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Diversity of Hemagglutination Phenotypes among P-Fimbriated Wild-Type Strains of *Escherichia coli* in Relation to *papG* Allele Repertoire

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Data regarding the hemagglutination (HA) patterns of the three variants (classes I, II, and III) of the *Escherichia coli* adhesin PapG are conflicting. These HA patterns usually have been assessed for each *papG* allele separately with recombinant strains in slide HA assays. We rigorously evaluated an alternative microtiter tray HA assay and then used it to assess the HA of four erythrocyte types (human A_1P_1 and OP_1, rabbit, and sheep erythrocytes) by multiple wild-type *E. coli* strains representing the four naturally occurring combinations of the *papG* alleles, i.e., class I plus III, class III only, class II plus III, and class II only. The microtiter tray HA assay displayed significantly better reproducibility of intraobserver (83%) and interobserver (86%) results than did slide HA assays (39 and 73%, respectively). Novel findings from the study of 32 wild-type P-fimbriated strains included reproducible determinations of phenotypic diversity among different *papG* categories, among strains within each *papG* category, and from day to day for individual strains. There was also substantial overlap of phenotypes between *papG* categories I plus III and III only and between II plus III and II only. A class III *papG* recombinant strain's HA pattern differed significantly from that of the wild-type class III strains. These data demonstrate that HA phenotypes of wild-type P-fimbriated *E. coli* strains can be reproducibly assessed by a microtiter HA assay and that they correspond broadly to *papG* genotype but in a more complex and varied fashion than previously recognized.

P fimbriae, the adhesins most clearly implicated in the pathogenesis of extraintestinal infection due to *Escherichia coli* (6, 15), mediate Gal(α1-4)Gal-specific binding to host epithelial surfaces (3, 26, 28, 31–33) via the tip adhesin molecule PapG (9, 39). A possible explanation for the subtle differences in binding preferences among P-fimbriated *E. coli* strains that were noted following the discovery of P fimbriae (5, 7) came with the later discovery that PapG occurs in three molecular variants (38, 40, 42). The PapG variants, sometimes categorized in classes I, II, and III, bind preferentially to different Gal(α1-4)Gal-containing compounds (25, 60, 61) and are encoded by distinct alleles of the adhesin gene *papG* (42) (Table 1).

Since receptor repertoire presumably determines a pathogen's host range (36, 60), efforts to define the three PapG variants' receptor specificities and clinical associations have been made (12, 13, 29, 30, 35, 36, 38, 50, 52, 57, 60, 61). Based largely on the study of single representatives of each *papG* class (often a recombinant strain), the concept that the three PapG variants can be differentiated phenotypically by their distinctive hemagglutination (HA) patterns with rabbit, sheep, and diverse human erythrocytes, which possess unique combinations of Gal(α1-4)Gal-containing glycolipids, has emerged (11, 12, 25, 29, 35, 38, 60, 61) (Table 1). Rabbit cells are reportedly agglutinated only by the class I variant (60, 61), sheep cells are agglutinated only by the class II and III variants (12, 42, 60, 61; but see reference 35), and human O cells are agglutinated only by the class I and II variants (35, 36; but see references 12 and 29). HA patterns of different erythrocyte types have been used in epidemiological studies to classify wild-type *E. coli* strains according to their PapG repertoire (2, 58) and in laboratory studies to define the receptor specificities and host ranges of the PapG variants (11, 12, 25, 35, 36, 60).

However, by using slide HA assays with single representatives of each PapG class, we recently found that the HA patterns of the three PapG classes overlap considerably, even with supposedly class-specific erythrocyte types (24). We also found an unacceptably high degree of irreproducibility of inter- and intraobserver results with slide HA assays (24), which challenged the validity of conclusions regarding the PapG variants previously derived by such assays. Others have interpreted the unexpected finding of diverse (slide) agglutination phenotypes among wild-type *E. coli* strains containing only *papG* allele III (13) as evidence that phenotype is an unreliable indicator of *papG* status. However, whether this apparent phenotypic diversity within class III was due to true biological diversity or rather to assay irreproducibility was not determined.

These conflicting findings prompted us to reexamine two hypotheses, namely, (i) that the different *papG* alleles confer sufficiently distinctive HA patterns with human, rabbit, and sheep erythrocytes to allow phenotypic differentiation of strains with different *papG* allele configurations and (ii) that such HA patterns are uniform among wild-type strains of the same *papG* allele configuration. Our experience with slide HA assays (24) prompted us first to evaluate an alternative microtiter tray (MT) HA assay to determine whether it could assess *E. coli* HA phenotypes more reproducibly.

MATERIALS AND METHODS

Strains and *papG* PCR assay. Representatives of each known naturally occurring *papG* allele configuration, i.e., those of classes I plus III, III, II plus III, and II (13, 60), plus several *papG*-negative strains, were arbitrarily selected from available collections of *papG* genotyped clinical isolates of *E. coli* (14, 18, 21, 43, 55, 59) (Table 2). In addition, because of previously reported phenotypic dis-

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TABLE 1. Characteristics of the three PapG variants, as reported in the literature[a]

PapG variant class	Operon of origin	Designation for corresponding *papG* allele	Postulated epidemiological association(s)	Postulated glycolipid receptor preference(s)	Erythrocyte types ostensibly agglutinated	Agglutination of digalactoside-coated latex beads	Phenotype(s)
I	*pap*	$papG_{J96}$	Diverse extraintestinal infections in humans (single clonal group)	Ceramide trihexoside (Gb_3Ose-Cer)	Human (including O and P_2^k but not p) and rabbit, not dog or sheep	Yes	P
II	*pap-3* (and others)	$papG_{IA2}$	Extraintestinal infections in humans, pyelonephritis and bacteremia more than cystitis	Globoside (Gb_4Ose-Cer)	Human (including O, +/− p), +/− sheep and rabbit	Yes	P; F?
III	*pap-2* = *prs* (and others)	$prsG_{J96}$, $prsG_{1442}$ (and others)	Canine UTI, human cystitis more than pyelonephritis, A_1 secretors?, Luke$^+$ humans?	Forssman glycolipid (Gb_5Ose-Cer)?, globo-A?, stage-specific embryonic antigen-4 (Luke antigen)?	Dog, sheep, +/− human (A_1, A_2?, O?, not p), not rabbit	+/−	F; ONAP?

