Use of Miniaturized Protein Arrays for *Escherichia coli* O Serotyping

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Serological typing of *Escherichia coli* O antigens is a well-established method used for differentiation and identification of O serotypes commonly associated with disease. In this feasibility study, we have developed a novel somatic antibody-based miniaturized microarray chip, using 17 antisera, which can be used to detect bound whole-cell *E. coli* antigen with its corresponding immobilized antibody, to assess the feasibility of this approach. The chip was tested using the related 17 control strains, and the O types found by the microarray chip showed 100% correlation with the O types found by conventional typing. A blind trial was performed in which 100 *E. coli* isolates that had been O serotyped previously by the conventional assay were tested by the array approach. Overall, the O serotypes of 88% of isolates were correctly identified by the microarray method. For several isolates, ambiguity of O-type designation by microarray arose due to increased sensitivity of this method, allowing signal intensities of cross-reactions to be quantified. Investigation of discrepancies between conventional and microarray O serotyping indicated that some isolates upon storage had become untypeable and, therefore, gave poor signal intensity when tested by the microarray or retested by conventional means. For all 20 serotype O26 and O157 isolates, the apparent discrepancy in O serotyping was analyzed further by a third independent test, which confirmed the microarray results. Therefore, the use of miniaturized protein arrays increases the speed and efficiency of O serotyping in a cost-effective manner, and these preliminary findings suggest the microarray approach may have a higher accuracy than those of traditional O-serotyping methods.

*Escherichia coli* bacteria of many O-antigen groups commonly occur as commensals in the colons of humans and animals. Nevertheless, many O serotypes are associated with a number of disease syndromes in both humans and animals, such that a subset of serotypes, often referred to as pathotypes, cause diarrhea, urinary tract infections, meningitis, or systemic disease (11, 18). Serological O-antigen typing is an established method used in routine laboratories to identify bacteria on isolation from clinical specimens (2, 9, 28). O serotyping has been useful in understanding the epidemiology of infections and allows differentiation between pathotypes. O-serotyping is based on the highly immunogenic variable lipopolysaccharides (LPS) on the bacterial cell surface of which about 170 forms are found in *E. coli* (23, 24, 32). Although O serotyping is a primary diagnostic tool in reference laboratories for identification of clinical and field isolates, these methods are time-consuming and costly and may be subjective due to cross-reactions, especially when polyclonal antisera are used.

In the last decade, microarray technology has become a powerful tool for rapid and parallel measurement of multiple nucleic acid-nucleic acid or protein-protein interactions (10, 33). More recently, protein microarray technology has been developed for the parallel identification, quantification, and functional analysis of different proteins. In principle, these applications will allow the substitution of single-plex systems. However, for acceptance, microarray approaches should meet certain prerequisites, such as robustness, reliability, appropriate pricing, low complexity, lower demands of experimental time and manpower, comparable sensitivity and specificity, and the possibility for high-throughput use (29). One such system, which has been developed recently and designed specifically for routine diagnostic laboratories, is the ArrayTube (AT) platform (12, 14, 15, 21, 26) (CLONDIAG Chip Technologies GmbH, Jena, Germany). These miniaturized arrays are mounted on the bottom of standard 1.5-ml microreaction tubes. Hybridization and analysis are performed using standard laboratory equipment. The hybridization signals are amplified by an enzyme-catalyzed precipitation reaction, and the kinetic measurement of the precipitation reaction at each spot is detected by specific changes in red light transmission, which is recorded using a photomager.

The aim of this study was to prove the principle that miniaturized arrays with immobilized O-antigen-specific antisera may be used to accurately serotype *E. coli* isolates under conditions used in routine diagnostic laboratories. For this feasibility study, 17 O antisera raised against some of the common *E. coli* pathogens associated with disease syndromes in humans (16, 18, 22, 25) and farm animals, such as cattle, sheep, pigs, poultry, and rabbits (1, 3, 7, 13, 16, 20, 30, 31), and contaminated food products (13, 16) were chosen. Approximately 20% of all *E. coli* strains received by the Veterinary Laboratories Agency (VLA) Enteric Bacteriology Reference Unit in the past few years expressed one of the 17 antisera selected for the miniaturized chip, which included important *E. coli* pathogenic serotypes, such as O157 and O26 (11).

