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

JAMES R. JOHNSON,* JENNIFER J. BROWN, AND PARVIA AHMED

*Medicine Service, Veterans Affairs Medical Center, and Department of Medicine, University of Minnesota, Minneapolis, Minnesota*

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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 A1P1 and OP1, 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 I and II 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-
crepancies between recombinant and wild-type class III strains (12, 35), recombinant clone III strain PS78-54 (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 II were j96-193f (5′-CTGGCTGTCAGGCTGGAATTT-3′) and j96-655r (5′-TGG CATECCCCAACTATCG-3′) (461-bp product), primers for allele II were i2a-383f (5′-GGGATAGCGGCCGCTTGTAG-3′) and i2a-572r (5′-CCGCGCC CAAAATGACTCG-3′) (190-bp product), and primers for allele III were prs-198f (5′-GGCCTTGCAATGATTACTCTGG-3′) and prs-455r (5′-CCACAA AATGACAATCGCAG-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).

**Table 1. Characteristics of the three PapG variants, as reported in the literature**

| 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              | papG396         | Diverse extraintestinal infections in humans (single clonal group) | Ceramide trihexoside (Gb3Ose-Cer)         | Human (including O and P, but not p) and rabbit, not dog or sheep | No                                      | No                                      | P            |
| II pap and others  | papGIA2         | Extraintestinal infections in humans, pyelonephritis and bacteremia more than cystitis | Globoside (Gb3Ose-Cer)                    | Human (including O, +/- p, or p; +/- sheep and rabbit) | Yes                                     | Yes                                     | P; F7        |
| III pap-2 = prs  | prsG194         | Canine UTI, human cystitis more than pyelonephritis, A1 secretors?, Luke + humans | Forssman glycolipid (Gb3Ose-Cer), globo-A?, stage-specific embryonic antigen-4 (Luke antigen) | Dog, sheep, +/- human (A2, A2, A2, O2, not p), not rabbit | +/-                                      | +/- ONAP?     |

* 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 papG396, papGIA2, and prsG194 are not according to standard conventions but appear in the literature as such. Gb3Ose-Cer, Gb3Ose-Cer, and Gb3Ose-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.

**HA assay reproducibility.** HA assay 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 (for LFP wells), or that were both > 12 (for FFN wells) were said to agree (A); those that differed by ≥ 2 (for LFP wells) were said to disagree. Comparisons were said to be indeterminate when one titrer was 1 or 12 and the other was 1 < 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 A1P1 and p erythrocytes with non-P adhesins, microscope slide HA assays (24) were used to determine each strain's mannose-resistant HA (MRHA) titer for human A1P1 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).

**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. Each control strain was amplified daily until all test strains had been assayed on two days each. Each day, duplicate dilution series of each test and control strain were titrated against (known) A1P1, A1P0, and P erythrocytes. Observers blinded to 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)
individual observers’ repeat assessments of the same strain (intraobserver) and (ii) results between observers (interobserver).

**Statistical methods.** Comparisons of proportions were tested by a χ² 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 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 as to the presence of HA occurred in only 4 to 7% of comparisons (Table 3).