[a] Data are based on data in references 12, 13, 17, 21 to 25, 29, 35, 36, 40, 42, 47, 50, 57, 60, and 61. The designations $papG_{J96}$, $papG_{IA2}$, and $prsG_{J96}$ are not according to standard conventions but appear the in literature as such. Gb_3Ose-Cer, Gb_4Ose-Cer, and Gb_5Ose-Cer are ceramide conjugates of globotriaose (i.e., ceramide trihexoside), globotetraose, and globopentaose, respectively. +/−, conflicting data in literature or reports of weak agglutination; ?, conflicting data. UTI, urinary tract infection; P, P fimbrial pattern; F, Forssman pattern.

crepancies between recombinant and wild-type class III strains (12, 35), recombinant class III strain P678-54 (pJFK102) (30) was included. Strains were stored at −70°C until used. Each strain's *papG* allele configuration was determined by an allele-specific PCR assay as previously described (16). Primers for allele I were j96-193f (5′-TCGTGCTCAGGTCCGGAATTT-3′) and j96-653r (5′-TGG CATCCCCCAACATTATCG-3′) (461-bp product), primers for allele II were ia2-383f (5′-GGGATGAGCGGGCCTTTGAT-3′) and ia2-572r (5′-CGGGCC CCCAAGTAACTCG-3′) (190-bp product), and primers for allele III were prs-198f (5′-GGCCTGCAATGGATTTACCTGG-3′) and prs-455r (5′-CCACCAA ATGACCATGCCAGAC-3′) (258-bp product) (16). *papG* genotypes were confirmed by at least one replicate PCR determination and by dot blot DNA probe hybridization with allele-specific probes, as previously described (22).

Serotypes and genomic fingerprints. O:H and O:K:H serotypes were determined at the *Escherichia coli* Reference Center at Pennsylvania State University and the International *Escherichia* and *Klebsiella* Centre (WHO), Copenhagen, Denmark, respectively, as previously described (48). For strain IA2 the published O6:H− serotype (4) was used.

Erythrocytes. Human A_1P_1 and OP_1 erythrocytes were from two of the investigators, and p erythrocytes were from the University of Minnesota Blood Bank rare donor collection. Sheep and rabbit erythrocytes were obtained as needed during the course of the study from commercial suppliers (Micropure Medical, Wayzata, Minn.; Remel, Lenexa, Kans.); no information was available regarding the donors. Erythrocytes were washed in phosphate-buffered saline, pH 7.4 (PBS), and stored in Alsever's solution (Gamma Biologicals, Houston, Tex.) at 4°C for up to 1 month. (No decrement in HA behavior was observed over this storage interval.)

MT HA assays. MT HA assays were done as previously described (26, 34, 47, 49), with modifications, in experiments designed primarily to assess reproducibility of assay results (experiment 1), to compare HA patterns of strains within a single *papG* category (experiment 2), and to compare HA patterns between *papG* categories (experiment 3). For all experiments, bacteria freshly harvested from lawns after overnight growth at 37°C on Luria-Bertani agar plates (41) were suspended in PBS plus 5% α-methyl-D-mannose (PBS-man; Sigma, St. Louis, Mo.). (Concentrations of approximately 10^{11} CFU/ml, as determined by quantitative culture, were routinely achieved by suspending the entire lawn from a single culture plate in 1 ml of PBS-man.) Serial 2× dilutions of these stock suspensions were prepared in PBS-man with 96-well V-bottom MTs (Sarstedt, Inc., Newton, N.C.) at 25 μl/well with 12 wells per dilution series. To each well was added 25 μl of erythrocyte suspension (5% in Alsever's solution; Gamma Biologicals). In all experiments, separate dilution series were prepared for each strain with each erythrocyte type. In addition, duplicate dilution series were prepared for each strain from the initial stock suspension (experiment 1.0) or a separately prepared stock suspension of the same strain (all experiments). Strains were sequenced randomly throughout. Erythrocytes were sequenced either randomly (experiment 1.1) or in a fixed order (i.e., A_1P_1, OP_1, rabbit, and sheep) (other experiments).

In all experiments, assays were incubated for 2 h at 4°C and then placed on an ice block and interpreted by one or more observers who were blinded to strain identity (all experiments, except for positive controls in experiment 3) and erythrocyte type (experiments 1 and 2). Two HA endpoints were recorded for each dilution series by inspection of top and bottom surfaces of the MT. The last well with full HA, i.e., with a carpet of erythrocytes covering the bottom of the well and no central cell button, was the last fully positive (LFP) well (Fig. 1). The first well without HA, i.e., with all the erythrocytes in a central button and no carpet, was the first fully negative (FFN) well (Fig. 1).

HA titer reproducibility. HA titer reproducibility was assessed by comparing HA endpoints from duplicate determinations done on the same day by the same observer or different observers, with LFP- and FFN-well endpoints being considered separately. Paired HA titers that differed by ≤1 twofold dilution, that were both <1 (for LFP wells), or that were both >12 (for FFN wells) were said to agree (A); those that differed by ≥2 twofold dilutions were said to disagree. Comparisons were said to be indeterminate when one titer was 1 or 12 and the paired titer was <1 or >12, respectively; conflicting when only one titration showed HA; and negative (N) when neither showed HA. Reproducibility rates were calculated as (A + N)/total.

Detection of P and non-P adhesins. Prior to experiment 2, to exclude strains with non-P adhesins, microscope slide HA assays (24) were used to determine each strain's mannose-resistant HA (MRHA) titer for human A_1P_1 and p erythrocytes (Table 2). If HA was observed with either cell type, inhibition of HA by pigeon egg white (PEW) and 3% bovine serum albumin (Sigma) in PBS was assessed at a bacterial concentration four times that required for HA (19, 20). Strains that exhibited PEW-inhibitable MRHA of A_1P_1 erythrocytes but no MRHA of p erythrocytes were considered to express P adhesins; those that exhibited PEW-inhibitable MRHA of both A_1P_1 and p erythrocytes were considered to express NPMR (formerly X) adhesins (19, 20).

HA pattern matching. In experiment 3, our ability to predict a strain's overall HA pattern for human, rabbit, and sheep erythrocytes on the basis of its *papG* genotype was assessed. Two of the eight NPMR adhesin-negative strains from each *papG* category, selected to represent different HA patterns observed within the category, were designated positive controls. The remaining six strains served as test strains for that *papG* category. Two test strains from each *papG* category (eight strains total) and all eight control strains were assayed daily until all test strains had been assayed on two days each. Each day, duplicate stock suspensions of each test and control strain were titrated against (known) A_1P_1, OP_1, rabbit, and sheep erythrocytes. Observers blinded to test strain identity matched each test strain to one or more (known) controls by direct inspection (observer A) or by analysis of quantitative HA titers as recorded by observer A (observers A to C), based on similarity of overall HA patterns between the test strain and the controls. After a washout period, the three observers independently repeated the latter analysis. Duplicate assessments by each of the three observers were combined to give the day's total score for matching of each test strain with each control strain. Concordance was defined as matching of a test strain to a control of the same *papG* category. Reproducibility was evaluated by comparing (i) an