MATERIALS AND METHODS

Bacterial strains, serotyping, and latex agglutination. Isolation, purification, and serotyping of *E. coli* strains are routinely done by the VLA Enteric Bacteriology Reference Unit, essentially following the procedures described by Sojka (28) and Guinee et al. (8). All *E. coli* strains included in this work were cultured...
were considered uncertain.

Four spots reacting. For analysis, a mean signal intensity value of all four spots for the majority of arrays performed (for each spot, i.e., probe position on the array. Average extinctions of local IconoClust software, which measures the signal intensity and local background reaction. After 10 min of staining, the resulting pictures were analyzed by the instructions. A picture was taken every minute for 10 min to record the staining steps. horseradish peroxidase conjugated with streptavidin (CLONDIAG) was added to start the staining reaction.

The latex agglutination assay was performed on cultures according to the manufacturer’s protocol (Oxoid).

Array design. The arrays are of 3- by 3-mm size and have an active surface area of 2.4 by 2.4 mm mounted onto the bottom of standard 1.5-mL microreaction tubes. Antisera (protein mixtures) were used at a final concentration of 0.5 mg/ml in 1× PBS buffer (pH 7.0) and spotted using a Microgrid II spotting machine (Genomic Solutions Ltd., United Kingdom). All probes, spotted redundantly four times on the array, were covalently coupled to the array surface by synthesizing an epoxy layer on a glass surface of the array (Fig. 1) (15, 33). After production, arrays were inserted into ArrayTube reaction vials (CLONDIAG) and used for serotyping assays. Ten control spots comprising of 5’ amino and 3’ biotin modified oligonucleotides (CLONDIAG) were also included.

Serotyping assay for miniaturized arrays. Prior to reaction with E. coli cells, each ArrayTube was washed three times with 500 μl of PBS-Tween wash buffer (1× PBS with 0.01% Tween 20 [PBS-Tween]). This was followed by a 15-min blocking step with 100 μl of freshly prepared 10% fetal calf serum (FCS) (10 μl 100% FCS plus 90 μl PBS-Tween) and then a 5-min wash in 500 μl PBS-Tween. All three steps were performed at 23°C, and the mixture was shaken at 550 rpm in a Thermomixer (Eppendorf). An aliquot of E. coli cells (30 to 50 μl) from an overnight LB broth that had been boiled for 1 h was diluted 1:1 in 1× PBS containing 0.01% Tween 20 and 1% FCS (PBS-F-Tween). The diluted culture was added into the AT, incubated at 23°C, and shaken at 400 rpm.

After 30 min of interaction, the sample was pipetted out and the ArrayTube was washed for 5 min in PBS-Tween. Then, 100 μl of anti-E. coli core LPS monomonal antibody WNI222-5 (4, 17), diluted to 52 ng/ml in PBS-F-Tween, was applied for 20 min at 23°C and 400 rpm, followed by two wash steps in PBS-Tween. Subsequently, 100 μl of a secondary biotinlated anti-mouse immunoglobulin G (IgG) (catalog no. B8520; Sigma), diluted 1:10,000 in PBS-F-Tween. Subsequently, 100 μl of anti-E. coli cells, each ArrayTube was washed three times with 500 μl of PBS-Tween wash buffer (1× PBS with 0.01% Tween 20 [PBS-Tween]). This was followed by a 15-min blocking step with 100 μl of freshly prepared 10% fetal calf serum (FCS) (10 μl 100% FCS plus 90 μl PBS-Tween) and then a 5-min wash in 500 μl PBS-Tween. All three steps were performed at 23°C, and the mixture was shaken at 550 rpm in a Thermomixer (Eppendorf). An aliquot of E. coli cells (30 to 50 μl) from an overnight LB broth that had been boiled for 1 h was diluted 1:1 in 1× PBS containing 0.01% Tween 20 and 1% FCS (PBS-F-Tween). The diluted culture was added into the AT, incubated at 23°C, and shaken at 400 rpm.

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Measurements and data analysis. For the array readout measurements, the ArrayTube reading device ATR01 (CLONDIAG) was applied in combination with IconoClust software (CLONDIAG) according to the manufacturer’s instructions. A picture was taken every minute for 10 min to record the staining reaction. After 10 min of staining, the resulting pictures were analyzed by the IconoClust software, which measures the signal intensity and local background for each spot, i.e., position on the array. Average extinctions of local backgrounds were subtracted from average extinctions of spots. We noted that for the majority of arrays performed (>95%), a positive reaction resulted in all four spots reacting. For analysis, a mean signal intensity value of all four spots was used for each O type. Mean values below 0.1 were considered negative, mean values above 0.3 were considered positive, and mean values between 0.1 and 0.3 were considered uncertain.