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

| *papG* category | Strain | Syndrome            | Locationa | Serotypeb | MRHA phenotypec | Use of strain: | Reference(s) |
|-----------------|--------|---------------------|-----------|-----------|-----------------|---------------|--------------|
|                 |        |                     |           |           |                 | Expt 1 Screen| Expt 2 and 3 |
| I plus III      | J96    | Pyelonephritis      | Seattle   | O4:H5     | P                |               |              |
|                 | BF1040 | Cystitis (adult)    | Ann Arbor | O4:H3     | NPMR            |               |              |
|                 | CP9    | Bacteremia          | Bethesda  | O4:K10, K54/96:H5 | P        | +             | 10           |
|                 | BF9043 | Cystitis (adult)    | Ann Arbor | O4:H3     | NPMR            | +             | 21           |
|                 | R28    | Cystitis (adult)    | Seattle   | O4:H3     | NPMR            | +             | 21, 54       |
|                 | 5184   | Cystitis (adult)    | Seattle   | O4:K10, K54/96:H5 | P        | +             | 55, 59       |
|                 | 5534   | 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:H5 | P        | +             | 43           |
| III             | U5     | Urosepsis           | Seattle   | O6:K5:H7  | P                | +             | 18           |
|                 | 2H25   | Urosepsis           | Seattle   | O18ac:K1:H7 | NPMR   | +             | 18           |
|                 | CA062  | Bacteremia          | Long Beach| O4:K3:H5  | P                |               |              |
|                 | BOS117 | Bacteremia          | Boston    | O6:K11,H18| P                |               |              |
|                 | CL14A  | Cystitis (child)    | Cleveland | O6:H   | P                |               |              |
|                 | 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:K+H+   | 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                 | Iowa City | O6:H    | ps-NPMR         | +             | 4             |
|                 | H16    | Urosepsis           | Seattle   | O1:K1:H7 | P                | +             | 18           |
|                 | H26    | Urosepsis           | Seattle   | O6:K2:H1 | P                | +             | 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 A1P1 erythrocytes but not p erythrocytes; NPMR, PEW-resistant HA of both A1P1 erythrocytes and p erythrocytes; ps-NPMR (pseudo-NPMR), PEW-inhibitable HA of both A1P1 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.
fimbriated published the MT HA assay’s reproducibility with wild-type P-

intraobserver results with the same dilution versus other erythrocytes,

other erythrocyte types (78.7 to 93.4% overall). (For sheep 80.1% overall, depending on the type of comparison) than did server and interobserver results in experiment 1.0 (77.2 to 90.8% overall). Reproducibility of results was 90.7% (185 of 204 determinations) overall. To-

were randomly distributed. Reproducibility of intraobserver assessments for duplicate dilution series (analysis 1.0.3) (Table 3) approximated shown). Reproducibility of interobserver assessments of the two observers and for the two endpoints used (not shown).

Factors contributing to the MT HA assay’s observed irreproduc-

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 pre-

pared 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. To-

gather 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 irreproduc-

ability 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 = 0.02 for reproducibility of intraobserver results with the same dilution and P = 0.003 for reproducibility of interobserver results with the same dilution.)

Experiment 2: screening for NPMR adhesins. Having estab-

lished the MT HA assay’s reproducibility with wild-type P-fimbriated E. coli, we next used the assay to assess papG cate-

gory-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-pos-

itive 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 de-

scribed 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. Al-

though the class I plus III strains typically gave only partial HA with any erythrocyte type, two strains (pattern 2) gave full HA of A₁P₁ and OP₁ 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 eryth-

rocytes. Of note, recombinant class III strain P678-54 (pJK102) was a marked outlier among the class III strains, reproducibly exhibiting much-higher-titer HA for sheep eryth-

rocytes (relative to that of any other erythrocyte type) than the

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

| Analysis | Type of comparison | Suspension | Dilution | % of comparisons with indicated status* from 544 pairwise HA titer comparisons | % Reproducibility (A + 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, 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

| papG category | HA pattern | HA titer of: | Type strain | Erythrocyte type | papG |
|---------------|------------|--------------|-------------|-----------------|------|
| I plus III    | 1          |              | A           | J96             | <1   |
|               |            |              | O           | <1              | 6    |
|               |            |              | R           | <1              | 9    |
|               |            |              | S           | <1              | 7    |
|               | 2          |              | A           | BF1023          | 3    |
|               |            |              | O           | 3               | 6    |
|               |            |              | R           | <1              | 9    |
|               |            |              | S           | <1              | 6    |
| III           | 1          |              | A           | BOS117          | <1   |
|               |            |              | O           | <1              | 7    |
|               |            |              | R           | <1              | 5    |
|               |            |              | S           | 5               | 10   |
|               | 2          |              | A           | CL14            | <1   |
|               |            |              | O           | <1              | 6    |
|               |            |              | R           | <1              | 6    |
|               |            |              | S           | <1              | 5    |
| III           | 3          |              | A           | CA062           | –c  |
|               |            |              | O           | –              | –    |
|               |            |              | R           | <1              | 6    |
|               |            |              | S           | <1              | 5    |
| II plus III   | 1          |              | A           | CL21            | 6    |
|               |            |              | O           | 6               | 8    |
|               |            |              | R           | 1               | 4    |
|               |            |              | S           | 3               | 8    |
|               | 2          |              | A           | AFR098          | 6    |
|               |            |              | O           | 6               | 7    |
|               |            |              | R           | <1              | 3    |
|               |            |              | S           | 3               | 9    |
|               | 3          |              | A           | BOS086          | 5    |
|               |            |              | O           | 5               | 7    |
|               |            |              | R           | <1              | 8    |
|               |            |              | S           | 1               | 9    |
| II            | 1          |              | A           | H26             | 8    |
|               |            |              | O           | 8               | 10   |
|               |            |              | R           | <1              | 8    |
|               |            |              | S           | <1              | 5    |
|               | 2          |              | A           | IA2             | 7    |
|               |            |              | O           | 7               | 9    |
|               |            |              | R           | <1              | 6    |
|               |            |              | S           | <1              | 5    |
|               | 3          |              | A           | CL01B           | 6    |
|               |            |              | 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 A1P1; O, human OP1; R, rabbit; S, sheep.

