APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1983, p. 826–831
0099-2240/83/100826-06$02.00/0

Vol. 46, No. 4

Use of Murine Myeloma Protein M467 for Detecting *Salmonella* spp. in Milk

A. MASON SMITH* AND CAROLYN JONES

Department of Microbiology, East Carolina University School of Medicine, Greenville, North Carolina 27834

Received 9 May 1983/Accepted 20 July 1983

This investigation introduces the use of an immunoglobulin A mouse myeloma protein for the detection of *Salmonella* spp. in milk. The immunoglobulin A protein M467 reacts with flagellin from a wide variety of serotypes. Two assays were developed which used an enzyme-linked immunosorbent assay (ELISA) and M467. Alkaline phosphatase was conjugated to M467 (M467-PH), and the presence of *Salmonella dublin* was detected by a competitive solid-phase ELISA and a membrane filtration ELISA. The competitive assay competed viable *Salmonella* spp. found in contaminated milk against polymerized flagellin or whole bacteria fixed to polyvinyl plates for binding by M467-PH. The membrane filtration method utilized a hydrophilic membrane for filtering the bacteria, which were then detected by the reaction with M467-PH and substrate. The sensitivity of the competitive solid-phase ELISA was 10^3 bacteria ml^{-1}, whereas the filter membrane assay required the media containing the bacteria to be cultured in enrichment medium for 4 h before the assay to ensure detection. Either assay could be run within a typical 8-h work day. The filter membrane assay was not suitable for milk due to the high level of natural alkaline phosphatase activity in the liquid food.

We have described in previous reports how monoclonal antibody (M467) of the immunoglobulin A isotype, derived from the MOPC-467 mouse plasmacytoma, bound flagellar protein associated with many species of *Salmonella* bacteria (3, 5). The M467 myeloma protein has been shown to react with structural peptides of the protein flagellin, and apparently these peptides have been highly conserved among *Salmonella* spp. (4). This cross-reactive property of M467 provided the opportunity to investigate the feasibility of its use in immunodiagnostic assays for the detection of *Salmonella* spp. in food products. Our immediate interest was the detection of *Salmonella* spp. in raw milk. The most reliable indication of the sanitary quality of milk was the standard plate count, which required 24 h of incubation. Definitive identification of *Salmonella* spp. required further chemical and immunological assays, all of which increased the total length of time necessary to hold the raw milk. It was important to design a diagnostic system that required less time and was reliable.

Although a radioimmunoassay method that uses M467 had been reported (4), the enzyme-linked immunosorbent assay (ELISA) was the method of choice for this study due to the innate problems associated with the use of isotopes in many laboratories. The ELISA method reported here was the basis for two separate assays, a solid-phase competitive assay done in microtiter plates and a membrane filtration system. Both methods proved to have the sensitivity to detect 10^3 *Salmonella* spp. ml^{-1} in either milk or culture media. Assuming that a food product originally contained $<10^3$ bacteria ml^{-1}, such numbers could be obtained by culturing in enrichment broth for a short period of time while still having the time to complete the assay in a single work day. The problem of high natural alkaline phosphatase activity in foods such as milk will be discussed.

MATERIALS AND METHODS

M467 preparation and alkaline phosphatase labeling. M467 monoclonal antibody was purified from ascites fluid from BALB/c mice carrying the MOPC-467 plasmacytoma as previously described (6). Flagellin was coupled to CH-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) through carbodiimide (Sigma Chemical Co., St. Louis, Mo.). The flagellin-Sepharose 4B was used to form an affinity column from which M467 was eluted with 0.2 M glycine-HCl–1.0 M NaCl buffer, pH 2.5. M467 preparations were tested for purity by immunoelectrophoresis, and anti-flagellin activity was tested by double diffusion in agar gel. Purified M467 was labeled with alkaline phosphatase (M467-PH) exactly as previously described (7). Type VII-S alkaline phosphatase, glutaraldehyde, and the substrate *p*-nitrophenyl phosphate were obtained

from Sigma. Working dilutions of M467-PH were determined by reacting various dilutions against 20 ng of flagellin fixed to polyvinyl wells as described below. The 20-ng concentration of flagellin was previously found to be optimal for providing a sensitive assay for the binding of M467 (4). Dilutions were used which produced an absorbance value at 405 nm (A_{405}) of approximately 1.0 after reaction with substrate when read on a Multiskan spectrophotometer (Flow Laboratories, McLean, Va.). All dilutions of M467-PH were made in phosphate-buffered saline (PBS), pH 7.4, containing 0.5% Tween 20 (PBS-T20; Sigma).

