CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Nov. 1996, p. 669–677 Vol. 3, No. 6
1071-412X/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Development of a Rapid and Specific Colony-Lift Immunoassay for Detection and Enumeration of *Campylobacter jejuni*, *C. coli*, and *C. lari*

BRANDT E. RICE,[1,2]* CHINTA LAMICHHANE,[3] SAM W. JOSEPH,[2] AND DAVID M. ROLLINS[1]

Enteric Diseases Program, Naval Medical Research Institute, Bethesda, Maryland 20889[1]*; Department of Microbiology, University of Maryland, College Park, Maryland 20742*[2]*; and Kirkegaard and Perry Laboratories, Gaithersburg, Maryland 20879*[3]

Received 8 April 1996/Returned for modification 15 May 1996/Accepted 11 July 1996

Contamination of retail poultry by *Campylobacter* spp. is a significant source of human diarrheal disease. We have developed a colony-lift immunoassay (CLI) for the detection of *Campylobacter jejuni*, *C. coli*, and *C. lari* isolated from such sources and grown on selective agar medium or on filter membranes. This technique has been successfully utilized to quantify *Campylobacter* colonies within 18 to 28 h after sampling. Hydrophobic, high-protein-binding membranes were prewet with methanol and used to imprint bacterial cells from the agar or filter membrane, while leaving colonies intact and viable. The membranes were air dried, peroxidase neutralized, blocked with bovine serum albumin in phosphate-buffered saline, and hybridized for 5 min with an affinity-purified, horseradish peroxidase-labeled goat anti-*Campylobacter* antibody preparation (Kirkegaard and Perry Laboratories). The membranes were washed briefly, exposed to a 3,3′,5,5′-tetramethylbenzidine membrane substrate, rinsed in deionized water, and allowed to dry. Lifted colonies of *Campylobacter* were identified by a blue color reaction on the membrane. Replicas of the membranes were made by marking the location of the *Campylobacter* colonies on clear transparencies, which were subsequently utilized to locate the original colony on the filter membrane or agar plate. The specificity of this antibody preparation has been evaluated against a wide range of *Campylobacter* spp., including American Type Culture Collection type and reference strains, retail poultry isolates, and isolates obtained from cloacal swabs of live commercial broiler chickens. Specificity against numerous non-*Campylobacter* spp. obtained from the same sources was also evaluated. The CLI provided a rapid and simple means for detection and enumeration of enteropathogenic *Campylobacter* organisms. We have successfully combined this CLI procedure with methods recently developed in our laboratories for retail meat and poultry sampling. Potentially, broader applications for use of this technique include detection and enumeration of campylobacters from clinical, veterinary, and environmental samples.

Advances in and refinements to isolation and growth techniques and epidemiologic tools, combined with increased awareness and surveillance, have enabled investigators to clearly demonstrate that *Campylobacter* spp. are one of the leading causes of human diarrheal disease throughout the world (4, 34, 35, 36). The majority of human cases of campylobacteriosis are sporadic in nature, resulting from infections after exposure to contaminated food and water (4, 26, 33, 34). Case control and retail surveillance studies in the United States have estimated that 48 to 70% of these sporadic infections are a direct result of the handling or consumption of raw or undercooked poultry (6, 12, 13).

The significance of this pathogen and the assimilation of a science-based method for safe food production, the Hazard Analysis and Critical Control Points Program, into meat and poultry processing facilities have created the need for rapid, qualitative, and quantitative methods for detecting enteropathogenic campylobacters in food. The fastidious nature of *Campylobacter* spp. and the inherent difficulties associated with its separation from a food matrix have been overcome by our laboratories (31). Following extraction from the food matrix, isolates were grown on selective agar plates or filter membranes, and a colony-lift immunoassay (CLI) method was used to detect and enumerate target colonies of *Campylobacter*.

We report here the development of this membrane-based enzyme-linked immunosorbent assay method for the identification and enumeration of *Campylobacter jejuni*, *C. coli*, and *C. lari* isolates. Optimal assay conditions for establishing maximum specificity and sensitivity in the identification of these target organisms were determined. The CLI technique provides a rapid and specific means of quantifying the level of *C. jejuni*, *C. coli*, and *C. lari* in food sources, with immediate applications for monitoring large-scale commercial poultry operations and the effect of intervention procedures in production and processing facilities. Furthermore, this technique has the potential to be applied to clinical, veterinary, and environmental samples.

MATERIALS AND METHODS

Bacterial isolates. The bacterial strains utilized in the development of the CLI included both *Campylobacter* and non-*Campylobacter* spp. These were either type or reference strains obtained from the American Type Culture Collection (ATCC) (Rockville, Md.) (Table 1), isolates from cloacal swabs of live broiler chickens from commercial production farms (Table 2), or isolates from phosphate-buffered saline (PBS) rinses of retail poultry products (Table 2). The non-*Campylobacter* isolates obtained from the poultry sources were chosen because they represented the most common breakthrough contaminants on the primary selective media.

Stock cultures of ATCC strains were grown and stored according to the procedures recommended by the supplier. Cloacal swabs were inoculated onto Campy blood agar plates (cBAP) (Remel, Lenexa, Kans.) and grown under microaerophilic conditions for 24 h at 42°C. Isolated colonies were subcultured once onto sheep blood agar plates (sBAP) (Remel). After 24 h of microaero-

* Corresponding author. Mailing address: Enteric Diseases Program, Naval Medical Research Institute, 8901 Wisconsin Ave., Bethesda, MD 20889-5055. Phone: (301) 295-0135. Fax: (301) 295-6641. Electronic mail address: rice@mail2.nmri.nnmc.navy.mil.

TABLE 1. Specificity and medium compatibility of the HRP-labeled goat anti-*Campylobacter* antibody (KPL) when used in the CLI with ATCC type and reference strains