c –, no agglutination.

The O-negative, A-positive (ONAP) phenotype was observed only once in experiment 2. Overall, A1P1 and OP1 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 A1P1 and OP1 cells disagreed (35 of 216 comparisons), in 66% of instances (67% for class III strains only) the reciprocal titer for OP1 cells actually exceeded that for A1P1 cells, reflecting more intense HA of OP1 than of A1P1 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 in a predictable fashion. 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 misclassified 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 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 for both *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 day 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

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, A1P1 and OP1 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 A1P1 and OP1 cells disagreed (35 of 216 comparisons), in 66% of instances (67% for class III strains only) the reciprocal titer for OP1 cells actually exceeded that for A1P1 cells, reflecting more intense HA of OP1 than of A1P1 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 misclassified 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 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 day 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 \( \text{papG} \)-positive test strains to controls according to overall HA phenotype with human, rabbit, and sheep erythrocytes

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

\(^a\) Data are from 12 matches per strain. Six strains (72 matches) were used for each of the \( \text{papG} \) 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 \( \text{papG} \) category (i.e., category I plus III versus other categories and category III versus other categories, etc.). \( p < 0.01 \) for all comparisons within \( \text{papG} \) categories (proportion with concordant phenotype versus proportion with each alternative phenotype).

\(^c\) \( p < 0.001 \), 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).

\(^g\) \( p < 0.005 \), I plus III versus II; nonconservative, other combinations. Row percent values reflect the proportion of each observer’s paired assessments that yielded a discrepancy.

\(^h\) 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 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 \( \text{papG} \) 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 OP1 cells at all on day 5 (Table 8), and BF1023 gave full HA of A\(_2\)P\(_1\) and OP\(_1\) cells on days 1 and 2 but not thereafter, despite equally-high-titer HA (Table 8). The class III strains gave intermediate HA of A\(_2\)P\(_1\), 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 \( \text{papG} \) 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 \( \text{papG} \) 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 \( \text{papG} \) 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                        | 15 (11)      | 10 (7)          | 25 (18) |

\(^a\) Discrepancies are defined as an individual observer’s assignment of a test strain to different \( \text{papG} \) categories in repeat analyses of the same HA titer data. Conservative, within same \( \text{papG} \) 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 (no. of comparisons) | % (within row) of interobserver discrepancies of indicated type with respect to deduced papG phenotype of test strains (no. of comparisons)* | Conservative | Nonconservative | Total |
|-------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|-------------|----------------|-------|
| I plus III (72) | 12 (17a) | 9 (13c) | 21 (29) | 21 (29a) | 21 (29) |
| III (60) | 6 (10b) | 4 (7f) | 10 (17) | 10 (17b) | 10 (17) |
| II plus III (72) | 3 (4e) | 3 (4f) | 6 (8) | 6 (8e) | 6 (8) |
| II (72) | 8 (11b) | 0 (0) | 8 (11) | 8 (11b) | 8 (11) |
| Total (276) | 29 (11) | 16 (6) | 45 (16) | 45 (16) | 45 (16) |

* 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.