Flagella preparation and fixation of antigens to polyvinyl tissue culture plates. Polymerized flagellin (POL) was prepared as previously described (5) without modification. POL was kept as a lyophilized preparation until used. For fixation to 96-well, flat-bottom polyvinyl tissue culture plates (3040 Microtest II; Falcon Plastics, Oxnard, Calif.), POL was suspended in deionized water at a concentration to deliver 20 ng 20 μl^{-1} unless otherwise noted in Results. The POL was allowed to air dry in a 37°C incubator. After drying, 0.1 ml of methanol was added and allowed to stand for 5 min followed by a 0.1 ml well $^{-1}$ wash with PBS. Finally, 0.3 ml of 10% bovine serum albumin (radioimmunoassay grade; Sigma) in barbitol-buffered saline, pH 8.6, was added to each well. After a 1-h incubation at room temperature (RT), the wells were washed with PBS. Bacteria were fixed to polyvinyl wells by pipetting 10^8 organisms in 50 μl of deionized water into each well. The cells were air dried overnight and were fixed by adding 0.1 ml of 0.15% glutaraldehyde in PBS. After a 5-min incubation at RT, the reaction was ended by the addition of 0.1 ml of 0.15 M glycine in PBS containing 1% bovine serum albumin. The plates were flicked dry and filled with PBS with 10% bovine serum albumin for 1 h at RT. The plates were finally washed twice with PBS and could then be immediately used or stored covered at 4°C for use at a later time.

Sources of bacteria and culture conditions. The *Salmonella* spp. used in this study were originally obtained from Michael Potter, National Cancer Institute, Bethesda, Md., with the exception of *Salmonella dublin*, which was obtained from the American Type Culture Collection, Rockville, Md. *Escherichia coli* strains tested were isolated from human patients or obtained from the American Type Culture Collection. *Proteus mirabilis* was originally isolated from a BALB/c mouse. All *Salmonella* spp. used in the study were cultured in brain heart infusion (BHI) broth containing 0.5% yeast extract (BHI-YE). Bacterial dilutions were standardized by using McFarland standards and a Beckman model 26 spectrophotometer (Beckman Instruments, Palo Alto, Calif.). Bacterial viability tests and counts were done on BHI agar plates after incubation at 37°C for 24 h.

Statistical methods. A_{405} values from the assay methods were subjected to a one-tailed Student *t*-test to determine the significance of absorbance value increases or decreases. Significance was assessed at the 95% confidence level. The data were also analyzed by regression plots at a confidence level of 99%.

Competitive ELISA. The competitive ELISA assays were designed to compete *Salmonella* spp. flagellar antigens of viable bacteria in milk or culture media with flagellar proteins in a solid phase for the binding of M467-PH. Bacteria were suspended in PBS-T20, BHI-YE, or raw milk. The media containing the bacteria were centrifuged at 30,000 × *g* in a model 21 Beckman refrigerated centrifuge for 10 min. The pellet was resuspended in 3 ml of a working dilution of M467-PH, agitated, and incubated at RT for 1 h. After incubation, the reactants were centrifuged at 30,000 × *g* for 10 min. The supernatant was sampled by pipetting 0.3 ml into each of five wells containing fixed POL. After a 3-h incubation at RT, the microplates were emptied by flicking. The wells were washed three times with PBS-T20, allowing 3 min for each wash. After the final wash, 0.2 ml of the substrate *p*-nitrophenyl phosphate at a concentration of 1 mg ml^{-1} in diethanolamine buffer (7) was added to individual wells and incubated at RT for 30 min. The reaction was terminated by the addition of 50 μl of 3 N NaOH, and the A_{405} of each well was read in a Multiskan spectrophotometer. The A_{405} values of the experimental samples were compared with control values obtained from wells containing POL and M467-PH without competing bacteria. The lowest number of bacteria per milliliter which could lower the A_{405} value a significant amount below control values was considered the sensitivity of the assay.