TABLE 2. Wild-type *E. coli* strains used

papG category	Strain	Syndrome	Location[a]	Serotype[b]	MRHA phenotype[c]	Use of strain in:			Reference(s)
						Expt 1	Screen only	Expt 2 and 3	
I plus III	J96	Pyelonephritis	Seattle	O4:K−:H5	P	+		+	10
	BF1040	Cystitis (adult)	Ann Arbor	O4:K3:H5	NPMR	+			21
	CP9	Bacteremia	Bethesda	O4:K10, K54/96:H5	P			+	21, 54
	BF9043	Cystitis (adult)	Ann Arbor	O4:K3:H5	P			+	21
	R28	Cystitis (adult)	Seattle	O4:K3:H5	NPMR		+		55, 59
	518[d]	Cystitis (adult)	Seattle	O4:K10, K54/96:H5	P			+	55, 59
	553[d]	Cystitis (adult)	Seattle	O4:H5	P			+	55, 59
	BF1023	Cystitis (adult)	Austin	O4:K10, K54/96:H5	P			+	21
	BF1056	Cystitis (adult)	Austin	O4:K10, K54/96:H5	P			+	21
	BOS038	Bacteremia	Boston	O4:K10, K54/96:H−	P			+	43
III	U5	Urosepsis	Seattle	O6:K53:H7	P	+		+	18
	2H25	Urosepsis	Seattle	O18ac:K1:H7	NPMR	+			18
	CA062	Bacteremia	Long Beach	O4:K3:H5	P			+	43
	BOS117	Bacteremia	Boston	O6:H16,H18	P			+	43
	CL14A	Cystitis (child)	Cleveland	O6:H−	P			+	14
	GH20	Cystitis (adult)	Seattle	O2:M	NPMR		+		55, 59
	1044	Cystitis (adult)	Seattle	O18:H15	P			+	55, 59
	R27+	Cystitis (adult)	Seattle	O6:H4,H32	NPMR		+		55, 59
	CL09A	Cystitis (child)	Cleveland	O6:M	P			+	14
	BOS002	Bacteremia	Boston	O6:M	P			+	43
	U7	Urosepsis	Seattle	O6:K+?:H−	P			+	18
II plus III	CL21	Cystitis (child)	Cleveland	O2:H−	P	+		+	14
	AFR098	Bacteremia	Nairobi	O2:H−	P	+		+	43
	BOS030	Bacteremia	Boston	O2:H−	P			+	43
	BOS034	Bacteremia	Boston	O2:H−	P			+	43
	CA002	Bacteremia	Long Beach	O4:K3:H5	NPMR		+		43
	BOS116	Bacteremia	Boston	O2:H−	NPMR		+		43
	BOS086	Bacteremia	Boston	U:H5	P			+	43
	CA032	Bacteremia	Long Beach	O2:M	ps-NPMR			+	43
	BOS119	Bacteremia	Boston	O2:H−	P			+	43
	BOS089	Bacteremia	Boston	O2:H−	P			+	43
II	IA2	UTI[e]	Iowa City	O6:H−	ps-NPMR	+		+	4
	H16	Urosepsis	Seattle	O1:K1:H7	P	+		+	18
	H26	Urosepsis	Seattle	O6:K2:H1	ps-NPMR	+		+	18
	BOS013	Bacteremia	Boston	O6:H16,H18	ps-NPMR	+		+	43
	R45	Cystitis (adult)	Seattle	O4:K12:H1	ps-NPMR	+		+	55, 59
	F14	Cystitis (adult)	Seattle	O6:M	ps-NPMR	+		+	55, 59
	CL01B	Cystitis (child)	Cleveland	O2:H−	ps-NPMR	+		+	14
	CL24	Cystitis (child)	Cleveland	O1:H7	ps-NPMR	+		+	14
papG negative	V21	Urosepsis	Seattle	O75:K5:H5	Negative	+			18
	V28	Urosepsis	Seattle	O8:K+:H−	Negative	+			18

[a] Locations: Austin, Tex.; Ann Arbor, Mich.; Bethesda, Md.; Boston, Mass.; Cleveland, Ohio; Iowa City, Iowa; Long Beach, Calif.; Nairobi, Kenya; Seattle, Wash.
[b] Seroantigens are shown where known; antigens not shown were not tested. K+, encapsulated (K antigen undetermined); K−, capsule minus; U, smooth (O antigen undetermined); M, motile (H antigen undetermined); H−, nonmotile.
[c] MRHA phenotypes were determined by slide HA assays. P, P blood group specific, i.e., PEW-inhibitable HA of A_1P_1 erythrocytes but not p erythrocytes; NPMR, PEW-resistant HA of both A_1P_1 erythrocytes and p erythrocytes; ps-NPMR (pseudo-NPMR), PEW-inhibitable HA of both A_1P_1 and p erythrocytes.
[d] Strains 518 and 553 are isolates from sequential cystitis episodes of the same subject and have indistinguishable pulsed-field gel electrophoresis fingerprints; hence, they likely represent the same strain. Only 518 had full O:K:H serotyping.
[e] UTI, urinary tract infection.

individual observers' repeat assessments of the same strain (intraobserver) and (ii) results between observers (interobserver).

Statistical methods. Comparisons of proportions were tested by a χ^2 test or Fisher's exact test.

RESULTS

Experiment 1: reproducibility of HA titers. Reproducibility of HA titers was assessed initially with two strains from each of the four *papG* categories plus two *papG*-negative strains (experiment 1.0), selected without regard for the presence or absence of NPMR adhesins. Reproducibility of same-day intraobserver HA titers for duplicate dilutions of the same bacterial suspension was 86.8% overall (analysis 1.0.1) (Table 3), and levels of reproducibility did not differ significantly between the two observers (87.9 versus 86.6%) or between the two endpoints used (86.8% for both LFP and FFN wells). Disagreement as to the presence of HA occurred in only 4 to 7% of comparisons (Table 3). For separately prepared stock suspensions of the same strain, agreement of intraobserver results was slightly worse, i.e., 81.8% overall (analysis 1.0.2) (Table 3)

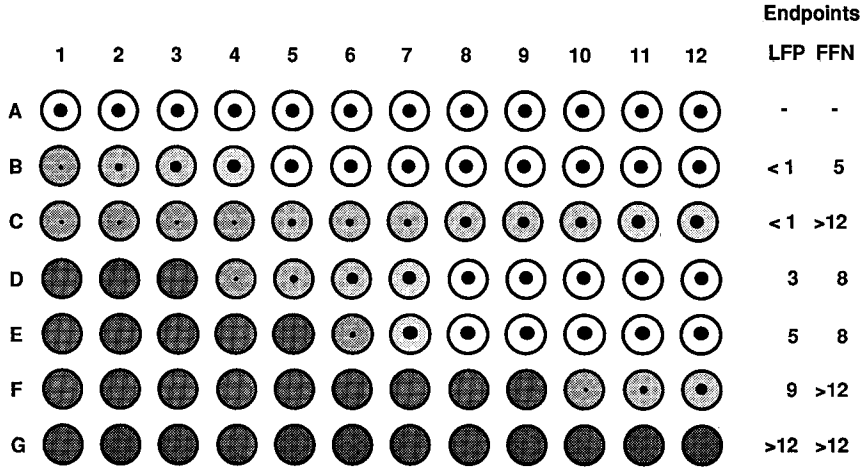

FIG. 1. Interpretation of results of MT HA assays. Circles represent wells in a 96-well V-bottom MT. Internal shading indicates erythrocytes. Numbers to the right are HA titers. Rows: A, all negative (no HA); B, partial HA only, changing to negative at low titer; C, partial HA only, but continuing with partial HA off scale to the right; D, slow transition from full HA to partial HA (wells 4 to 7) to negative; E, more brisk transition; F, high-titer full HA, continuing with partial HA off scale to the right; G, very-high-titer full HA, never changing even to partial HA.