† Versus category I plus III, P = 0.03 (III), 0.03 (II plus III), and 0.47 (II).
‡ Versus category I plus III, P = 0.38 (III), 0.33 (II plus III), and 0.003 (II).
§ Versus category I plus III, P = 0.10 (III), 0.002 (II plus III), and 0.01 (II).
¶ 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 interobserver 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 AP1, OP1, 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 | Reciprocal HA titer of a duplicate suspension (tube A or B) on day 4: | 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 | Tube A | Tube B |
| I plus III     | J96                     |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               | A                       |                 | <1    | 8     | 1     | 8     | 1     | 8     | 1     | 8     | 1     | 7     | 1     | 7     | 1     | 8     | 1     | 7     |
|               | O                       |                 | <1    | 8     | 1     | 8     | 1     | 8     | 1     | 8     | 1     | 8     | 1     | 8     | 1     | 8     | 1     | 7     |
|               | R                       |                 | <1    | 8     | 1     | 7     | 1     | 7     | 1     | 5     | 1     | 6     | 1     | 7     | ND    | ND    | <1    | 5     | 1     | 6     | 1     | 7     |
|               | S                       |                 | <1    | 8     | 1     | 8     | 1     | 8     | 1     | 8     | 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     | 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     |
|               | R                       |                 | 1     | 5     | 1     | 4     | 1     | 5     | 2     | 6     | 1     | 5     | 1     | 5     | 1     | 6     | 1     | 6     | 1     | 5     | 1     | 7     | 1     | 6     |
|               | S                       |                 | 1     | 5     | 1     | 4     | 1     | 5     | 1     | 5     | 1     | 4     | 1     | 4     | 1     | 6     | 1     | 6     | 1     | 5     | 1     | 7     | 1     | 6     |
|               |                         |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| III           | CL14                    |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               | A                       |                 | <1    | 5     | 1     | 5     | 1     | 6     | 1     | 6     | 1     | 6     | 1     | 6     | 1     | 7     | 1     | 6     | 1     | 6     | 1     | 7     | 1     | 6     |
|               | O                       |                 | <1    | 5     | 1     | 5     | 1     | 5     | 1     | 5     | 1     | 5     | 1     | 6     | 1     | 7     | 1     | 6     | 1     | 6     | 1     | 6     | 1     | 6     |
|               | R                       |                 | 2     | 6     | 2     | 6     | 1     | 5     | 1     | 6     | 1     | 6     | 1     | 6     | 1     | 5     | 1     | 5     | 1     | 6     | 1     | 6     | 1     | 6     |
|               | S                       |                 | 2     | 7     | 4     | 7     | 1     | 6     | 1     | 6     | 1     | 6     | 1     | 7     | 1     | 4     | 1     | 4     | 1     | 6     | 1     | 8     | 7     | 10    |
|               | CA062 or U5°             |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               | A                       |                 | <1    | 2     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               | O                       |                 | <1    | 2     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               | R                       |                 | <1    | 2     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               | S                       |                 | <1    | 5     | 1     | 5     | 1     | 5     | 1     | 5     | 1     | 5     | 1     | 5     | 1     | 5     | 1     | 4     | 1     | 4     | 1     | 3     | 6     | 2     |
|               |                         |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| II plus III   | BOS030                  |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               |                         |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               | CL21                    |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               |                         |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| II            | IA2                     |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               |                         |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|               | H16                     |                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |

* ND, not done; –, no agglutination.

a A and O, human A1P2 and OP1; R, rabbit; S, sheep.

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).
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 the same reagents did not (e.g., Table 8), which argues against instability of erythrocytes as an explanation. For dual-allele (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 A1P1 and OP1 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 A1 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 II only strains. The biological basis for and epidemiological implications of these findings remain to be determined.

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REFERENCES

1. Arthur, M., C. Campanelli, R. D. Arbeit, C. Kim, S. Steinbach, C. E. Johnson, R. H. Rubin, and R. Goldstein. 1989. Structure and copy number of gene clusters related to the pap P-adhesin operon of uropathogenic Escherichia coli. Infect. Immun. 57:314–321.