PCR amplification of somotype O26-specific wzy and wxy genes. For amplification of somotype O26-specific wzy and wxy genes, the primers used were O26 wzy F (5’-GGCCTGCAATTGTTAGTA-3’), O26 wxy R (5’-TTTCCCCCGGAAATTATCCAG-3’), O26 wzy F (5’-TTAATTTGGGGGAAAAAGATG-3’), and O26 wxy R (5’-GACTTCTAGGTGATCCCGGTA-3’). PCRs were set up using Taq polymerase (Promega) and manufacturer’s protocols. The conditions for PCR amplification were as follows: 1 cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s; and 1 cycle at 72°C for 4 min. The PCR products were checked using a 1% agarose gel with subsequent ethidium bromide staining.

FIG. 1. Layout of the miniaturized protein array used in this study. The position of each somatic antibody probe is shaded on the array.

RESULTS

Array design and optimization. The miniaturized microarrays were designed with 17 different somatic antisera that were raised against E. coli pathogenic to both humans and animals. The series of reactions by which E. coli, bound by its somatic O antigen to the corresponding antiserum, was detected are outlined in Fig. 2. The antisera were freeze-dried and resuspended in the PBS spotting solution prior to spotting at the specified concentration (see Materials and Methods). Only highly purified antisera that do not contain high-molecular-weight additives required for storage and are present at concentrations able to form stable covalent interactions between the amino group of the protein antiserum and the epoxy glass surface can be used for spotting (33). Optimization included determining
the optimal spotting concentration for the somatic antisera and the optimal dilutions for the primary and secondary antibodies to ensure strong signal intensity with minimal nonspecific cross-hybridization reaction. The concentration range assessed for spotting somatic antisera was 0.01 to 1.0 μg/μl, with 0.5 μg/μl showing the strongest signal; higher antisera concentrations resulted in nonspecific reactions (data not shown). Similarly, a concentration range of 10 ng/ml to 20 μg/ml was used for the primary anti-\textit{E. coli} core LPS antibody (WN1222-5), and a dilution range of 1:500 to 1:10,000 in PBS-F-Tween was used for the biotin-conjugated secondary anti-mouse IgG antibody during development of the assay procedure. It was found, using a control \textit{E. coli} strain, that the optimal concentration for the primary antibody was 52 ng/ml and a dilution of 1:10,000 was optimal for the secondary antibody; antibody concentrations and dilutions above or below the optimal dilution resulted in nonspecific cross-hybridization or no detectable reaction at all (Fig. 3).

**Specificity test.** For initial validation purposes, all 17 control strains, which had been used for antiserum production, were assayed using the miniaturized microarrays and the assay procedure described above in Materials and Methods. In all cases, the strains reacted with their corresponding somatic antiserum with minimal cross-reaction. Generally, all four spots showed very similar signal intensity values, with less than 10% variability in signal intensity (Fig. 3b), and could be clearly identified using the IconoClust software (Fig. 3d). Furthermore, we performed experiments with mixed cultures whereby \textit{E. coli} control strains from up to six different serotypes were added to the AT and correctly identified (data not shown).

**Blind trial.** A blind trial comprising 100 clinical isolates that had been serotyped previously by the VLA Enteric Bacteriology Reference Unit and stored on Dorset egg culture medium slopes were assayed using the miniaturized microarrays. The serotypes of these isolates were unknown during the trial. After initial data analysis, approximately 74 strains were identified as the same O serotype by both conventional and microarray serotyping methods, and the isolates showing poor correlation are shown in Table 1. These isolates could be divided into three categories. The first category (six isolates) comprised isolates for which the somatic O type determined by microarray matched the conventional serotyping result, but due to cross-reaction with more than one antiserum, the result was not clearly discerned. The second category comprised 10 isolates for which there was no match between the serotype found by the conventional method and the microarray results. The last category comprised 10 isolates that had consistently shown very low signal hybridization intensity (\( < 0.3 \)) and, therefore, could not be positively identified by microarray. Strains from the latter group were sent for repeat serotyping using the conventional method, and the results showed that the majority of strains on repeat typing were untypeable (Table 2).