Membrane filtration and M467-PH for detection of *Salmonella* spp. Hydrophilic HVLP filters (Millipore Corp., Bedford, Mass.) were used due to their low nonspecific protein-binding property. The 25-cm-diameter, 0.45-μm filter membranes were held in a vertical-wall Plexiglas funnel attached to a vacuum flask. Various concentrations of *Salmonella* spp. suspended in 20 ml of either PBS or BHI-YE were applied to the filters with sufficient vacuum to remove the liquid. The filters with the trapped bacteria were then washed under vacuum with 20 ml of PBS-T20. The membranes were carefully removed with forceps and placed in individual sterile 35-mm plastic petri dishes. Subsequently, 0.3 ml of M467-PH was applied to the surface of the individual membranes. The 0.3 ml volume covered the entire surface of an HVLP filter with the plate held on a level surface. After a 30-min incubation at RT. the individual membranes were returned to the funnel and washed under vacuum with 20 ml of PBS-T20 for each filter. The membranes were then placed in sterile petri dishes and were covered with 0.3 ml of substrate. After incubation for an additional 30 min, each dish containing a filter was tilted and the incubation mixture was removed in 0.1-ml aliquots, which were placed in five separate wells and read in a Multiskan spectrophotometer. Control values were obtained by applying 0.3 ml of M467-PH diluted in PBS-T20 to a membrane while processing simultaneously with the membranes containing known *Salmonella* spp. A_{405} values significantly higher than the control were taken as an indication of the presence of *Salmonella* spp.

RESULTS

Sensitivity of detection of flagellar antigens in solid-phase M467-PH binding. Results obtained from published data provided the basis for experiments to determine the sensitivity of a competitive solid-phase assay for the presence of *Salmonella* spp. in food products (4). Each

preparation of M467-PH was titrated by reacting various dilutions in microwells containing *S. milwaukee* POL. The working dilutions which provided an A_{405} value of 0.9 to 1.0 ranged from 1:2,000 to 1:4,000. The concentration at which flagellar protein could be detected with a competitive solid-phase system was determined by first reacting various concentrations of POL with a working dilution of M467-PH. After incubation, the reaction mixture was placed into wells containing fixed *S. milwaukee* bacteria. The concentration of POL providing sufficient competition to reduce the A_{405} a significant amount from control binding in wells containing only bacteria and M467-PH was consistently 0.125 ng. These results were highly reproducible, using different plates and different preparations of POL.

From these results it was concluded that small quantities of POL as the antigen in a solid-phase system could be used in a competitive system to detect viable bacteria in raw milk. Results of the following experiments show the sensitivity levels achieved in this competitive system.

Specificity of the M467-flagellin reaction. Our laboratory had tested M467 reactivity against more than 40 different *Salmonella* spp. as well as a number of other species of flagellated bacteria. Reactivity has been determined by precipitation in agar gel as well as in a competitive radioimmunoassay (4). Of the representative species listed, (Table 1), only *S. paratyphi*-A and *S. typhi* have not shown reactivity with M467.

TABLE 1. *Salmonella* spp. and other flagellated bacteria tested for reactivity with M467

Type[a]	Flagellar antigen (H1-H2)
Positive	
S. aberdeen	i; 1,2
S. adelaide	f,g; —[b]
S. anatum	e,h; 1,6
S. canoga	g,s,t; —
S. donna	l,v; 1,5
S. dublin	g,p; —
S. enteritidis	g,m; —
S. greenside	z; e,n,x
S. milwaukee	f,g; —
S. mississippi	b; 1
S. newington	e,h; 1,6
S. newport	e,h; 1,2
S. schottmuelleri	b; 1,2
Negative	
S. paratyphi-A subsp. *durazzo*	a; —
S. typhi (NCTC 8385)	d; —
E. coli (five strains tested)	
P. mirabilis	

[a] According to Kauffman-White classification.
[b] —, No H2 phase.

However, attempts in our laboratory to isolate and purify flagellar protein from these organisms have not been successful. We have tested five strains of *E. coli* whereas another laboratory has tested an additional number, and none reacted with M467 (3). *P. mirabilis* has also proven to be nonreactive with M467. Two additional organisms found frequently in raw milk, *Enterobacter cloacae* and *Citrobacter freundii*, were not bound by M467.