2. Arthur, M., C. E. Johnson, R. H. Rubin, R. D. Arbeit, C. Campanelli, C. Kim, S. Steinbach, M. Agarwal, R. Wilkinson, and R. Goldstein. 1989. Molecular epidemiology of adhesin and hemolysin virulence factors among uropathogenic Escherichia coli. Infect. Immun. 57:303–313.

3. Bock, K., M. E. Breimer, A. Brignole, G. C. Hansson, K.-A. Karlsson, G. Larson, H. Leffler, B. E. Samuelsson, N. Strömberg, C. Svanborg Edén, and J. Thorin. 1985. Specificity of binding of a strain of uropathogenic Escherichia coli to Galα1-3Gal-containing glycosphingolipids. J. Biol. Chem. 260:8545–8551.

4. Clegg, S. 1982. Cloning of genes determining the production of mannose-resistant fimbriae in a uropathogenic strain of Escherichia coli belonging to serogroup O6. Infect. Immun. 38:739–744.

5. de Man, P., B. Cedergren, S. Enerback, A. C. Larsson, H. Leffler, A. L. Lundell, B. Nilsson, and C. Svanborg Edén. 1987. Receptor-specific agglutination tests for detection of bacteria that bind globo-series glycolipids. J. Clin. Microbiol. 25:401–406.