Analysis of the results showed several discrepant isolates were from serogroups O26 and O157 (Table 1). Both these groups showed \( \sim 70\% \) correlation between the two serotyping methods (Fig. 4). However, as these are important enteric pathogens (18), a third method was used to confirm the serotyping results for all strains within these groups. For samples identified to be of serotype O26 from conventional serotyping, a PCR was performed using primers designed specifically.
FIG. 3. Optimization of the primary and secondary antibody concentrations used in the assay. The primary and secondary antibodies were used in a range of concentrations/dilutions, including 20 μg/ml of WN1222-5 and 1:500 dilution of biotin-conjugated secondary anti-mouse IgG antibody (a), 52 ng/ml of WN1222-5 and 1:10,000 dilution of biotin-conjugated secondary anti-mouse IgG antibody (b), and 10 ng/ml WN1222-5 and 1:10,000 dilution of biotin-conjugated secondary anti-mouse IgG antibody (c) to determine the optimal concentration that would result in high signal intensities for spots with minimal nonspecific cross-hybridization, using a control E. coli strain. The control spots at the sides are clearly distinguishable for panels b and c but not for panel a. (d) Result of analyzing the array image from panel b with the IconoClust software. Abbreviation: B, blank.
against the O26 \textit{wzx} and \textit{wxy} genes, while for O157 samples the O157-specific Oxoid latex agglutination kit was used. The PCR results confirmed that all six O26 isolates positively identified O157-specific genes, while the three microarray-negative isolates did not harbor either of these genes (data not shown). Similarly, of the presumptive O157 isolates, the seven strains that had been positively identified by microarray were also positive by latex agglutination, while the four microarray-negative isolates were also negative by latex agglutination (data not shown).

Therefore, it can be concluded that although initial comparison of the two approaches showed between 40 and 100\% correlation for each serogroup investigated and 74\% correlation overall between the two methods (Fig. 4), more detailed analysis revealed at least 88\% of isolates had been correctly identified by the microarray approach (Table 2).

### DISCUSSION

Between 1994 and 2002, there were 8,500 to 13,400 cases of human gastroenteritis due to \textit{E. coli} reported in the United Kingdom, of which approximately 0.5 to 1\% were due to \textit{E. coli} O157 (\textit{www.hpa.org.uk/infections/topics_az/ecoli/menu.htm}). Although there are no data available on the most prevalent \textit{E. coli} serotypes responsible for human and animal infection in the United Kingdom or worldwide (except for \textit{E. coli} O157), O serotyping is nonetheless the method used routinely by laboratories as a presumptive guide to distinguish between pathogenic and commensal \textit{E. coli}. However, due to the time and cost of conventional serotyping assays, we have developed a protein array that can produce O-serotyping results in a fraction of the time and that precludes any possible subjectivity in the estimation of the extent of agglutination due to automation of the analysis software. In this feasibility study, we immobilized 17 somatic antisera, raised against \textit{E. coli} pathogenic to both humans and animals and used routinely for O serotyping in the VLA Enteric Bacteriology Reference Unit, in four replicates on the array surface. The 17 control strains were successfully used to validate the O-serotyping protocol for use with miniaturized microarrays. Studies using control strains identified the O serotype for each strain accurately, and even the serotypes of multiple control strains added together could be clearly distinguished. These results indicate that the miniaturized microarrays can be used to identify not only strains grown in a single purified culture but also strains from mixed cultures of \textit{E. coli}.

A blind trial was performed with 100 \textit{E. coli} clinical isolates that had previously been received by the VLA Enteric Bacteriology Reference Unit, O serotyped, and stored on Dorset egg culture medium slopes. Overall, 88\% of the isolates were correctly identified by the microarray method. Isolates that showed discrepancy included those that could not be distinguished due to cross-reactivity. It has long been established that cross-reactivity can occur between different \textit{E. coli} O types (5). Although cultures belonging to certain O-antigen groups react specifically, in some instances cross-reactions occur due to structural relatedness between the sugar moieties of the O antigens, resulting in cross-reactive epitopes that are recognized by the somatic antibody (5). The antisera used for conventional serotyping are all polyclonal, and without very extensive cross absorption, the possibility of cross-reactions remains. Furthermore, for microarrays, the photoimager amplifies and records all enzyme-catalyzed precipitating reactions, thus detecting and quantifying both specific reactions and nonspecific cross-reactions. The use of monoclonal antibodies may circumvent this problem; however, as these antibodies are highly specific, any mutation in the O antigen or modification, such as glycosylation or lipidation, may render it undetectable.