Bacterial strain	ATCC isolate identification no.	Medium[a]	Reaction with anti-*Campylobacter* antibody[b]
Type strains			
Campylobacter jejuni subsp. *jejuni*	33560	sBAP	Positive
Campylobacter jejuni subsp. *doylei*	49349	sBAP	Positive
Campylobacter coli	33559	sBAP	Positive
Campylobacter lari	35221	sBAP	Positive
Campylobacter hyointestinalis	35217	sBAP	Positive
Campylobacter fetus subsp. *venerealis*	19438	sBAP	Negative
Campylobacter fetus subsp. *fetus*	27374	sBAP	Negative
Campylobacter upsaliensis	43954	sBAP	IG[c]
Campylobacter sputorum subsp. *sputorum*	35980	sBAP	Negative
Campylobacter sputorum subsp. *bubulus*	33562	sBAP	Negative
Helicobacter pylori	43504	sBAP	Negative
Helicobacter mustelae	43772	sBAP	Negative
Helicobacter cinaedi	35683	sBAP	Negative
Helicobacter fennelliae	35684	sBAP	IG
Aeromonas jandaei	49568	NA	Negative
Aeromonas hydrophila	7966	NA	Negative
Alcaligenes eutrophus	17697	sBAP	Negative
Aquaspirillum serpens	12638	NA	IG
Aquaspirillum itersonii subsp. *itersonii*	12639	NA, MH	Negative
Arcobacter butzleri	49616	sBAP	Negative
Bacillus cereus	14579	NA, sBAP, MH	Negative
Escherichia coli	11775	sBAP	Negative
Klebsiella oxytoca	13182	sBAP	Negative
Klebsiella pneumoniae subsp. *pneumoniae*	13883	sBAP	Negative
Legionella pneumophila subsp. *pneumophila*	33152	CYE	Negative
Proteus mirabilis	29906	sBAP	Negative
Proteus vulgaris	13315	sBAP	Negative
Pseudomonas aeruginosa	10145	sBAP	Negative
Reference strains			
Campylobacter jejuni subsp. *jejuni*	35918	sBAP	Positive
	35925	sBAP	Positive
	43430	sBAP	Positive
	43437	sBAP	Positive
Campylobacter coli	43133	sBAP	Positive
	43134	sBAP	Positive
	43136	sBAP	Positive
	43742	sBAP	Positive
	43475	sBAP	Positive
	43476	sBAP	Positive
	43480	sBAP	Positive
	43481	sBAP	Positive
Campylobacter lari	35222	sBAP	Positive
	35223	sBAP	Positive
	43675	sBAP	Positive

[a] Medium abbreviations: NA, nutrient agar (Oxoid); MH, Mueller-Hinton agar (Remel); CYE, charcoal yeast extract agar (ATCC medium 1088).
[b] CLI performed using optimized protocol.
[c] IG, inadequate growth for CLI.

philic growth, isolated colonies were harvested and the cells were resuspended in brain heart infusion broth (Oxoid, Basingstoke, Hampshire, England) supplemented with 15% glycerol and slowly frozen. Frozen stock cultures were stored at −80°C until needed. At that time, samples were thawed, plated on sBAP, and incubated for 24 h at 42°C under microaerophilic conditions. Either frozen stock isolates from PBS rinses of poultry products were prepared as described for cloacal swabs of commercial broilers or the rinses were initially filtered through 0.2-μm-pore-size Millipore-MF filter membranes (Millipore Corp., Bedford, Mass.) which were incubated faceup on cBAP and subsequently treated as described for the cloacal swab isolates. Alternatively, for PBS rinses of poultry products, CLIs, as described below, were performed directly from primary spread plates (cBAP) or from filter membranes placed faceup on cBAP (filter membrane transfer) following a 24-h microaerophilic incubation (see Fig. 3).

Bacterial characterization. Biochemical and serological analyses of *Campylobacter* spp. were performed according to the methods of Lior et al. (16, 17). Non-*Campylobacter*, gram-negative isolates from retail poultry and cloacal swabs were identified by using API 20E test strips according to the directions of the manufacturer (BioMerieux Vitek, Inc., Hazelwood, Mo.).

Comparison of membranes, antisera, membrane substrates, and agar media. Dot immunoblots were utilized initially to compare results obtained with different membranes, antisera, and membrane substrates. Three membranes were evaluated: (i) Immobilon-P (0.45 μm), a polyvinylidene fluoride (PVDF) mem-

TABLE 2. Specificity and medium compatibility of the HRP-labeled goat anti-*Campylobacter* antibody (KPL) when used in the CLI with cloacal swab isolates from commercial broiler chickens and from retail poultry isolates

Bacterium and serotype	Isolate	Medium	Reaction with anti-*Campylobacter* antibody[a]
Commercial broiler isolates			
Campylobacter jejuni			
Lior 1	FICJ-1	sBAP, cBAP	Positive
Lior 2	F1BCB	sBAP, cBAP, MCCDA, CVA	Positive
Lior 4	FICJ-4	sBAP, cBAP	Positive
Lior 17	FICJ-17	sBAP, cBAP, MCCDA	Positive
Lior 36	FICJ-36	sBAP, cBAP	Positive
Gram-positive coccus, NFC[b]	SWC	sBAP, cBAP	Negative
Escherichia coli	LYC	sBAP, cBAP	Negative
Retail poultry isolates			
Campylobacter jejuni	112-7MS	cBAP	Positive
	112-5MS	cBAP	Positive
	1114-4	cBAP	Positive
	1019-22	cBAP	Positive
	1019-26	cBAP	Positive
Aeromonas hydrophila	1-B	sBAP, cBAP	Negative
Chromobacterium sp.	5	sBAP, cBAP	Negative
Escherichia coli	1-A	sBAP, cBAP	Negative
	4	sBAP, cBAP	Negative
	114-NM	sBAP, cBAP	Negative
Fluorescent *Pseudomonas* group	044	sBAP, cBAP	Negative
Gram-positive coccus, NFC	2	sBAP, cBAP	Negative
Hafnia alvei	1C	sBAP, cBAP	Negative
Proteus mirabilis	888	sBAP, cBAP	Negative
Pseudomonas aeruginosa	114-M	sBAP, cBAP	Negative
	FD-4	sBAP, cBAP	Negative

[a] CLI performed using optimized protocol.
[b] NFC, not further characterized.

brane which binds proteins primarily via hydrophobic and ionic interactions (Millipore); (ii) Zeta-Probe (0.45 μm), a high tensile-strength cationized nylon membrane carrying a high-density quatenary amine (Bio-Rad, Hercules, Calif.) which despite being designed primarily for DNA blotting, does have protein-blotting applications; and (iii) Hybond-ECL nitrocellulose (0.45 μm), which binds proteins via hydrophobic and electrostatic interactions and is marketed specifically for chemiluminescent Western blotting (immunoblotting) applications (Amersham Corp., Arlington Heights, Ill.). Strips of each of the membranes measuring approximately 0.5 by 7.0 cm were used. Bacterial cells were grown as described above, suspended in PBS, and standardized to an optical density of 0.25 at 280 nm. PVDF membranes were prewetted in 100% methanol prior to spotting of the whole-cell suspensions. The other membranes were not prewetted. Two microliters of each of the bacterial suspensions was applied to the membrane strips. After the cell suspensions were applied, the membranes were allowed to dry.

