johnson JR. 2003. Medical and economic impact of extraintestinal infections due to Escherichia coli: focus on an increasingly important endemic problem. Microbes Infect 5:449–456. https://doi.org/10.1016/S1286-4579(03)00049-2.
6. Mathers AJ, Peirano G, Pitout JD. 2015. Escherichia coli ST131: the quintessential example of an international multiresistant high-risk clone. Adv Appl Microbiol 90:109–154. https://doi.org/10.1016/bs.aambs.2014.09.002.
7. Poolman JT, Wacker M. 2016. Extraintestinal pathogenic Escherichia coli, a common human pathogen: challenges for vaccine development and progress in the field. J Infect Dis 213:6–13. https://doi.org/10.1093/infdis/jiv429.
8. Orskov I, Orskov F. 1985. Escherichia coli in extra-intestinal infections. J Hyg (Lond) 95:551–575. https://doi.org/10.1017/S0022172400060678.
9. Ihssen J, Kowarik M, Dilettoso S, Tanner C, Wacker M, Thöny-Meyer L. 2010. Production of glycoprotein vaccines in Escherichia coli. Microb Cell Fact 9:61. https://doi.org/10.1186/1475-2859-9-61.
10. Huttner A, Hatz C, van den Dobbelsteen G, Abbanat D, Hornacek A, Frolich R, Dreyer AM, Martin P, Davies T, Fae K, van den Nieuwenhof I, Thoelen S, de Valliere S, Kuhn A, Bernasconi E, Viereck V, Kavvadias T, Kling K, Ryu G, Hulder T, Groger S, Scheiner D, Alaimo C, Harbarth S, Poolman J, Fonck VG. 2017. Safety, immunogenicity, and preliminary clinical efficacy of a vaccine against extraintestinal pathogenic Escherichia coli in women with a history of recurrent urinary tract infection: a randomised, single-blind, placebo-controlled phase 1b trial. Lancet Infect Dis 17:528–537. https://doi.org/10.1016/S1473-3099(17)30108-1.
11. Schuerman L, Wysocki J, Tejedor JC, Knuf M, Kim KH, Poolman J. 2011. Prediction of pneumococcal conjugate vaccine effectiveness against invasive pneumococcal disease using opsonophagocytic activity and antibody concentrations determined by enzyme-linked immunosorbent assay with 22F adsorption. Clin Vaccine Immunol 18:2161–2167. https://doi.org/10.1128/CVI.05313-11.
12. Henckaerts I, Durant N, De Grave D, Schuerman L, Poolman J. 2007. Validation of a routine opsonophagocytosis assay to predict invasive pneumococcal disease efficacy of conjugate vaccine in children. Vaccine 25:2518–2527. https://doi.org/10.1016/j.vaccine.2006.09.029.
13. Jódar L, Butler J, Carlone G, Dagan R, Goldblatt D, Käyhty H, Klugman K, Plikaytis B, Siber G, Kohberger R, Chang I, Cherian T. 2003. Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. Vaccine 21:3265–3272. https://doi.org/10.1016/S0264-410X(03)00230-5.
14. Romero-Steiner S, Frasch CE, Carlone G, Fleck RA, Goldblatt D, Nahm MH. 2006. Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. Clin Vaccine Immunol 13:165–169. https://doi.org/10.1128/CVI.13.2.165-169.2006.
15. Miajlovic H, Smith SG. 2014. Bacterial self-defence: how Escherichia coli evades serum killing. FEMS Microbiol Lett 354:1–9. https://doi.org/10.1111/1574-6968.12419.
16. Burns SM, Hull SI. 1999. Loss of resistance to ingestion and phagocytic killing by O(−) and K(−) mutants of a uropathogenic Escherichia coli O75:K5 strain. Infect Immun 67:3757–3762.
17. Cross A, Artenstein A, Que J, Fredeking T, Furer E, Sadoff JC, Cryz SJ, Jr. 1994. Safety and immunogenicity of a polyvalent Escherichia coli vaccine in human volunteers. J Infect Dis 170:834–840. https://doi.org/10.1093/infdis/170.4.834.
18. Hu BT, Yu X, Jones TR, Kirch C, Harris S, Hildreth SW, Madore DV, Quataert SA. 2005. Approach to validating an opsonophagocytic assay for Streptococcus pneumoniae. Clin Diagn Lab Immunol 12:287–295. https://doi.org/10.1128/CDLI.12.2.287-295.2005.
19. Burton RL, Nahm MH. 2006. Development and validation of a fourfold multiplexed opsonization assay (MOPA4) for pneumococcal antibodies. Clin Vaccine Immunol 13:1004–1009. https://doi.org/10.1128/CVI.00112-06.
20. Kim KH, Yu J, Nahm MH. 2003. Efficiency of a pneumococcal opsonophagocytic killing assay improved by multiplexing and by coloring colonies. Clin Diagn Lab Immunol 10:616–621. https://doi.org/10.1128/CDLI.10.4.616-621.2003.
21. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. 2005. ICH harmonized tripartite guideline validation of analytical procedures: text and methodology Q2(R1). International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland.
22. DeSilva B, Smith W, Weiner R, Kelley M, Smolec J, Lee B, Khan M, Tacey R, Hill H, Celniker A. 2003. Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic

assessments of macromolecules. Pharm Res 20:1885–1900. https://doi.org/10.1023/B:PHAM.0000003390.51761.3d.
23. World Health Organization. 2009. Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines. WHO Expert Committee on Biological Standardization, World Health Organization, Geneva, Switzerland.
24. van Lookeren Campagne M, Wiesmann C, Brown EJ. 2007. Macrophage complement receptors and pathogen clearance. Cell Microbiol 9:2095–2102. https://doi.org/10.1111/j.1462-5822.2007.00981.x.
25. Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS. 2010. The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. Infect Immun 78:704–715. https://doi.org/10.1128/IAI.00881-09.
26. Shimanovich AA, Buskirk AD, Heine SJ, Blackwelder WC, Wahid R, Kotloff KL, Pasetti MF. 2017. Functional and antigen-specific serum antibody levels as correlates of protection against shigellosis in a controlled human challenge study. Clin Vaccine Immunol 24:e00412-16. https://doi.org/10.1128/CVI.00412-16.
27. Roberts PJ, Ford JM. 1982. A new combined assay of phagocytosis and intracellular killing of Escherichia coli by polymorphonuclear leukocytes. J Immunol Methods 49:193–207. https://doi.org/10.1016/0022-1759(82)90277-0.
28. Fleck RA, Romero-Steiner S, Nahm MH. 2005. Use of HL-60 cell line to measure opsonic capacity of pneumococcal antibodies. Clin Diagn Lab Immunol 12:19–27. https://doi.org/10.1128/CDLI.12.1.19-27.2005.
29. Rose CE, Romero-Steiner S, Burton RL, Carlone GM, Goldblatt D, Nahm MH, Ashton L, Haston M, Ekstrom N, Haikala R, Kayhty H, Henckaerts I, Durant N, Poolman JT, Fernsten P, Yu X, Hu BT, Jansen KU, Blake M, Simonetti ER, Hermans PW, Plikaytis BD. 2011. Multilaboratory comparison of Streptococcus pneumoniae opsonophagocytic killing assays and their level of agreement for the determination of functional antibody activity in human reference sera. Clin Vaccine Immunol 18:135–142. https://doi.org/10.1128/CVI.00370-10.
30. de Roux A, Schmöle-Thoma B, Schmöele-Thoma B, Siber GR, Hackell JG, Kuhnke A, Ahlers N, Baker SA, Razmpour A, Emini EA, Fernsten PD, Gruber WC, Lockhart S, Burkhardt O, Welte T, Lode HM. 2008. Comparison of pneumococcal conjugate polysaccharide and free polysaccharide vaccines in elderly adults: conjugate vaccine elicits improved antibacterial immune responses and immunological memory. Clin Infect Dis 46:1015–1023. https://doi.org/10.1086/529142.
31. Miernyk KM, Butler JC, Bulkow LR, Singleton RJ, Hennessy TW, Dentinger CM, Peters HV, Knutsen B, Hickel J, Parkinson AJ. 2009. Immunogenicity and reactogenicity of pneumococcal polysaccharide and conjugate vaccines in Alaska native adults 55-70 years of age. Clin Infect Dis 49:241–248. https://doi.org/10.1086/599824.
32. Fratamico PM, Yan X, Liu Y, DebRoy C, Byrne B, Monaghan A, Fanning S, Bolton D. 2010. Escherichia coli serogroup O2 and O28ac O-antigen gene cluster sequences and detection of pathogenic E. coli O2 and O28ac by PCR. Can J Microbiol 56:308–316. https://doi.org/10.1139/W10-010.
33. Li D, Liu B, Chen M, Guo D, Guo X, Liu F, Feng L, Wang L. 2010. A multiplex PCR method to detect 14 Escherichia coli serogroups associated with urinary tract infections. J Microbiol Methods 82:71–77. https://doi.org/10.1016/j.mimet.2010.04.008.
34. Burton RL, Nahm MH. 2012. Development of a fourfold multiplexed opsonophagocytosis assay for pneumococcal antibodies against additional serotypes and discovery of serological subtypes in Streptococcus pneumoniae serotype 20. Clin Vaccine Immunol 19:835–841. https://doi.org/10.1128/CVI.00086-12.
35. van den Dobbelsteen GP, Fae KC, Serroyen J, van den Nieuwenhof IM, Braun M, Haeuptle MA, Sirena D, Schneider J, Alaimo C, Lipowsky G, Gambillara-Fonck V, Wacker M, Poolman JT. 2016. Immunogenicity and safety of a tetravalent E. coli O-antigen bioconjugate vaccine in animal models. Vaccine 34:4152–4160. https://doi.org/10.1016/j.vaccine.2016.06.067.