($P = 0.03$ versus agreement of intraobserver results with the same suspension), but again levels of agreement were similar for the two observers and for the two endpoints used (not shown). Reproducibility of interobserver assessments of the same dilution series (analysis 1.0.3) (Table 3) approximated that of intraobserver assessments for duplicate dilution series from the same stock suspension (analysis 1.0.1) (Table 3).

Each of the two blinded observers next scored newly prepared assays twice in succession, with reassortment of MTs between scorings (experiment 1.1). Erythrocytes and strains were randomly distributed. Reproducibility of intraobserver results was 90.7% (185 of 204 determinations) overall. Together with the results of analyses 1.01, 1.02, and 1.03 (Table 3), this finding suggested that both interpretive and technical factors contribute to the MT HA assay's observed irreproducibility of results.

Differences in absolute bacterial concentrations of duplicate stock suspensions of the same strain prepared on a given day were usually small and explained few of the observed same-day HA titer discrepancies (not shown). Sheep erythrocytes consistently exhibited lower rates of reproducibility of intraobserver and interobserver results in experiment 1.0 (77.2 to 80.1% overall, depending on the type of comparison) than did other erythrocyte types (78.7 to 93.4% overall). (For sheep versus other erythrocytes, P was 0.02 for reproducibility of intraobserver results with the same dilution and P was 0.003 for reproducibility of interobserver results with the same dilution.)

Experiment 2: screening for NPMR adhesins. Having established the MT HA assay's reproducibility with wild-type P-fimbriated *E. coli*, we next used the assay to assess *papG* category-specific HA phenotypes attributable to PapG adhesins per se in the absence of NPMR adhesins. Strains representing the four *papG* categories were screened by slide HA assays for expression of NPMR adhesins (Table 2). NPMR adhesin-positive strains were excluded and replaced by alternative strains from the same *papG* category to give eight wild-type strains per category. Of the wild-type strains screened, 2 of 10 from category I plus III, 3 of 11 from category III, 2 of 10 from category II plus III, and 0 of 8 from category II expressed an NPMR adhesin. Of strains expressing a P but no NPMR adhesin, seven of the eight class II strains, but only one from another *papG* category (a category II plus III strain), exhibited a pseudo-NPMR HA pattern ($P < 0.001$) (Table 1). The only non-pseudo-NPMR class II strain was strain H16 (Table 2), which subsequently exhibited other phenotypic anomalies, as described below.

Experiment 2: HA phenotypes according to *papG* category. Within each *papG* category, both consistency and variability of phenotype were encountered. The most common HA pattern in each *papG* category is shown in Table 4 as pattern 1. Although the class I plus III strains typically gave only partial HA with any erythrocyte type, two strains (pattern 2) gave full HA of A_1P_1 and OP_1 erythrocytes. The class III strains varied mainly with respect to their complete (pattern 1) or partial (patterns 2 and 3) HA of sheep erythrocytes and their partial (patterns 1 and 2) or absent (pattern 3) HA of human erythrocytes. Of note, recombinant class III strain P678-54 (pJFK102) was a marked outlier among the class III strains, reproducibly exhibiting much-higher-titer HA for sheep erythrocytes (relative to that of any other erythrocyte type) than the

TABLE 3. Reproducibility of same-day results of MT HA assay (experiment 1.0)

Analysis	Type of comparison	Suspension	Dilution	% of comparisons with indicated status[a] from 544 pairwise HA titer comparisons					% Reproducibility (A + N)
				A	D	C	I	N	
1.0.1	Intraobserver	Same	Different	73.2	8.3	4.6	0.4	13.6	86.8
1.0.2	Intraobserver	Different	Different	68.6	10.8	7.0	0.4	13.2	81.8
1.0.3	Interobserver	Same	Same	70.8	9.7	4.4	0.4	14.7	85.5

[a] A, titers agree by ±1 twofold dilution or both titers are either <1 or >12; D, titers disagree by ≥2 twofold dilutions; C, conflicting, i.e., HA seen in one titration but not in the other; I, indeterminate, i.e., HA was seen in both titrations but the difference in titer was undefined; N, negative, i.e., no HA was seen in either titration.

TABLE 4. *papG*-category-specific HA patterns from experiment 2[a]

papG category	HA pattern	Erythrocyte type[b]	Type strain	HA titer of:	
				LFP well	FFN well
I plus III	1	A	J96	**<1**	6
		O		**<1**	6
		R		<1	9
		S		<1	7
	2	A	BF1023	**3**	7
		O		**3**	6
		R		<1	9
		S		<1	6
III	1	A	BOS117	**<1**	**7**
		O		**<1**	**5**
		R		<1	6
		S		**5**	**10**
	2	A	CL14	**<1**	**6**
		O		**<1**	**6**
		R		<1	5
		S		**<1**	**7**
	3	A	CA062	–[c]	–
		O		–	–
		R		<1	6
		S		**<1**	**5**
II plus III	1	A	CL21	6	9
		O		6	8
		R		**1**	**4**
		S		3	8
	2	A	AFR098	6	8
		O		6	7
		R		**<1**	**3**
		S		3	9
	3	A	BOS086	5	8
		O		5	7
		R		**<1**	**8**
		S		1	9
II	1	A	H26	8	10
		O		8	10
		R		**<1**	**8**
		S		<1	5
	2	A	IA2	7	10
		O		7	9
		R		**<1**	**6**
		S		<1	5
	3	A	CL01B	6	10
		O		7	11
		R		**<1**	**2**
		S		<1	4

[a] Comparisons between strains within a *papG* category reflect same-day HA pattern differences (boldface); comparisons between different *papG* categories are of uncertain significance, since assays for different categories were done on different days. (Eight strains were tested per category; only one representative of each observed HA pattern is listed in the table.)
[b] A, human A_1P_1; O, human OP_1; R, rabbit; S, sheep.
[c] –, no agglutination.

wild-type class III strains, i.e., by ≥6 twofold dilutions, versus a median of 1.5 twofold dilutions (range, −1 to >7) ($P < 0.001$). The class II plus III strains usually gave full HA of rabbit cells (pattern 1), but some gave only partial HA, with either lower (pattern 2) or similar (pattern 3) titers relative to those of other cells. The class II strains varied mostly with respect to intensity of HA of rabbit versus sheep cells, i.e., higher (pattern 1), the same (pattern 2), or lower (pattern 3) intensity.