Initially, membrane strips were hybridized separately with various dilutions of three different antibody preparations. These antibody preparations included anti-*Campylobacter* hyperimmune rabbit serum, prepared as previously described (25); serum obtained from a 6-week-old commercial broiler chicken which had been experimentally colonized with *C. jejuni* at 2 days of age (30); and an affinity-purified, horseradish peroxidase (HRP)-labeled goat anti-*Campylobacter* antibody (Kirkegaard and Perry Laboratories [KPL], Gaithersburg, Md.). The first two antisera were prepared in our laboratories. Membrane strips were hybridized following the Western blot protocol described below. Rabbit or chicken serum immunoglobulin G antibodies were detected by utilizing a secondary isotype-specific, HRP-labeled goat anti-rabbit (KPL) or goat anti-chicken (KPL) antibody diluted to 0.2 μg/ml in blocking buffer. These membrane strips were subsequently hybridized for 1.5 h at room temperature and washed three times as described below. Dot blots were developed via exposure to either a one-component 3,3′,5,5′-tetramethylbenzidine (TMB) membrane substrate (KPL) for 5 min or a 4-chloro-1-naphthol (4C1N) substrate (Sigma, St. Louis, Mo.) for 10 to 20 min.

Finally, a variety of growth and selective agar media were evaluated to determine the nature of the effect, if any, of their composition on the CLI reaction. The media included nutrient agar (Oxoid), sBAP (Remel), cBAP (Remel), CVA (Remel), charcoal yeast extract agar (ATCC medium 1088), Mueller-Hinton agar (Remel), and MCCDA (Oxoid). Tables 1 and 2 indicate the various types of media evaluated for each of the bacterial strains used in the CLI assay.

Analysis of antigen preparations by SDS-PAGE and Western immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed to evaluate the reaction profile of the commercially available, HRP-labeled anti-*Campylobacter* antibody (KPL) against three different antigen preparations from a single, Lior 2, biotype I isolate of *C. jejuni* obtained from a cloacal swab of a commercial broiler chicken (F1BCB) (see Fig. 1 and Table 2). The first preparation consisted of surface outer membrane antigens that were extracted by a glycine-HCl acid extraction process (18). The second antigenic preparation consisted of intact *Campylobacter* cells that were formalin fixed (2). The third preparation consisted of unprocessed *C. jejuni* whole cells that were grown under microaerophilic conditions from frozen stock cultures on sBAP for 24 h and suspended in buffered saline. Total protein concentrations of each of the antigen preparations were determined and standardized by using the Bio-Rad dye binding assay. Antigens were separated by using SDS-polyacrylamide gels consisting of a 4% stacking gel and a 12% separating gel and were transferred to 0.45-μm-pore-size Hybond-ECL nitrocellulose (Amersham) according to the method of Towbin et al. (37), using a semidry transfer cell (Bio-Rad).

The transfer membrane was subsequently blocked for 1.5 h in a blocking buffer consisting of 3% nonfat dry milk (Difco, Detroit, Mich.), 0.02% Tween 20 (Sigma), and 0.01% thimerosal (Sigma) in 10 mmol of PBS (pH 7.4) (Sigma). The blots were rinsed three times in PBS–0.02% Tween 20 and hybridized for 1.5 h with a 0.5-μg/ml concentration of the anti-*Campylobacter* antiserum (KPL) prepared in blocking buffer. The Western immunoblots were again rinsed three times in PBS, treated with enhanced chemiluminescence (ECL) reagents (Amersham), and used to expose Kodak AR imaging film (Eastman Kodak, Rochester, N.Y.).

CLI protocol. The optimized CLI protocol is shown below (see Fig. 2). Circular (83-mm-diameter) Immobilon-P PVDF membranes (Millipore) were prewetted in 100% methanol. While still damp with methanol, the membranes were laid on agar plates or filter membranes containing putative *Campylobacter* colonies. Following the colony lift, the membranes were allowed to dry (10 to 15 min), and 2 μl of a positive-control, *C. jejuni* whole-cell preparation (suspended in 50% methanol) was spotted onto the edge of the membrane and allowed to dry. Blocking and hybridization steps were performed either in glass trays (for five or more membranes) or in large, disposable plastic weigh boats. To inactivate extraneous peroxidases, the membranes were incubated for 10 to 15 min either in commercial peroxidase buffer (KPL) or in 1 to 3% hydrogen peroxide

in PBS. The membranes were then transferred to blocking buffer consisting of either a commercially prepared blocking solution (KPL) or 3% bovine serum albumin plus 0.02% Tween 20 (Sigma) suspended in PBS. Blocking was carried out for 10 to 15 min, followed by a 5-min hybridization in blocking buffer containing 0.5 μg of the affinity-purified HRP-labeled goat anti-*Campylobacter* antibody (KPL) per ml. After the hybridization was complete, the membranes were rinsed twice for a minimum of 5 min in either a commercially prepared plate wash solution (KPL) or 0.02% Tween 20 in PBS and allowed to dry. Lastly, the membranes were reacted with the one-component, TMB membrane peroxidase system (KPL) for exactly 2 min, after which the reaction was stopped by rinsing the membranes in deionized water. The membranes were then air dried for 30 to 60 min to allow fading of any nonspecific background reactions. At this time, a replica of the positive blue colonies was marked onto a clear transparency which could be utilized to locate the position of the target colony or colonies easily on the plate or filter membrane and to quantify the total number of *Campylobacter* colonies.

The experiments reported here were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (13a).

RESULTS

Selection of membrane, antiserum, and membrane substrate. Initially, comparisons of membranes, antisera, and membrane substrates were performed with five different Lior serotypes (Lior 1, 2, 4, 17, and 36) of *C. jejuni* that had been isolated from cloacal swabs of commercial broiler chickens (Table 2). These strains were chosen because they represented the five most common serotypes found in commercial broiler chickens during an extensive evaluation of flocks on farms in the mid-Atlantic United States (32). In addition, two non-*Campylobacter* contaminants frequently isolated on cBAP were used as negative controls: one *Escherichia coli* strain (isolate LYC) and a gram-positive coccus (isolate SWC) that was not further characterized (Table 2).

The three types of membranes were evaluated on the basis of substrate color retention, background color reaction, and durability. Considering these factors, the PVDF membrane proved superior for the CLI. The Zeta-Probe membranes did not retain the positive color reaction of the colony imprints as well as the nitrocellulose or PVDF membranes. The hydrophobic PVDF membrane required minimal blocking and retained less background color than either of the two hydrophilic membranes. Finally, the nitrocellulose membrane was brittle and far less durable than either the PVDF or the Zeta-Probe membranes.

To evaluate the antiserum preparations, identically prepared membrane strips were hybridized with various dilutions of each of the antisera ranging from 1:1,000 to 1:10,000 for the chicken and hyperimmune rabbit antisera and from 1.0 to 0.05 μg/ml for the affinity-purified, HRP-labeled goat anti-*Campylobacter* antibody (KPL). Furthermore, membrane strips were run in duplicate, allowing for the evaluation of the two different substrates at each antibody concentration.