6. Donnenberg, M. S., and R. A. Welch. 1996. Virulence determinants of uropathogenic Escherichia coli, p. 135–174. In H. L. T. Mobley and J. W. Warren (ed.), Urinary tract infections: molecular pathogenesis and clinical management. ASM Press, Washington, D.C.
27. García-Martínez, J., A. J. Martínez-Murcia, F. Rodríguez-Valera, and A. Zorruño. 1996. Molecular evidence supporting the existence of two major groups in uropathogenic Escherichia coli. FEMS Microbiol. Lett. 14:231–244.
28. Johnston, L. M., K. Plos, B.-I. Marklund, and C. Svanborg. 1997. Clonal distribution of the O-galactosyl-1-4-galactosyl-specific adhesin (P adhesin) from fimbriated Escherichia coli. Infect. Immun. 65:76–81.
29. Hull, S. L., and R. A. Hull. 1989. Linkage and duplication of copies of genes encoding P fimbriae and hemolysin in the chromosome of a uropathogenic Escherichia coli isolate. In E. H. Kass and C. Svanborg Edén (ed.), Host-foreign interactions in urinary tract infections. The University of Chicago Press, Chicago, Ill.
30. Hultgren, S. J., S. Abraham, M. Caparon, P. Falk, J. W. St. Germe, and S. Normark. 1993. Pili and non-pili bacterial adhesins: assembly and function in cell recognition. Cell 73:887–901.
31. Johanson, T. K., V. Väisänen, P. Kallio, E.-L. Nurminaho-Lassila, H. Ranta, A. Siitonen, J. Eho, B. S. Svenson, and C. Svanborg Edén. 1982. Role of pili in the adhesion of Escherichia coli to human urinary tract epithelial cells. Scand. J. Infect. Dis. 33:26–31.
32. Karr, J., P. Baker, F. Palm, R. Hull, S. Hull, J. Karr, H. Leffler, C. Svanborg Edén, and G. Larson. 1989. Binding specificities of wild-type and cloned Escherichia coli strains that recognize globo-A. Infect. Immun. 57:3349–3359.
33. Karr, W. S., M. L. Greenberg, and W. E. Stamm. 1984. Escherichia coli strains causing urosepsis. J. Infect. Dis. 150:589–602.
34. Karr, J. F., B. Nowicki, L. D. Truong, R. A. Hull, J. J. Mould, and S. L. Hull. 1990. Pap-2-encoded fimbriae adhere to the P blood group-related glycosphingolipid stage-specific embryonic antigen 4 in the human kidney. Infect. Immun. 58:4055–4062.
35. Karr, J. F., B. Nowicki, L. D. Truong, R. A. Hull, and S. L. Hull. 1989. Purified P fimbriae from two cloned gene clusters of a single pyelonephri- togenic strain adhere to unique structures in the human kidney. Infect. Immun. 57:3594–3600.
36. Korhonen, T. K., V. Väisänen, P. Kallio, E.-L. Nurminaho-Lassila, H. Ranta, A. Siitonen, J. Eho, B. S. Svenson, and C. Svanborg Edén. 1982. The role of pili in the adhesion of Escherichia coli to human urinary tract epithelial cells. Infect. Immun. 37:286–291.
37. Leffler, H., and C. Svanborg Edén. 1980. Chemical identification of a glyco- sphingolipid receptor for Escherichia coli attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. FEMS Microbiol. Lett. 8:127–134.
38. Lindberg, F. P., B. Lund, and S. Normark. 1984. Genes of pyelonephrito- genic Escherichia coli required for digalactoside-specific agglutination of Menigh cells. EMBO J. 3:1617–1173.
39. Lindstedt, R., N. Baker, P. Falk, R. Hull, S. Hull, J. Karr, H. Leffler, C. Svanborg Edén, and G. Larson. 1989. Binding specificities of wild-type and cloned Escherichia coli strains that recognize globo-A. Infect. Immun. 57:3349–3359.
40. Lindstedt, R., G. Larson, P. Falk, U. Jodal, H. Leffler, and C. Svanborg Edén. 1991. The receptor repertoire defines the host range for attaching Escherichia coli strains that recognize globo-A. Infect. Immun. 59:1086–1102.
41. Low, D., E. N. Robinson, Jr., Z. A. McGee, and S. Falkow. 1987. The frequency of expression of pyelonephritis-associated pili is under regulatory control. Mol. Microbiol. 1:335–346.
42. Lund, B., F. Lindberg, M. Bäga, and S. Normark. 1985. Globo-side-specific adhesions of uropathogenic Escherichia coli are encoded by similar trans- conformatable gene clusters. Bacteriol. 162:1293–1301.
43. Lund, B., F. Lindberg, B.-I. Marklund, and S. Normark. 1987. The PapG protein is the α-(1→4)-β-D-glucopyranose-binding adhe- sin of uropathogenic Escherichia coli. Proc. Natl. Acad. Sci. USA 84:5989– 5907.
44. Lund, B.-I. Marklund, N. Strömberg, F. Lindberg, K.-A. Karlsson, and S. Normark. 1988. Uropathogenic Escherichia coli can express serologically identical pili of different receptor binding specificities. Mol. Microbiol. 2:255–263.
45. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. Manioudi, B. L., J. M. Tenen, C. Garcia, A. Hamers, M. Baga, F. Lindberg, W. Gaaster, and S. Normark. 1992. Horizontal gene transfer of the Esche- richia coli pap and prs pili operons as a mechanism for the development of tissue-specific adhesive properties. Mol. Microbiol. 6:2225–2242.
47. Maslow, J. N., T. S. Whittam, C. F. Gilks, R. A. Wilson, M. E. Mulligan, K. S. Adams, and R. D. Arbeit. 1995. Clonal relationships among bloodstream isolates of Escherichia coli. Infect. Immun. 63:2409–2417.
48. Moberly, H. L. T., K. G. Jarvis, P. J. Edword, D. I. Whittle, C. V. Lockatell, R. G. Russell, D. E. Johnson, M. S. Donnemager, and J. W. Warren. 1993. Isolates of pyelonephritis due to Escherichia coli: the role of the ααββ-glucoside binding adhesin in the susceptibility of a wild-type strain. Mol. Microbiol. 10:143–155.
49. Nowicki, B., M. Rhen, V. Väisänen-Rhen, A. Pere, and T. K. Korhonen. 1984. Immunofluorescence microscopy of fimbrial phase variation in Escherichia coli K571. Bacteriol. 160:691–695.
50. Nowicki, B., M. Rhen, V. Väisänen-Rhen, A. Pere, and T. K. Korhonen. 1985. Fractionation of a bacterial cell population by absorption to erythrocytes and yeast cells. FEMS Microbiol. Lett. 26:35–40.
51. Orino, K., and M. Naiki. 1990. Two kinds of P-fimbriated variants of uro- pathogenic Escherichia coli recognizing Forssman glycosphingolipid. Micro- biol. Immunol. 34:607–615.
52. Orskov, F., and I. Orskov. 1984. Serotyping of Escherichia coli. Methods Microbiol. 14:43–111.
53. Orskov, I., A. Birch-Andersen, P. J. Duguid, J. Stenderup, and F. Orskov. 1985. An adhesive protein capsule of Escherichia coli. Infect. Immun. 47: 991–1000.
54. Otto, G., T. Sandberg, B. I. Marklund, P. Ullery, and C. Svanborg Edén. 1993. Virulence factors and pap genotype in Escherichia coli isolates from women with acute pyelonephritis, with or without bacteremia. Clin. Infect. Dis. 17:448–455.
55. Plos, K., T. Carter, S. Hull, R. Hull, and C. Svanborg Edén. 1990. Frequency and organization of pap homologous DNA in relation to clinical origin of uropathogenic Escherichia coli. J. Infect. Dis. 161:518–524.
56. Plos, K., H. Connell, U. Jodal, B. I. Marklund, S. Mårild, B. Wettergren, and C. Svanborg Edén. 1995. Intestinal carriage of P-fimbriated Escherichia coli and the susceptibility to urinary tract infection in young children. J. Infect. Dis. 171:625–631.
57. Roberts, A. J., B.-I. Marklund, D. Dver, D. Haslam, M. B. Kaack, G. Baskin, M. Louis, R. Mollby, J. Winberg, and S. Normark. 1994. The Galα1-4Gal- specific tip adhesin of Escherichia coli P-fimbriae is needed for pyelonephritis.
to occur in the normal urinary tract. Proc. Natl. Acad. Sci. USA 91:11889–11893.