The O-negative, A-positive (ONAP) phenotype was observed only once in experiment 2. Overall, A_1P_1 and OP_1 erythrocytes gave equivalent results (i.e., A or N) in 181 (84%) of the 216 paired comparisons, including 83% of the 108 comparisons involving class III strains. When titers for A_1P_1 and OP_1 cells disagreed (35 of 216 comparisons), in 66% of instances (67% for class III strains only) the reciprocal titer for OP_1 cells actually exceeded that for A_1P_1 cells, reflecting more intense HA of OP_1 than of A_1P_1 cells (not shown).

Experiment 3.0: comparison of HA phenotypes between *papG* categories. We next sought to determine whether, despite the phenotypic diversity within each *papG* category, the different *papG* categories could still be distinguished phenotypically from one another when they were compared on the same day. This was done by matching (unknown) test strains with positive controls based on overall similarity of HA patterns with the four erythrocyte types (as detailed in Materials and Methods).

Phenotype-based *papG* category assignments segregated according to underlying *papG* status in a predictable, statistically significant fashion (Table 5). Within each *papG* category, in 48 to 71% of assessments (67% [185 of 276 assessments] overall) the assigned category corresponded to the test strain's underlying *papG* genotype. For test strains in each *papG* category, concordant matches were significantly more frequent than were matches to any other single *papG* category ($P < 0.01$ for all comparisons) (Table 5). Both of the HA endpoints used (LFP and FFN) were needed for optimal pattern matching between test strains and controls (not shown).

Discrepancies between actual and inferred *papG* categories (33% of assessments overall) followed predictable patterns. When misclassified, class I plus III strains were designated as either class III or class II, but the latter misclassification occurred only with matches to class II control strain H16, not IA2 (Table 5). Class III strains were miscategorized almost exclusively as class I plus III, and when a match to class II occurred, it again was with control strain H16, not IA2. In contrast, if misclassified, the class II plus III strains were almost always designated as class II only and class II strains were designated as class II plus III (Table 5). For both the class II plus III and the class II strains, assessment as being of class II almost always resulted from a match with class II control strain IA2, rather than H16 (Table 5) ($P < 0.001$). Thus, if the class I plus III and the class III strains were considered together to belong to family A and the class II plus III and class II strains were considered to belong to family B, correspondence of HA phenotype with *papG* family occurred for 83% of assessments for family A and 99.7% for family B ($P < 0.001$ for phenotype-genotype correspondence by family).

Experiment 3.0: diversity of HA phenotype within *papG* categories. HA patterns differed significantly not only between *papG* categories but also within each *papG* category. Among the class I plus III test strains, although strain 518 matched exclusively (12 of 12 comparisons) the class I plus III controls, on the same days strain 553 matched other controls more frequently (5 of 12 comparisons [$P = 0.04$]), and although strain CP9 matched predominantly the class II control strain H16 (8 of 12 comparisons), paired strain BOS038 exhibited no such matches ($P = 0.001$). Among the class III only test strains, strain 1044 matched moderately well the class III controls (5 of 12 comparisons), but paired strain BOS0025 matched the class

TABLE 5. Matching of *papG*-positive test strains to controls according to overall HA phenotype with human, rabbit, and sheep erythrocytes

papG category	Positive control strain(s)	% (within column) of HA phenotype matches for test strain(s) within *papG* category[a]:			
		I plus III	III	II plus III	II
I plus III	J96, BF1023	48[b,c]	23[c]	0[c]	0[c]
III	CL14, CA062 (or U5)[h]	26[d]	71[b,d]	1[d]	0[d]
II plus III	BOS030, CL21	6[e]	3[d]	70[b,e]	22[e]
II	IA2	0[f]	0[f]	23[f]	71[b,f]
	H16	20[g]	3[g]	6[g]	7[b,g]

[a] Data are from 12 matches per strain. Six strains (72 matches) were used for each of the *papG* categories I plus III, II plus III, and II; five strains (60 matches total) were used for category III.
[b] $P < 0.001$ for all overall comparisons of genotype-phenotype correspondence according to *papG* category (i.e., category I plus III versus other categories and category III versus other categories, etc.). $P < 0.01$ for all comparisons within *papG* categories (proportion with concordant phenotype versus proportion with each alternative phenotype).
[c] $P = 0.004$, I plus III versus III. $P < 0.001$, I plus III versus II plus III, versus II, or versus all others combined.
[d] $P < 0.001$, III versus all others (singly or combined).
[e] $P < 0.001$, II plus III versus all others (singly or combined).
[f] $P < 0.001$, II versus all others (singly or combined).
[g] $P = 0.003$, I plus III versus III. $P = 0.03$, I plus III versus II plus III or versus II. $P < 0.001$, I plus III versus all others combined.
[h] Strain U5 was substituted for CA062 as a second class III control on days 3 to 6 due to sparse HA with CA062.

III controls exclusively (12 of 12 comparisons [$P = 0.03$]). Class III test strains BOS117 and CL09 exhibited similar levels of divergence when they were tested in parallel (they matched class III controls in 4 of 12 and 11 of 12 comparisons, respectively [$P = 0.009$]). Among the class II plus III test strains, matches with the class II plus III controls were significantly more frequent for strain CA032 (12 of 12 comparisons) than for paired strain BOS086 (1 of 12 comparisons [$P < 0.001$]) and for strain BOS119 (12 of 12 comparisons) than for paired strain BOS098 (3 of 12 comparisons [$P < 0.001$]). Among the class II test strains, a match to either of the class II controls was more common with strain BOS013 (11 of 12 comparisons) than with paired strain H26 (7 of 12 comparisons [$P = 0.03$]), and a match specifically to control strain IA2 was significantly more common with strain CL24 (9 of 12 comparisons) than with paired strain CL01B (4 of 12 comparisons [$P = 0.005$]).

Experiment 3.0: reproducibility of HA pattern assessments. The reproducibility of the phenotype matching system for overall HA patterns with human, rabbit, and sheep erythrocytes (Tables 6 and 7) approximated that of the underlying HA titer assay itself (Table 3). Discrepancies in experiment 3.0 with respect to overall HA pattern matches occurred in 18% of pairwise intraobserver comparisons and were similarly frequent for the three observers (who had considerable, moderate, and little experience with the assay system, respectively) (Table 6). Most intraobserver and interobserver discrepancies were conservative, i.e., they stayed within the same *papG* family (class I plus III and class III only, family A, versus class II plus III and class II only, family B) (Tables 6 and 7). Interobserver discrepancies (Table 7) occurred significantly more often with family A strains (23%) than with family B strains (10%).