When TMB was utilized as the substrate, all three antibody preparations produced a positive blue reaction with each of the five *C. jejuni* serotypes throughout the range of dilutions. However, at dilutions of 1:5,000 or less, both the hyperimmune rabbit serum and the chicken serum showed nonspecific reactions with both of the negative-control isolates (data not shown). The 4C1N peroxidase substrate system was far less sensitive than the TMB substrate. When 4C1N was used, all five *C. jejuni* isolates were consistently identified by either the chicken or the hyperimmune rabbit serum samples only at dilutions of 1:5,000 or less. The direct immunoassay utilizing the HRP-labeled goat anti-*Campylobacter* antibody and 41CN resulted in only weakly positive reactions to each of the *C. jejuni* strains even at the highest concentration tested (1.0 μg/ml) (data not shown). Given these results, development of the CLI protocol proceeded using PVDF membranes, affinity-purified HRP-labeled anti-*Campylobacter* antibody (KPL), and TMB membrane substrate (KPL).

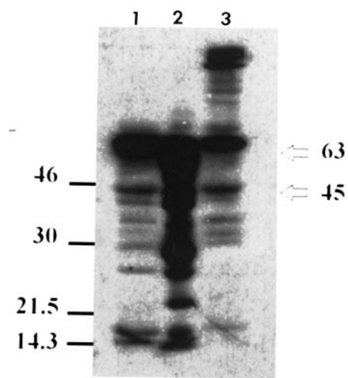

FIG. 1. ECL Western blot of *C. jejuni* F1BCB antigen preparations hybridized with HRP-labeled, goat anti-*Campylobacter* antibody (KPL). Positions of molecular mass markers (Amersham) (in kilodaltons) are indicated on the left. The 63-kDa flagellin and 45-kDa major outer membrane protein are indicated. Lane 1, whole-cell preparation; lane 2, 0.2 M glycine-extracted proteins (18) from F1BCB; lane 3, formalin-fixed whole cells (2). Proteins (ca. 7.6 μg per well) were separated via SDS-PAGE with a 12% separating gel overlaid by a 4% stacking gel and transferred to nitrocellulose. The Western blot was hybridized at a concentration of 0.2 μg/ml. Following addition of ECL reagents, Kodak AR imaging film (Eastman Kodak Co.) was exposed for 2 min.

Antibody recognition profile determined by Western immunoblotting. Although the antibody preparation had been affinity purified by the manufacturer (KPL), its reactive profile had not been extensively evaluated. Using ECL Western blot techniques, the KPL anti-*Campylobacter* antibody was evaluated for its immunoreactivity to three different antigen preparations of the same Lior 2 serotype strain of *C. jejuni* (F1BCB) (Fig. 1). These preparations included a whole-cell preparation (Fig. 1, lane 1), glycine-extracted cell surface proteins (lane 2), and a formalin-fixed whole-cell preparation (lane 3). The antibody showed a noticeably enhanced response to the glycine-extracted cell surface proteins (Fig. 1, lane 2). Furthermore, in each of the *C. jejuni* antigen preparations, the antibody reacted predominantly to the 63- and 45-kDa bands. Previous studies in our laboratory (data not shown) and also studies described by others (3, 8, 20–22, 24, 27, 38) have putatively identified these two antigens as flagellin and a major outer membrane porin, respectively. Other immunoreactive whole-cell antigens included a low-molecular-mass antigen of ca. 14 kDa, in addition to several antigens in the 29- to 44-kDa range.

Effect of membrane hydrophobicity and methanol wetting. Although the dot blots provided useful data with respect to the selection of membrane, antibody, and substrate, the experimental conditions were quite different from those encountered for the CLI. CLI experiments were performed initially to determine how these new conditions would affect the sensitivity and specificity of the assay and the viability of the lifted bacterial colonies.

The hydrophobic nature of PVDF membranes requires that these membranes be wetted in methanol prior to the colony lift to allow bacterial proteins to bind. For classical Western blotting applications, PVDF is typically wetted in methanol and equilibrated in Western transfer buffer containing 20% (vol/vol) methanol prior to electrotransfer of proteins. For the CLI, the membranes were used either directly out of the prewetting methanol solution or after equilibration in a PBS solution. The binding of the cells to the membrane and the viability of the postlift bacterial colonies were determined under both conditions. A series of sequential lifts was performed on well-iso-

lated *C. jejuni* colonies. Separate membranes were used to lift the colonies within 15 s after removal from 100% methanol or after equilibration for 5 to 10 min in PBS. The membranes were allowed to dry, blocked, hybridized, and developed as described above. The CLI results for the two membrane-wetting techniques were comparable (data not shown). The use of 100% methanol kept the postlift drying time to a minimum, roughly 5 to 10 min as opposed to 30 min, or more, for the membranes which were equilibrated in PBS. It was important to ensure that the membrane was dried prior to initiation of the blocking and hybridization steps in order to minimize the nonspecific background coloration of the membrane after exposure to the TMB substrate. Brief exposure of the *Campylobacter* colonies to 100% methanol did not prevent the successful subculture of these colonies for subsequent biochemical and serological analyses.

Optimization of the CLI procedure. A series of checkerboard-type assays was performed to optimize both the antibody concentration and the hybridization time with respect to nonspecific background reactions, color intensity, and color retention for the CLI. The strains of *Campylobacter* utilized in these studies included *C. jejuni* F1BCB, *C. coli* ATCC 33559, and *C. lari* ATCC 35221. These bacteria were grown on agar media and lifted with membranes which had been prewetted in 100% methanol. Whole membranes were divided into sections, each having contacted about 20 to 80 colonies of *Campylobacter*. Hybridization times were varied from 1 to 20 min, with a series of antibody concentrations ranging from 0.067 to 1.5 μg/ml (data not shown). It was determined that a 5-min hybridization with an antibody concentration of 0.5 μg/ml provided reproducible and sensitive detection of each of the *Campylobacter* isolates tested. Furthermore, these parameters resulted in optimal color intensity and the most persistent color retention on the membranes. A flow chart depicting the entire optimized CLI protocol is shown in Fig. 2.

Evaluation of antibody specificity using the CLI. By using the optimized CLI protocol (Fig. 2), 28 ATCC type strains of *Campylobacter* spp., *Helicobacter* spp., and other related or potential water- or foodborne contaminants, in addition to 15 ATCC reference strains of *Campylobacter* spp. (Table 1), were evaluated for cross-reactivity to the HRP-labeled goat anti-*Campylobacter* antibody (KPL). The inocula were adjusted so that colonies were isolated on the agar surface, and where possible, a separate region of the same plate was inoculated with a positive-control *C. jejuni* strain. Of the 48 ATCC cultures, only the *C. jejuni*, *C. coli*, *C. lari*, and *C. hyointestinalis* strains reacted positively with the HRP-labeled goat anti-*Campylobacter* antibody. None of the non-*Campylobacter* strains were detected.