Selection of solid-phase antigens for maximum reproducible results. From previous experience, we have found that fixing POL by air drying followed by methanol fixation to polystyrene wells provides a stable solid-phase component for a binding assay. We have kept POL-bound microculture plates at 4°C for 2 weeks without detectable loss of sensitivity. It had also been reported that M467 bound POL from various *Salmonella* spp. with varying avidity (4). We have concluded from a large number of such experiments that more reproducible results can be obtained by using POL from a "Hi" inhibitor such as *S. milwaukee* as the solid-phase antigen. Table 2 presents the results of experiments in which POL from the "Hi" inhibitor *S. milwaukee* and a "Lo" inhibitor, *S. typhimurium*, were used as solid-phase antigens with *S. dublin* and *S. milwaukee* viable bacteria as competitors for the binding of M467-PH. When 20 ng of *S. milwaukee* POL was used as the solid-phase antigen, it was possible to detect 10^3 *S. dublin* bacteria ml^{-1} (Table 2). When 20 ng of *S. typhimurium* POL was the solid-phase antigen, the sensitivity of the assay was reduced to $>10^8$ bacteria ml^{-1}. Also, it was apparent (Table 2) that the sensitivity diminished when the concentration of the "Hi" inhibitor POL in each well was reduced from 20 to 10 ng. An increase in the concentration of POL above 20 ng $well^{-1}$ of a "Hi" inhibitor will increase the A_{405} value, but the sensitivity of the assay is not increased (data not shown). It was concluded from the data that, when M467-PH was reacted with viable *Salmonella* spp. in low numbers and subsequently added to POL from a *Salmonella* spp. for which M467 has a high avidity, inhibition of binding of M467-PH to the solid-phase antigen can be readily detected.

Detection of *S. dublin* by a competitive ELISA.

TABLE 2. Sensitivity of the competitive binding assay for detection of viable *Salmonella* spp.

Solid-phase POL (ng fixed $well^{-1}$)	Competitive *Salmonella* sp.	Minimum no. of bacteria detected
S. milwaukee (20)	*S. dublin*	10^3
S. milwaukee (20)	*S. milwaukee*	10^3
S. milwaukee (10)	*S. dublin*	10^6
S. typhimurium (20)	*S. dublin*	10^8

Having shown that the solid-phase inhibition system was sensitive enough to be practical, experiments were designed to detect *Salmonella* spp. in a liquid food product. Raw milk adulterated by the addition of *S. dublin* was added to either PBS-T20 or BHI-YE and centrifuged. The bacteria in the pellet were resuspended in a working dilution of M467-PH and were bound by the antibody, which was always in excess. The supernatant from a second centrifugation contained unbound M467-PH which, when placed in wells containing fixed POL, bound the POL antigen. The A_{405} values from the experimental wells after reaction with the substrate were compared with those of controls with M467-PH and POL antigen without competition from viable bacteria. The data in Table 3 indicate that *S. dublin* in the milk samples diluted in PBS-T20 as well as in BHI-YE could be detected at a concentration of 10^3 bacteria ml^{-1}. A further experiment was done in which raw milk samples of 2 ml containing 10, 10^2, and 10^3 *S. dublin* bacteria were placed in 20 ml of BHI-YE and incubated at 37°C for periods of 3, 4, and 5 h. We found that by culturing the milk for 4 h or longer the numbers of bacteria had increased such that they could be detected in each of the original cultures by the competitive assay. In an additional experiment, the pellets produced after incubation periods of 3, 4, and 5 h were fixed in 0.7% formalinized saline overnight before the addition of M467-PH. Although the A_{405} values were lower, the 10^3 ml^{-1} sensitivity level of the assay was not affected.

Detection of *S. dublin* by membrane filtration and M467-PH. As an additional method for the detection of *Salmonella* spp., a membrane filtration system was studied. A number of commercially available membranes were tested for low nonspecific retention of the M467-PH reagent. The hydrophilic HVLP filters from Millipore Corp. consistently afforded the lowest background activity and were used throughout the experiments.