Experiment 3.0: day-to-day variability of HA phenotypes. Several test strains exhibited day-to-day shifts in HA pattern during experiment 3.0, such as class III only strain 1044's matching predominantly (5 of 6 comparisons) the class I plus III controls on day 1 (versus 1 of 6 comparisons on day 2 [$P = 0.08$), in contrast to its matching predominantly (5 of 6 comparisons) the class III controls on day 2 (versus 0 of 6 comparisons on day 1 [$P = 0.015$]). Similar shifts were seen among the control strains, as tested in duplicate on six successive days in experiment 3.0 (Table 8). The class I plus III controls failed to exhibit the more intense HA of rabbit erythrocytes seen in experiment 2 (Table 3). Strain J96 did not agglutinate OP_1 cells at all on day 5 (Table 8), and BF1023 gave full HA of A_1P_1 and OP_1 cells on days 1 and 2 but not thereafter, despite equally-high-titer HA (Table 8). The class III strains gave intermittent HA of A_1P_1, OP_1, and rabbit cells and variable intensity HA of sheep cells (Table 8). The class II plus III strains varied with respect to their levels of HA of rabbit cells (full, days 1, 2, and 6; partial, days 3 to 5). Of the class II strains, IA2 gave inconsistent HA of rabbit cells and H16 never did exhibit its HA pattern from experiment 2 (Table 3), instead usually appearing more like one of the family A control strains (Table 8). (Of note, although some of these day-to-day shifts occurred simultaneously in both control strains within a *papG* category, others were specific to individual strains.)

Experiment 3.1: phenotype matching with randomly selected test strains. To more realistically simulate the evaluation of unselected wild-type strains, on two different days eight test strains were selected randomly from the pool (without regard to *papG* category) and matched phenotypically to control strains with respect to their overall HA patterns for human, rabbit, and sheep erythrocytes (as in experiment 3.0) by

TABLE 6. Intraobserver discrepancies in matching of test strains to positive controls according to MT HA phenotype

Observer (no. of comparisons)	% (within row) of intraobserver discrepancies of indicated type with respect to deduced *papG* phenotype of test strains (no. of comparisons)[a]		
	Conservative	Nonconservative	Total
A (46)	5 (11)	2 (4)	7 (15)
B (46)	4 (9)	5 (11)	9 (20)
C (46)	6 (13)	3 (7)	9 (20)
Total (138)	15 (11)	10 (7)	25 (18)

[a] Discrepancies are defined as an individual observer's assignment of a test strain to different *papG* categories in repeat analyses of the same HA titer data. Conservative, within same *papG* family, i.e., class I plus III versus III or II plus III versus II; nonconservative, other combinations. Row percent values reflect the proportion of each observer's paired assessments that yielded a discrepancy. For all comparisons between observers, *P* was less than 0.10.

TABLE 7. Interobserver discrepancies in matching of test strains to positive controls according to MT HA phenotype

papG category of test strain (total no. of comparisons)	% (within row) of interobserver discrepancies of indicated type with respect to deduced *papG* phenotype of test strains (no. of comparisons)[a]		
	Conservative	Nonconservative	Total
I plus III (72)	12 (17)[b]	9 (13)[c]	21 (29)[d,e]
III (60)	6 (10)[b]	4 (7)[c]	10 (17)[d,e]
II plus III (72)	3 (4)[b]	3 (4)[c]	6 (8)[d,e]
II (72)	8 (11)[b]	0[c]	8 (11)[d,e]
Total (276)	29 (11)	16 (6)	45 (16)

[a] Discrepancies are defined as assignments of a test strain to a *papG* category other than that to which the majority of assignments were made the same day for that particular test strain (among six determinations by three observers). Conservative, within the same *papG* family, i.e., category I plus III versus III or II plus III versus II; nonconservative, other combinations. Row percent values reflect the proportion of interobserver comparisons within each *papG* category that yielded a discrepancy.

[b] Versus category I plus III, $P = 0.30$ (III), 0.03 (II plus III), and 0.47 (II).

[c] Versus category I plus III, $P = 0.38$ (III), 0.13 (II plus III), and 0.003 (II).

[d] Versus category I plus III, $P = 0.10$ (III), 0.002 (II plus III), and 0.01 (II).

[e] $P = 0.003$ (I plus III and III combined versus II plus III and II combined).

observers blinded to test strain identity. Reproducibility of global HA pattern matching was better in experiment 3.1 than in experiment 3.0, for both intraobserver discrepancies (4% [2 of 48 comparisons] versus 18% [25 of 138 comparisons] [$P = 0.017$]) and interobserver discrepancies (6% [6 of 96 comparisons] versus 16% [45 of 276 comparisons] [$P = 0.015$]). All intraobserver or interobserver discrepancies involved family A test strains, and five of eight strains were conservative with respect to *papG* family (not shown).

Phenotype-genotype concordance was only slightly lower in experiment 3.1 than in experiment 3.0, both overall (57% [55 of 96 comparisons] versus 67% [185 of 276 comparisons] [$P = 0.11$]) and according to family (84% [81 of 90 comparisons] versus 92% [253 of 276 comparisons] [$P = 0.05$]). As in experiment 3.0, there were examples of statistically significant strain-strain differences with respect to assessed HA phenotype within each *papG* category, as well as significant day-to-day shifts for individual strains (not shown). However, in contrast to experiment 3.0's result for any class I plus III strain, on day 1 of experiment 3.1 two of the class I plus III test strains (518 and 553) matched unequivocally the class II plus III controls (6 of 6 and 5 of 6 assessments, respectively). (On the same day a third class I plus III test strain, BOS038, was assessed exclusively as class III only.) Although in experiment 3.1 the class II test strains always matched a class II control, two (H26 and CL24) matched exclusively class II only control H16, whereas on the same day a third (F14) matched exclusively class II only control IA2. In contrast to class II only control strain H16's tendency in experiment 3.0 to match test strains from family A, in experiment 3.1 all 16 matches with H16 involved test strains from family B (not shown).

DISCUSSION

We rigorously evaluated an MT HA assay and found it to exhibit a high degree of reproducibility of same-day intra- and interobserver results despite blinding of observers to strain and erythrocyte type. We then used the assay to assess the HA phenotypes of wild-type clinical *E. coli* isolates representing all four naturally occurring combinations of the three *papG* alleles, plus a recombinant class III strain. We found that despite statistically significant overall differences in the HA pattern of the four *papG* categories, there was reproducible overlap of phenotypes between class I plus III and class III strains and between class II plus III and class II strains, phenotypic diversity among strains within each *papG* category that were tested on the same day, and day-to-day phenotypic variability for individual strains.

We found that the MT HA assay exhibits significantly better reproducibility of same-day results than we obtained by conventional slide HA assays (24), which are the more commonly used phenotyping methods for extraintestinal pathogenic *E. coli*. When HA titer reproducibility results from the present study are calculated as in our previous study of slide HA assays (A/[total − N]) (24), the MT HA assay's overall reproducibility of same-day intraobserver results for different suspensions was 83% (1,169 of 1,408 comparisons; experiments 1 to 3), versus the slide assay's 39% (50 of 127 comparisons; experiments R2 to R4 [24] [$P < 0.001$]); its reproducibility of same-day interobserver results was 85.8% (398 of 464 comparisons; experiment 1), versus the slide assay's 73% (87 of 120 comparisons; experiments B1 and B2 [24] [$P = 0.001$]). The MT HA assay's reproducibility was high both for individual HA titers (experiments 1 to 3) and for assessment of overall HA patterns with A_1P_1, OP_1, rabbit, and sheep erythrocytes combined (experiment 3).