Pure cultures of cloacal swab isolates from commercial broiler chickens were tested. The same five *C. jejuni* Lior serotypes (Lior 1, 2, 4, 17, and 36) and two non-*Campylobacter* strains which were previously tested via dot blots were lifted directly from agar medium and reevaluated with the optimized CLI protocol (Table 2). Each of the *Campylobacter* strains produced a positive reaction with HRP-labeled, goat anti-*Campylobacter* antibody in the CLI, confirming that the lifting protocol and membrane binding conditions did not adversely affect the detection of these different Lior serotypes. Neither of the non-*Campylobacter* strains was detected.

Isolates from retail poultry products were also tested. The CLI results using pure cultures of five representative *C. jejuni* strains that were subcultured from these original retail sample cultures are listed in Table 2. Each of the campylobacters was CLI positive. Furthermore, 11 non-*Campylobacter* background contaminants from retail poultry rinses were identified and subcultured for direct CLI evaluation. CLIs using pure cultures of these common contaminants were consistently negative (Table 2).

Effect of agar media on CLI results. Each of the agar media tested was used successfully in the CLI; however, the blood-containing media (sBAP, cBAP, and CVA) resulted in increased levels of nonspecific background coloration of the membranes when reacted with the TMB substrate. This effect was likely a result of peroxidases released from the erythrocytes in the media and subsequently retained on the membrane. A peroxidase-blocking step was incorporated after the membranes were allowed to dry in order to reduce this background. Interestingly, the addition of this step not only reduced background but also appeared to enhance color retention of the *Campylobacter* colonies. Peroxidase blocking was therefore added as a permanent step in the CLI protocol regardless of the presence or absence of blood in the agar medium.

Evaluation of optimized CLI with retail poultry samples. To determine the applicability of the optimized CLI procedure (Fig. 2), retail poultry rinses and experimentally inoculated poultry products were cultured directly on cBAP or filtered through Millipore-MF membranes (0.2-μm pores) which were then placed faceup onto cBAP (31). Following 18 to 24 h of incubation, the resultant pinpoint colonies that are typical of *Campylobacter* spp. were difficult to identify or quantify by direct visual observation, particularly when grown on the filter membranes (Fig. 3A and C). However, distinct colony imprints were readily enumerable on the CLI membrane (Fig. 3B and D). Although not readily apparent in Fig. 3, these cultures are often heavily contaminated, but the CLI procedure has repeatedly proven effective in detecting the target colonies of *Campylobacter* that are often much smaller and/or overgrown by contaminants.

DISCUSSION

A CLI method was developed for the specific detection of enteropathogenic *Campylobacter* spp. To optimize this procedure, various membranes, antisera, membrane substrates, and bacteriological media were compared. Several factors were taken into consideration for selecting the best membrane for use in the CLI, including substrate color retention, background, and durability. PVDF was determined to be the membrane of choice. The hydrophobic nature of nonwetted PVDF membranes minimized blocking requirements, confined the immunologic reactions to the surface of the membrane, and allowed the development of a more rapid assay.

The use of immunological methods for the identification of enteropathogenic *Campylobacter* spp. is hampered by the serologic diversity of these organisms. The cell surface antigens of *Campylobacter* spp. are complex, and relatively few have been characterized in detail (8, 23). To date, the best-described proteins include the 58- to 66-kDa flagellin (8, 20, 21, 24, 38), the 41- to 45-kDa major outer membrane proteins (4, 8, 22, 38), and several proteins in the 27- to 36-kDa range (5, 14, 27, 28). Immunologic cross-reactivity among *Campylobacter* strains has been described (3, 4, 18, 29). However, the nature of this apparent antigenic cross-reactivity has not been extensively evaluated among enteropathogenic *Campylobacter* spp., and some of the antigenic homology among these proteins may be due to conserved internal domains (7, 19). Thus, a unique or conserved surface-exposed antigen on enteropathogenic *Campylobacter* spp., which could be valuable in immunodiagnosis, has yet to be identified and characterized. In the absence of this information, the potential use of monoclonal antibodies

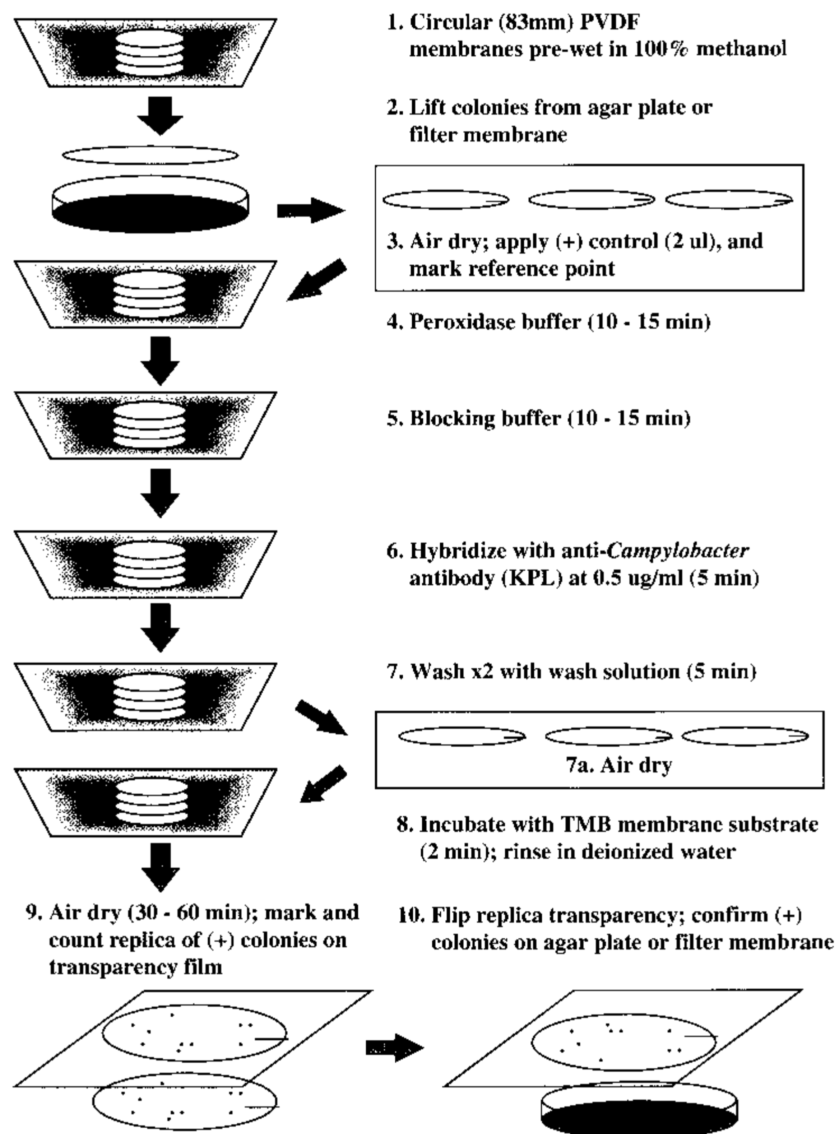

FIG. 2. Flow chart of optimized CLI protocol.

is limited. Thus, we focused our efforts on polyclonal antibody preparations for the development of the CLI.