The pathogenic serogroups O26 and O157 both showed \textsim 70\% correlation in results between the two O-serotyping methods. However, as these are important pathogens, the apparent discrepancy in O serotyping was analyzed further by a third independent test, which then confirmed the microarray serotyping results. Therefore, although the same antibodies were used for both conventional and microarray serotyping,
the latter method appears to be more accurate. The discrepancy in results may have arisen from a difference in the time of incubation of the antigen with antibody and inclusion of wash steps. In conventional serotyping, the incubation time is between 8 and 24 h, and this method has no wash steps (8, 28). In comparison, for the microarray serotyping assay, the antigen-antibody agglutination step is 30 min long. In addition, the latter method includes several washing steps for removal of any unbound reagent or substrate that would help increase specificity. A third group of isolates included in this study was untypeable by the microarray assay. The majority of these samples had been stored on Dorset egg culture medium slopes for between 2 and 6 years. Retyping of these isolates using the conventional method confirmed our findings for the majority of isolates (Table 2).

This study has shown the potential of using protein arrays for serotyping, and the assay developed in this study can also be used for other organisms that are routinely serotyped, such as *Salmonella*. A major advantage of serotyping using the miniaturized protein arrays is that very small quantities of antisera are required for spotting onto arrays. Since well-characterized sera are precious, this may be a significant advantage of using protein arrays for serotyping over conventional methods. In our current study, printed arrays have been stored at room temperature and used up to 1 year after production. However, shelf life studies are currently being performed to establish the longevity of the arrays.

In addition, this study has indicated that the microarray approach, using a single miniaturized AT system, has the potential to accurately serotype up to 10 *E. coli* isolates in less than 2 hours in comparison to 2 or 3 days with the conventional O-serotyping method. Therefore, although the costs of consumables are similar for both methods, the reduction in man-hours using the array system will ultimately result in significant cost savings. In the future, array strips or even microtiter plates could be used instead of array tubes, so up to 96 *E. coli* strains could be O serotyped simultaneously, which would increase the speed of tests even further, especially if the procedure became fully automated (CLONDIAG). Moreover, due to the available working space of the array being 2.4 by 2.4 mm in size, future studies could comprise 10 marker spots plus up to 215 different somatic antibodies printed singly, which would enable all currently known *E. coli* serotypes, including those of medical and veterinary importance, to be typed using one chip.

Recent advancements of molecular techniques have led to adaptation of bacterial typing to various formats, including multiplex assay formats, which are generally more rapid and simpler than conventional methods. Multiplex PCR assays developed for bacterial serotyping include typing *Listeria monocytogenes* serotypes 1/2a and 4b (35); *E. coli* O103 (6); seven *Streptococcus pneumoniae* serotypes (19); and *Actinobacillus pleuropneumoniae* serotypes 1, 2, and 8 (27). A disadvantage of the multiplex PCR assays is that gene sequences of polysaccharide capsules or O-antigen gene clusters are not yet known for many bacterial serotypes. Therefore, the development of a protein chip using somatic antisera for serotyping, as performed in this study, has the distinct advantage of precluding the dependence on knowing gene sequences. A similar ap-

![FIG. 4. Comparison of conventional serotyping with the array system. The percentages of correlation of results from a blind trial using the AT system to serotype strains that had previously been serotyped using a conventional method are shown. A total of 100 strains were included. The result of the total initial correlation and correlation for each individual serotype is shown. The total number of isolates and number in each serotype are given in parentheses above the bars.](http://cvi.asm.org/)

566 ANJUM ET AL. CLIN. VACCINE IMMUNOL.
proach has been used for serotyping pneumococci, whereby latex beads coated with pneumococcal capsular polysaccharide have been used (34). This method is simpler and more specific and efficient than the Quellung method historically used for pneumococcal serotyping, showing advantages similar to those of the E. coli protein chip used in this study. A comparison of the cost of using the latex bead serotyping method, which includes use of fluorescent dyes and a flow cytometer, against the array method, which includes an array printer (Microgrid II; Genomic Solutions Ltd., United Kingdom) and a customized scanner (CLONDIAG), shows the costs to be roughly equivalent. However, these costs do not reflect the complexity involved in production of ArrayTube reaction vials, which makes the use of customized preprinted miniaturized arrays, available from CLONDIAG, the most convenient and likely option.

In conclusion, in this pilot study, we have demonstrated the principle and feasibility of using protein chips for O serotyping with a subset of E. coli antisera. Future work will focus on developing arrays for comprehensive coverage of all E. coli strains serotyped in reference laboratories.

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