The MT HA assay's documented reproducibility indicates that a valid method for assessing the HA phenotypes of the P-adhesin variants exists, quite the opposite of our pessimistic conclusion regarding slide HA assays (24). Whereas others have studied uropathogenic *E. coli* adhesins using MT HA assays (20, 45, 55, 56), the present study to our knowledge represents the first formal, quantitative assessment of the performance characteristics of MT HA assays in this application. It supersedes prior work in its use of blinding (56), randomization, replicate determinations, interobserver and intraobserver comparisons, and statistical testing; its detailed assessment of phenotype stability over time; and its use of a diverse sample of wild-type clinical isolates from each *papG* category, with careful exclusion of strains coexpressing NPMR adhesin. In addition, our pattern-matching system (experiment 3) is unique in its use of same-day positive controls for each *papG* category, which compensates for day-to-day phenotypic shifts due to erythrocyte variability (24), and in its use of two endpoints per titration, which enhances discriminating power.

Our findings with respect to the PapG variants' phenotypes challenge several prevailing assumptions, the first being that the three *papG* alleles confer sufficiently distinctive receptor specificities that they can be distinguished phenotypically based on differential HA patterns of human, rabbit, and sheep erythrocytes (11, 25, 60). We found that in the combinations in which the three *papG* alleles occur in nature, they do not confer sufficiently distinct HA phenotypes for accurate stratification of strains according to *papG* allele genotype. Although differing significantly from one another in the aggregate, the four *papG* categories overlapped substantially with respect to types of erythrocytes agglutinated and relative HA titers for each erythrocyte type. Control strains from different *papG* categories were often phenotypically indistinguishable (Table 8).

This phenotypic overlap between strains possessing different *papG* variants fits with our previous slide HA assay-based findings for the individual *papG* alleles (24). In the present study we observed pyenotypic merging between *papG* categories I plus III and III only and between categories II plus III and II only but little overlap between these two larger groups. These two aggregate groups, i.e., classes I plus III and III and classes II plus III and II, might be considered phenotypic families,

TABLE 8. Reproducibility and stability of HA phenotype of control *E. coli* strains (experiment 3)

papG category	Positive control strain	Erythrocyte type[b]	Reciprocal HA titer of a duplicate suspension (tube A or B) on day[a]: 1				2				3				4				5				6			
			Tube A		Tube B		Tube A		Tube B		Tube A		Tube B		Tube A		Tube B		Tube A		Tube B		Tube A		Tube B	
			LFP well	FFN well	LFP well	FFN well	LFP well	FFN well	LFP well	FFN well	LFP well	FFN well	LFP well	FFN well	LFP well	FFN well	LFP well	FFN well	LFP well	FFN well	LFP well	FFN well	LFP well	FFN well	LFP well	FFN well
I plus III	J96	A	<1	8	<1	8	<1	9	<1	9	<1	6	<1	7	<1	8	<1	8	<1	7	<1	7	<1	8	<1	8
		O	<1	8	<1	8	<1	8	<1	8	<1	6	<1	6	<1	8	<1	8	–	–	–	–	<1	8	<1	8
		R	<1	8	<1	7	<1	7	<1	7	<1	5	<1	6	<1	7	ND	ND	<1	5	<1	6	<1	7	<1	7
		S	<1	8	<1	8	<1	8	<1	8	<1	7	<1	8	<1	9	ND	ND	<1	6	<1	8	1	9	<1	8
	BF1023	A	3	7	2	6	3	7	3	7	<1	6	<1	5	<1	7	<1	6	<1	6	<1	8	<1	6	<1	6
		O	3	7	2	6	2	7	3	7	<1	6	<1	5	1	7	<1	6	<1	6	<1	6	<1	6	<1	6
		R	1	5	<1	4	1	5	2	6	<1	5	<1	5	<1	6	<1	6	<1	5	<1	7	<1	6	<1	5
		S	1	5	<1	4	1	5	1	5	<1	4	<1	4	<1	6	<1	6	<1	5	<1	7	<1	6	<1	6
III	CL14	A	<1	5	<1	5	<1	6	<1	6	<1	6	<1	6	–	–	–	–	<1	6	<1	6	<1	7	<1	6
		O	<1	5	<1	5	<1	5	<1	5	<1	5	<1	6	–	–	<1	2	<1	6	<1	5	<1	6	<1	6
		R	2	6	2	6	<1	5	<1	6	<1	6	<1	6	<1	5	<1	5	<1	6	<1	7	3	6	3	7
		S	2	7	4	7	<1	6	1	6	<1	6	<1	7	<1	4	<1	4	<1	6	<1	8	7	10	7	10
	CA062 or U5[c]	A	<1	2	–	–	–	–	–	–	–	–	–	–	<1	2	–	–	–	–	<1	5	<1	2	<1	2
		O	–	–	–	–	–	–	–	–	–	–	–	–	<1	2	–	–	–	–	–	–	<1	2	<1	2
		R	<1	2	–	–	1	2	–	–	<1	6	<1	5	<1	5	<1	5	<1	5	–	–	–	–	–	–
		S	<1	5	<1	4	<1	5	<1	5	1	5	1	5	<1	5	<1	5	<1	4	1	4	3	6	2	5
II plus III	BOS030	A	6	10	6	9	8	10	8	10	7	10	7	9	7	9	7	9	6	9	6	10	8	10	ND	ND
		O	6	10	7	10	7	9	7	9	7	9	6	9	7	10	8	10	6	9	5	8	8	10	ND	ND
		R	1	4	1	4	1	4	1	3	<1	3	<1	3	<1	2	<1	3	1	3	<1	4	1	3	ND	ND
		S	3	6	3	6	3	6	3	6	3	6	3	5	3	6	3	6	2	6	3	6	4	7	ND	ND
	CL21	A	7	10	6	9	7	9	6	9	5	8	5	8	7	9	7	9	6	8	6	8	6	9	7	9
		O	7	10	6	9	6	9	5	9	5	8	5	8	7	9	7	9	6	8	6	8	6	8	6	8
		R	1	3	1	3	1	3	1	3	<1	5	<1	4	<1	3	<1	3	<1	3	<1	3	1	3	<1	4
		S	3	7	3	7	3	6	3	6	3	6	3	6	3	6	3	6	2	6	3	6	2	7	4	7
II	IA2	A	6	9	6	8	6	9	7	10	5	8	5	8	5	8	5	8	7	9	6	8	9	10	8	10
		O	7	9	7	9	6	9	6	9	5	8	5	8	7	9	7	9	7	9	5	7	9	10	8	10
		R	<1	2	<1	2	<1	2	<1	2	–	1	<1	2	–	–	–	–	–	–	<1	5	–	–	–	–
		S	<1	5	<1	5	1	5	2	5	<1	5	<1	6	<1	6	<1	6	<1	6	<1	5	<1	7	<1	6
	H16	A	<1	8	<1	9	<1	9	<1	9	<1	8	<1	6	<1	8	<1	7	<1	7	<1	7	6	9	<1	9
		O	<1	8	<1	8	<1	9	<1	9	<1	8	<1	6	<1	8	<1	8	<1	8	<1	7	6	9	<1	9
		R	<1	5	<1	2	–	–	–	–	<1	6	<1	5	<1	2	–	–	<1	6	<1	7	–	–	–	–
		S	<1	6	<1	6	1	5	1	5	<1	7	<1	6	<1	6	<1	6	<1	6	<1	6	3	6	<1	5