54. Russo, T. A., Y. Liang, and A. S. Cross. 1994. The presence of K54 capsular polysaccharide increases the pathogenicity of Escherichia coli in vivo. J. Infect. Dis. 169:112–118.

55. Russo, T. A., A. Stapleton, S. Wenderoth, T. M. Hooton, and W. E. Stamm. 1995. Chromosomal restriction fragment length polymorphism analysis of Escherichia coli strains causing recurrent urinary tract infections in young women. J. Infect. Dis. 172:440–445.

56. Schulz, K. F., I. Chalmers, R. J. Hayes, and D. G. Altman. 1995. Empirical evidence of bias: dimensions of methodological quality associated with estimates of treatment effects in controlled trials. JAMA 273:408–412.

57. Senior, D., N. Baker, B. Cedergren, P. Falk, G. Larson, R. Lindstedt, and C. Svanborg Eden. 1988. Globo-A—a new receptor specificity for attaching Escherichia coli. FEBS Lett. 237:123–127.

58. Stapleton, A., T. M. Hooton, C. Fennell, P. L. Roberts, and W. E. Stamm. 1995. Effect of secretor status on vaginal and rectal colonization with fim-briated Escherichia coli in women with and without recurrent urinary tract infection. J. Infect. Dis. 171:717–720.

59. Stapleton, A., S. Moseley, and W. E. Stamm. 1991. Urovirulence determinants in Escherichia coli isolates causing first-episode and recurrent cystitis in women. J. Infect. Dis. 163:773–779.

60. Strömberg, N., B. I. Marklund, B. Lund, D. Ilver, A. Hamers, W. Gaastra, K. A. Karlsson, and S. Normark. 1990. Host-specificity of uropathogenic Escherichia coli depends on differences in binding specificity to Galα1-4Gal-containing isoreceptors. EMBO J. 9:2001–2010.

61. Strömberg, N., P. G. Nyholm, I. Pascher, and S. Normark. 1991. Saccharide orientation at the cell surface affects glycolipid receptor function. Proc. Natl. Acad. Sci. USA 88:9340–9344.

62. van der Woude, M. W., B. A. Braaten, and D. A. Low. 1992. Evidence for global regulatory control of pilus expression in Escherichia coli by Lrp and DNA methylation: model building based on analysis of pap. Mol. Microbiol. 6:2429–2435.