In the first studies, 10^3, 10^6, and 10^8 *S. dublin* bacteria in PBS were placed on individual membranes and processed. The system was found to be sensitive enough to detect 10^8 bacteria ml^{-1}. Therefore, due to this low sensitivity, various concentrations of both *S. dublin* and *S. milwaukee* from 10^3 to 10^7 ml^{-1} were cultured in 20 ml of BHI-YE for 4 h at 37°C. At the end of 4 h, dilutions were made from each culture and were plated on BHI-YE agar. An increase in numbers was noted in all cultures. A mean of 5×10^3 bacteria ml^{-1} was determined from five different cultures of *S. dublin* originally inoculated with 10^3 bacteria. Four-hour cultures originally containing 10^3 bacteria ml^{-1} filtered through HVLP filters were detectable (Table 4) when compared with A_{405} values of controls of media, M467-PH, and substrate. Based on a number of experiments, the M467-PH–plus–substrate background A_{405} level would vary from 0.2 to 0.5.

An innate problem encountered with the alkaline phosphatase enzyme system was found when working with raw milk. Milk without *S. dublin* was diluted in PBS-T20 or BHI-YE and filtered through HLVP filters. Extremely high-background A_{405} values were noted which were due to natural alkaline phosphatase activity in raw milk since heating the milk at 92°C for 3 min significantly lowered the background. However, enough sensitivity in the *Salmonella* spp. detection system was lost due to the heating to make the assay not feasible for raw milk.

DISCUSSION

The major purpose of this work was to develop a relatively rapid, sensitive immunodiagnostic assay for the detection of *Salmonella* spp. in food preparations. The results of this investigation have shown that such a screening assay is feasible for liquid foods such as milk, and it is reasonable to assume that *Salmonella* spp. from extracts of solid foods could also be detected utilizing the monoclonal antibody M467 derived from a mouse plasmacytoma and the ELISA system. We have developed two types of assays, a solid-phase competitive assay done in microplates and a direct-type assay done on membrane filters.

The M467 protein is unique in that it has been shown to recognize epitopes associated with a large number of different serotypes of *Salmonella* spp., in both phase 1 and phase 2 (4). To attempt to develop a commercial polyvalent anti-H antiserum with the broad specificity of M467 would be extremely difficult due to the

TABLE 3. Detection of viable *S. dublin* in raw milk by the competitive ELISA[a]

Milk diluent	A_{405}[b] at given concn of *S. dublin* (ml^{-1}):				
	10^8	10^4	10^3	10^2	None
PBS-T20	0.42 ± 0.01	0.61 ± 0.03	0.65 ± 0.01	0.92 ± 0.02	0.91 ± 0.02
BHI-YE	0.30 ± 0.01	1.20 ± 0.04	1.33 ± 0.02	1.48 ± 0.03	1.47 ± 0.03

[a] Each well contained 20 ng of *S. milwaukee* POL as the solid-phase antigen.
[b] Mean ± SEM of five wells for each concentration of *S. dublin*.

TABLE 4. Detection of *S. dublin* and *S. milwaukee* by membrane filtration after incubation (4 h) in BHI-YE

Salmonella sp.:	A_{405}[a] after incubation at given preculture concn of *Salmonella* sp.:					
	Control[b]	10^3	10^4	10^5	10^6	10^7
S. dublin	0.45 ± 0.12	0.65 ± 0.09	0.67 ± 0.06	0.70 ± 0.07	0.93 ± 0.02	1.6 ± 0.03
S. milwaukee	0.47 ± 0.03	0.61 ± 0.06	0.65 ± 0.09	0.67 ± 0.02	0.81 ± 0.05	1.4 ± 0.01

[a] Mean ± SEM of five replications of substrate M467 mixture from three membranes for each concentration of *S. dublin* and *S. milwaukee*.
[b] Control = M467 + substrate + medium.

large number of serological types. In addition, *Salmonella* spp. are subject to many variational changes (1). Thus far, we have found only *S. typhi* and *S. paratyphi* to be nonreactive with M467. It is highly probable that, in a situation where *S. typhi* was suspected of being a food contaminant, more strenuous biochemical tests would be used for positive identification. Importantly, M467 has not been shown to react with some other common flagellated bacteria such as *E. coli*. We have concluded that M467 is sufficiently specific for use in food microbiology. The major questions to be answered by further studies are whether there are additional *Salmonella* spp. that will not react with M467 and whether there are other flagellated organisms that would cross-react with the immunoglobulin A protein. We propose that the M467 protein be used for preliminary screening of batches of food products; when a positive reaction is obtained, additional diagnostic measures could be used to define the contaminating organism.