Chicken (30) and rabbit (25) antiserum preparations were chosen for evaluation because specific reactivity to *C. jejuni* had been previously demonstrated with these sera. Although each of the antiserum preparations produced positive reactions to the five Lior serotype isolates of *C. jejuni* screened via dot blots, the chicken and rabbit antisera prepared in our laboratories showed significant cross-reactions with the non-*Campylobacter* contaminants when used in the CLI protocol. These cross-reactions likely resulted because neither of these serum preparations was affinity purified. Furthermore, even low levels of cross-reacting antibodies may have been amplified significantly because of the need to utilize an HRP-labeled, anti-isotypic secondary antibody for detection. For these reasons, the rabbit and chicken antisera were not further tested.

Alternatively, the goat anti-*Campylobacter* antibody preparation (KPL) had been affinity purified and produced strong reactivity to *C. jejuni* antigens as observed with Western immunoblotting (Fig. 1). Two additional advantages to using the commercially prepared antibody were that it saved time and reagents, since it could be utilized in a direct assay, and that it offered the advantage of having surpassed the manufacturer's quality assurance evaluations to guarantee lot-to-lot consistency. Thus, this antibody preparation was chosen for further evaluation of specificity and sensitivity and ultimately for use in the optimized CLI.

The TMB membrane substrate (KPL) proved to be more sensitive than 4C1N. This sensitivity allowed the use of greater dilutions of the polyclonal antisera, which was important to minimize potential cross-reactivity with non-*Campylobacter* strains. This enhanced sensitivity also reduced the volume of reagent required and allowed for a more rapid development time. Unlike the 4C1N, which first must be dissolved in methanol, the TMB membrane substrate was provided as a single component, which eliminated the need for additional reagents and simplified the procedure. All of these factors made the CLI using the TMB substrate a more rapid, sensitive, and

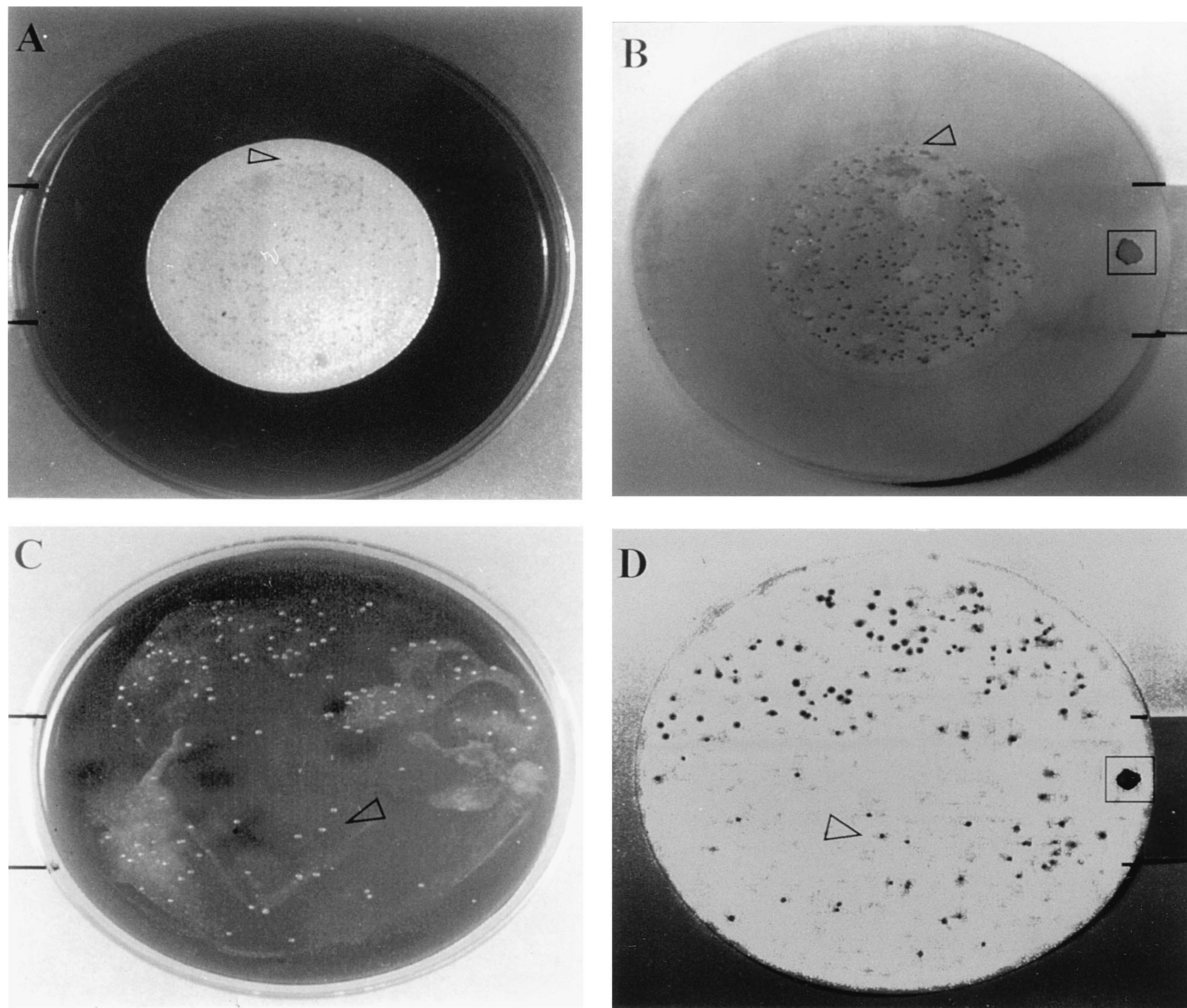

FIG. 3. Filter membrane and direct plate growth of *C. jejuni* with corresponding CLIs. Samples of retail poultry washes were either inoculated directly onto cBAP (Remel) or filtered through 0.2-μm-pore-size Millipore-MF filter membranes which were transferred faceup onto cBAP. Following a 24-h microaerophilic incubation at 42°C, a CLI was performed on both cultures, the filter membrane transfer and the selective agar plate. (A) Culture of retail poultry wash grown on filter membrane transfer on cBAP; (B) positive CLI imprint of *Campylobacter* organisms from filter membrane shown in panel A; (C) culture of retail poultry wash grown on cBAP; (D) positive CLI imprint of *Campylobacter* organisms from agar plate shown in panel C. Representative *Campylobacter* colonies (A and C) and the mirror image location of the corresponding colorimetric positive reaction of the colony imprints on the CLI (B and D) are indicated (arrowheads). The positive-control reaction is also shown (boxed areas), and lines at the borders of the plates and CLI imprints are used for orientation.

cost-effective procedure, qualities which would undoubtedly be important in large-scale commercial applications of this technology.