[a] ND, not done; –, no agglutination.
[b] A and O, human A_1P_1 and OP_1; R, rabbit; S, sheep.
[c] As the second class III control, CA062 was used on days 1 and 2 and U5 was used on days 3 to 6.

internally similar but usually dissimilar to one another. A possible explanation for phenotypic convergence between *papG* categories I plus III and III or between II plus III and II is phase variation (37, 45, 62), with decreased or absent expression by dual-allele strains of one allele.

A second common assumption challenged by our findings is that *papG* genotype equates with phenotype, i.e., that strains of the same genotype have a uniform and stable MR adherence phenotype (11, 25, 60). Corollaries are that phenotype assessments not only are definitive for a given strain but also can be generalized to others of the same *papG* genotype (12, 60, 61) and that phenotype testing may even be dispensable (8, 52). We observed substantial diversity of phenotype within each *papG* category, even for the pure genotypes (classes II only and III only), where differential expression of *papG* alleles of alternate classes should not cause shifts in HA patterns. In addition, there was substantial day-to-day variability of phenotype for individual strains, including the class II only and class III only strains. That these findings were not artifactual is suggested by their reproducibility in replicate, blind determinations by the same observer (experiments 2 and 3) and different observers (experiment 3).

This reproducible phenotypic diversity among strains with the same *papG* allele(s) (and no NPMR adhesin), even when they are tested on the same day and with the same reagents, is consistent with the diversity of agglutination patterns reported among class III only strains (13) and extends this phenomenon to include the other *papG* categories. Such diversity may arise from subtle variations in receptor specificity within each *papG* category due to sequence differences within *papG* (42). There

may also be strain-strain differences in P-fimbrial expression not involving PapG, unrecognized secondary adhesins, or differences in nonadhesin background properties.

Some strains clearly changed their phenotype considerably from day to day, even when other strains tested in parallel with the same reagents did not (e.g., Table 8), which argues against instability of erythrocytes as an explanation. For dual-allele strains (classes I plus III and II plus III), such shifts may occur by alternating expression of alleles of different *papG* classes (37, 45, 46, 62). A similar process may also occur in single-allele strains (class III only or II only), if multiple copies of *pap* are present (1, 10, 51), that contain subtly different versions of *papG*, albeit of the same class.

The observed phenotypic heterogeneity within *papG* categories suggests that in epidemiological studies phenotypes may need to be directly assessed, since phenotype presumably is more closely related to pathogenesis than is genotype but cannot be predicted reliably based on genotype. Additionally, conclusions regarding *papG*-specific adherence phenotypes derived from the study of single representatives of a particular *papG* category should be validated in a larger population. (Of note, since we tested only eight representatives of each *papG* category, broad generalization from our data may be premature and additional strains should be studied.) Finally, the day-to-day shifts in relative intensity of HA of human, rabbit, and sheep erythrocytes seen with individual strains argue that one day's HA testing is insufficient to define a given strain's P-adhesin-based phenotypic repertoire.

A third prevalent assumption regarding the *papG* variants which is challenged by our findings is that phenotypes associated with *papG* alleles are best studied for each allele in isolation, preferably with the use of recombinant strains (12, 36, 60, 61). We found that each of the two naturally occurring multiple allele *papG* categories (I plus III and II plus III) exhibits a characteristic range of HA phenotypes that in the aggregate differ significantly from the phenotypes of the single-allele *papG* categories (III only and II only). Thus, since both class I plus III and class II plus III strains are significant pathogens (13, 21, 23), the phenotypes associated with these combined *papG* alleles merit study.

In addition, a recombinant strain expressing J96's class III *papG* allele exhibited a reproducibly different HA phenotype than did wild-type class III strains, which calls into question the generalizability of previous in vitro observations regarding the class III *papG* variant derived through study of this or similar recombinant strains (12, 29, 30, 60, 61). Simple quantitative differences in the level of P-adhesin expression between recombinant and wild-type strains does not seem to explain our findings, since the recombinant class III only strain exhibited a selective HA titer increase for sheep erythrocytes, rather than the across-the-board increases for all cell types that would be expected from enhanced expression alone. This strain's outlier phenotype may be due instead to actual receptor specificity differences or to other factors such as host strain background. Whatever the explanation, our findings suggest that the class III recombinant strain may not be a reliable surrogate for wild-type class III strains for in vitro assays or in vivo models (38). Alternative approaches, such as knockout mutants of wild-type strains (44, 53), might allow more valid assessments of the receptor specificities and adherence phenotypes of allele III.

(Of note, no class I or II recombinant strain was included in present study because no phenotypic discrepancies between wild-type and recombinant strains for these adhesins have been reported in the literature. There is no known wild-type class I only strain against which a class I recombinant strain can be compared, and in our previous study of slide HA assays, a class II recombinant strain and its wild-type parent exhibited similar HA patterns with diverse erythrocytes [24].)

The similar behaviors of human A_1P_1 and OP_1 erythrocytes indicate that these cells give redundant information in the MT HA assay: either alone would suffice. The absence of the ONAP phenotype (as previously described for some class III strains) (35, 36, 57) fits with the concept that globo-A is not a necessary receptor on human erythrocytes for the class III adhesin and that alternative broadly prevalent receptors (such as Luke antigen, or stage-specific embryonic antigen 4) are sufficient (29). Our findings also reconfirm that the class III adhesin does not agglutinate only sheep or canine erythrocytes (24, 35). These observations are consistent with reports that the class III *papG* variant predominates among cystitis isolates from children (13) and women (22) and can be as prevalent as the class II variant among certain bacteremia isolates (17). In contrast, they conflict with the concept that class III strains narrowly target dogs (60) or humans of the A (36) or A_1 secretor (12) phenotypes. Further study is needed of the clinical implications of class III strains' diverse and variable adherence capabilities (14).

In summary, we found that individual HA titers for human, rabbit, and sheep erythrocytes, as well as overall HA patterns for these erythrocyte types combined, can be reproducibly assessed for P-fimbriated *E. coli* by an MT HA assay. Wild-type clinical isolates exhibit reproducible diversity of HA phenotypes both between and within the four naturally occurring *papG* categories, day-to-day phenotypic variability for individual strains, and phenotypic overlap between class I plus III and class III only strains and between class II plus III and class II only strains. The biological basis for and epidemiological implications of these findings remain to be determined.

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