We have shown that a competitive solid-phase assay done in microtiter plates, using the ELISA system, provides the sensitivity to detect 10^3 *Salmonella* spp. ml^{-1} in a reproducible manner. Although many laboratories have the equipment needed for the solid-phase assay as standard equipment, it may be that the average number of assays run in a given day would not warrant the use of 96-well plates. An alternative using the same methodology as with the microplates are individual polystyrene tubes (10 by 75 mm) with POL bound in the solid phase. This single-tube method was used in our laboratory before the microplate and also allows one to use any spectrophotometer capable of reading A_{405}.

An optional method we have developed for detecting *Salmonella* spp. is membrane filtration, using a hydrophilic membrane. The sensitivity of the filtration system for the initial detection of *Salmonella* spp. in numbers below 10^8 was not acceptable. However, by using an enrichment medium and the minimum incubation time of 4 h, the numbers of bacteria per milliliter could be increased to a level detectable by the membrane filtration system. Many human illnesses associated with food-borne bacterial pathogens are caused by $<10^3$ organisms ml^{-1}. Therefore, it is assumed that further studies could produce methodology utilizing M467 that could increase the sensitivity that we have achieved with the ELISA system. We have shown, for example, that prior incubation of the medium containing the *Salmonella* spp. in enrichment medium increased the number of organisms to a level detectable by both diagnostic methods described.

We have found that the ELISA used with the membrane system is not acceptable when used with foods such as milk which contain high levels of alkaline phosphatase. Likewise, in experiments not described, we have used horseradish peroxidase-labeled M467 and found it unsuitable when used with foods with high levels of peroxidase activity. It is likely that a system utilizing polystyrene balls (2) coated with POL would provide an alternative to the membrane system since the balls could be removed from the presence of endogenous alkaline phosphatase and be washed before the reaction with substrate. Laboratories equipped for the use of radioisotopes might use the radioimmunoassay with labeled M467 to circumvent the problems of natural alkaline phosphatase and peroxidase activity in some foods.

We have described two methods which use the "natural" monoclonal antibody M467 to detect the presence of *Salmonella* spp. Further work with M467 should lead to improved methodology with increased sensitivity as well as additional uses for the immunoglobulin A myeloma protein in the food industry.

ACKNOWLEDGMENTS

This work was supported in part by Altadena Dairy of City of Industry, Calif.

We express appreciation to John McGonagle for the design and construction of a Plexiglas support for the filter membranes. We also thank Debbie Robbins and Holly Pulley for the preparation of this manuscript.

LITERATURE CITED

1. **Edwards, P. R., and W. H. Ewing.** 1970. The genus *Salmonella*, p. 92–167. *In* P. R. Edwards and W. H. Ewing (ed.), Identification of Enterobacteriaceae, 8th ed. Burgess Publishing Co., Minneapolis.
2. **Freed, R. C., M. L. Erenson, R. F. Reiser, and M. S. Bergdoll.** 1982. Enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxins in food. Appl.

Environ. Microbiol. **44**:1349–1355.
3. **Potter, M.** 1971. Antigen-binding myeloma proteins in mice. Ann. N.Y. Acad. Sci. **190**:306–321.
4. **Smith, A. M., J. S. Miller, and D. S. Whitehead.** 1979. M467: a murine IgA myeloma protein that binds a bacterial protein. I. Recognition of common antigenic determinants on *Salmonella* flagellins. J. Immunol. **123**:1715–1720.
5. **Smith, A. M., and M. Potter.** 1976. A BALB/c mouse myeloma protein that binds *Salmonella* protein. J. Immunol. **114**:1847–1850.
6. **Smith, A. M., J. Slack, and M. Potter.** 1977. Restriction in the immune response to flagellar proteins in inbred mice. Eur. J. Immunol. **7**:497–500.
7. **Voller, A., D. Bidwell, and A. Bartlett.** 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506–512. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 1st ed. American Society for Microbiology, Washington, D.C.