Despite the use of selective media, the overwhelming bacterial load in the poultry rinse samples often would allow some breakthrough contaminating organisms to persist. Even to the trained eye, at this early stage of bacterial growth, the colony morphology of some of these breakthrough contaminants was virtually indistinguishable from that of typical *Campylobacter* colonies. This problem was especially acute when the isolates were grown on the filter membranes. Thus, identifying the specificity of the HRP-labeled, goat anti-*Campylobacter* antibody to these contaminants was imperative if the assay was to be used with confidence. Fortunately, on the basis of colony morphology, microscopic examination of wet-mount preparations, Gram stains, and biochemical identification, it appeared that only a few bacterial species accounted for the majority of these breakthrough organisms, and each of these noncampylobacters reacted negatively with the goat anti-*Campylobacter* antibody (KPL).

The objectives for the development of the CLI were rapid identification and enumeration of *C. jejuni*, *C. coli*, and *C. lari*, the three most common causes of human campylobacteriosis. However, the HRP-labeled goat anti-*Campylobacter* antibody did cross-react with *C. hyointestinalis* when used in the optimized CLI protocol, despite having undergone affinity purification. The nature of the antigen(s) responsible for this cross-reactivity has not been investigated. Although *C. hyointestinalis* has been documented as an infrequent cause of human diarrheal disease (10), this species is associated primarily with veterinary infections, particularly proliferative enteritis in pigs (9). To our knowledge, there have been no reports of the

association of this bacterium with poultry or retail meat products. *C. hyointestinalis* can be differentiated from other *Campylobacter* species by its sensitivity to cephalothin, resistance to nalidixic acid, ability to grow at 25°C, and ability to grow anaerobically in the presence of 0.1% trimethylamine-*N*-oxide hydrochloride (TAMO) (10).

Since its inception in 1975 (11), the colony lift has been used extensively with radiolabeled DNA probes, and, more recently, with colorimetric and chemiluminescent detection techniques, for the genotypic identification of bacterial colonies. Subsequently, these DNA probe-based colony lift techniques have been utilized for the specific detection and enumeration of human pathogens isolated from both clinical and food samples. However, to our knowledge, there are limited reports in the literature on the use of a CLI for detection and enumeration of target organisms. The detection of group D *Salmonella typhimurium* from poultry by using a CLI has been reported (15), and a commercial kit for this process is available (Check Point; KPL). In addition, a CLI using a monoclonal antibody for the identification of *Listeria monocytogenes* has been described (1). However, we believe that our use of the CLI for the rapid, specific identification and enumeration of *C. jejuni*, *C. coli*, and *C. lari* from retail poultry products is the first such report described in the literature. This CLI method can be performed more simply, rapidly, and efficiently than DNA probe-based techniques.

Despite the numerous advances in isolation methods, *Campylobacter* spp. remain fastidious, labor-intensive organisms to culture and identify. Until now, adequate means of quantifying this pathogen in naturally occurring sources have not been developed. A significant advantage of the CLI technique is that it can successfully detect and enumerate *C. jejuni*, *C. coli*, and *C. lari* after a minimal period of growth, and it can greatly assist those with little experience in identifying these pathogens. Importantly, brief exposure of the *Campylobacter* colonies to 100% methanol did not adversely affect the successful subculture of identified *Campylobacter* colonies, allowing for subsequent biochemical and serological analyses. Thus, in combination with our modified bacterial sampling techniques (31), the CLI appears to be vastly superior to standard methods for quantitation, e.g., most probable number and five-colony pick methods. Specifically, the CLI is less labor-intensive and resource intensive and is more rapid, specific, sensitive, and accurate. A large-scale statistical evaluation of our quantitative methods, which incorporate the CLI, in comparison with the most probable number technique is under way in our laboratories.

In conclusion, the CLI method provides a powerful tool for the rapid and specific identification of *C. jejuni*, *C. lari*, and *C. coli*, the causative agents of the majority of human campylobacteriosis in the United States and throughout the world. In addition to its use in monitoring retail poultry and food products, potential applications include rapid screening of clinical, veterinary, and environmental culture samples.

ACKNOWLEDGMENTS

This work was supported by USDA contract FSIS-15-W-94, University of Maryland subcontract 2542801, USDA CSREES grant 92-COOP-1-7520, Interagency agreement 22U592CRS228051513, and Naval Medical Research Institute grants 62787A 001.01.EOX1289 and AA 1761319.B992 00# 6422300688923C.

We thank E. Weiss for his advice and critical review of the manuscript.

REFERENCES

1. **Bhunia, A. K., and M. G. Johnson.** 1992. Monoclonal antibody specific for *Listeria monocytogenes* associated with a 66-kilodalton cell surface antigen. Appl. Environ. Microbiol. **58:**1924–1929.
2. **Black, R. E., M. M. Levien, M. L. Clements, C. R. Young, A.-M. Svennerholm, and J. Holmgren.** 1987. Protective efficacy of a killed whole vibrio oral cholera vaccine with and without the B subunit of cholera toxin. Infect. Immun. **55:**1116–1120.
3. **Blaser, M. J., J. A. Hopkins, and M. L. Vasil.** 1984. *Campylobacter jejuni* outer membrane proteins are antigenic for humans. Infect. Immun. **43:**986–993.
4. **Blaser, M. J., D. N. Taylor, and R. A. Feldman.** 1983. Epidemiology of *Campylobacter jejuni* infections. Epidemiol. Rev. **5:**157–176.
5. **De Melo, M. A., and J.-C. Pechere.** 1990. Identification of *Campylobacter jejuni* surface proteins that bind to eukaryotic cells in vitro. Infect. Immun. **58:**1749–1756.
6. **Deming, M. S., R. V. Tauxe, P. A. Blake, S. E. Dixon, B. S. Fowler, T. S. Jones, E. A. Lockamy, C. M. Patton, and R. O. Sikes.** 1987. *Campylobacter* enteritis at a university: transmission from eating chicken and from cats. Am. J. Epidemiol. **126:**526–534.
7. **Dubreuil, J. D., M. Kostrzynska, S. M. Logan, L. A. Harris, J. W. Austin, and T. J. Trust.** 1990. Purification, characterization, and localization of a protein antigen shared by thermophylic campylobacters. J. Clin. Microbiol. **28:**1321–1328.
8. **Dunn, B. E., M. J. Blaser, and E. L. Snyder.** 1987. Two-dimensional gel electrophoresis and immunoblotting of *Campylobacter* outer membrane proteins. Infect. Immun. **55:**1564–1572.
9. **Gebhart, C. J., G. E. Ward, K. Chang, and H. J. Kurtz.** 1983. *Campylobacter hyointestinalis* (new species) isolated from swine with lesions of proliferative ileitis. Am. J. Vet. Res. **44:**361–367.
10. **Goossens, H., and J.-P. Butzler.** 1992. Isolation and identification of *Campylobacter* spp., p. 93. *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
11. **Grunstein, M., and D. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72:**3961–3965.
12. **Harris, N. V., D. Thompson, D. C. Martin, and C. M. Nolan.** 1986. A survey of *Campylobacter* and other bacterial contaminants of pre-market and retail poultry and meats, King County, Washington. Am. J. Public Health **76:**401–406.
13. **Harris, N. V., N. S. Weiss, and C. M. Nolan.** 1986. The role of poultry and meats in the etiology of *Campylobacter jejuni/coli* enteritis. Am. J. Public Health **76:**407–411.

13a. **Institute of Laboratory Animal Resources.** 1986. Guide for the care and use of laboratory animals. U.S. Department of Health and Human Services publication (NIH) 86-23. Institute of Laboratory Animal Resources, National Research Council, Washington, D.C.

14. **Kervella, M., J.-M. Pages, Z. Pei, G. Grollier, M. J. Blaser, and J.-L. Fauchere.** 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect. Immun. **61:**3440–3448.
15. **Lamichhane, C. M., S. W. Joseph, E. T. Mallinson, and M. Blankfard.** 1994. Rapid assay for identifying group D *Salmonella* in poultry, p. 115. *In* Proceedings of the 43rd Western Poultry Disease Conference. University of California, Davis.
16. **Lior, H.** 1984. New extended biotyping scheme for *Campylobacter jejuni*, *Campylobacter coli*, and "*Campylobacter laridis*." J. Clin. Microbiol. **20:**636–640.
17. **Lior, H., D. L. Woodward, J. A. Edgar, L. J. LaRoche, and P. Gill.** 1982. Serotyping of *Campylobacter jejuni* by slide agglutination based on heat labile antigenic factors. J. Clin. Microbiol. **15:**761–768.
18. **Logan, S., and T. J. Trust.** 1983. Molecular identification of surface protein antigens of *Campylobacter jejuni*. Infect. Immun. **42:**675–682.
19. **Logan, S., and T. J. Trust.** 1986. Location of epitopes on *Campylobacter jejuni* flagella. J. Bacteriol. **168:**739–745.
20. **Nachamkin, I., and A. M. Hart.** 1986. Common and specific epitopes of *Campylobacter* flagellin recognized by monoclonal antibodies. Infect. Immun. **53:**438–440.
21. **Nachamkin, I., and X.-H. Yang.** 1988. Isoelectric focusing of *Campylobacter jejuni* flagellin: microheterogeneity and restricted antigenicity of charged species with a monoclonal antibody. FEMS Microbiol. Lett. **49:**235–238.
22. **Newell, D. G., H. McBride, and A. D. Pearson.** 1984. The identification of outer membrane proteins and flagella of *Campylobacter jejuni*. J. Gen. Microbiol. **130:**1201–1208.
23. **Newell, D. G., and I. Nachamkin.** 1992. Immune responses directed against *Campylobacter jejuni*, p. 201–206. *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
24. **Nuijten, P. J. M., B. A. M. van der Zeijst, and D. G. Newell.** 1990. Localization of immunogenic regions on the flagellin proteins of *Campylobacter jejuni* 81116. Infect. Immun. **59:**1100–1105.
25. **Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell.** 1993. Colonization of broiler chickens by waterborne *Campylobacter jejuni*. Appl. Env. Microbiol. **59:**987–998.
26. **Pearson, A. D., and T. D. Healing.** 1992. The surveillance and control of

Campylobacter infection. Communicable Dis. Rep. **12:**R133–R139.
27. **Pei, Z., and M. J. Blaser.** 1993. PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. J. Biol. Chem. **268:**18717–18725.
28. **Pei, Z., R. T. Ellison III, and M. J. Blaser.** 1991. Identification, purification and characterization of major antigenic proteins of *Campylobacter jejuni*. J. Biol. Chem. **266:**16363–16369.
29. **Rautelin, H., and T. U. Kosunen.** 1993. An acid extract as a common antigen in *Campylobacter coli* and *Campylobacter jejuni* strains. J. Clin. Microbiol. **17:**700–701.
30. **Rice, B. E., D. M. Rollins, and S. W. Joseph.** 1995. Immune response enhancement and colonization reduction in broiler chickens immunized with a modified prototype oral vaccine initially developed for human prophylaxis against enteropathogenic *Campylobacter*, abstr. C3/11, p. 50. *In* 8th International Workshop on Campylobacters, Helicobacters, and Related Organisms. Central Veterinary Laboratory (Weybridge), Addlestone, Surrey, United Kingdom.
31. **Rollins, D. M., L. H. Lee, B. E. Rice, C. Lamichhane, and S. W. Joseph.** Development of a quantitative methodology to estimate the number of enteropathogenic *Campylobacter* on fresh poultry products. *In* D. G. Newell and J. Ketley (ed.), *Campylobacter* VIII, in press. Plenum Publishing Corp., New York.
32. **Rollins, D. M., M. Shahamat, B. E. Rice, and S. W. Joseph.** Unpublished data.
33. **Skirrow, M. B.** 1987. A demographic survey of *Campylobacter*, *Salmonella*, and *Shigella* infections in England. Epidemiol. Infect. **99:**647–657.
34. **Tauxe, R. V.** 1992. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 9–10. *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
35. **Tauxe, R. V., N. Hargrett-Bean, C. M. Patton, and K. W. Wachsmuth.** 1988. *Campylobacter* isolates in the United States, 1982–1986. Morbid. Mortal. Weekly Rep. **37:**1–3.
36. **Taylor, D. N.** 1992. *Campylobacter* infections in developing countries, p. 20–30. *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
37. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
38. **Wenman, W. M., J. Chai, T. J. Louie, C. G. Goudreau, H. Lior, D. G. Newell, A. D. Pearson, and D. E. Taylor.** 1985. Antigenic analysis of *Campylobacter* flagellar protein and other proteins. J. Clin. Microbiol. **21:**108–112.