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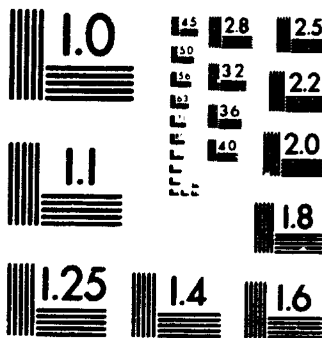

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DEVELOPMENT OF SIMPLE AND ECONOMICAL METHODS FOR CULTURING CAMPYLOBACTER AND FOR PRODUCING ANTI-CAMPYLOBACTER ANTIBODIES FOR ENZYME IMMUNOASSAY

by

Vandana Chandan, B.Sc.

A thesis submitted to
the faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of
Master of Science

Department of Biology
Carleton University
Ottawa, Ontario

December 06, 1991

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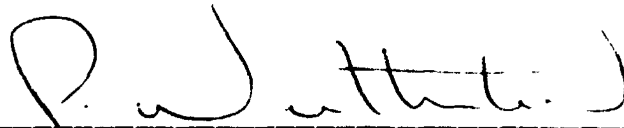
Thesis Supervisor



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ABSTRACT

This thesis critically examines two essential steps required for the development of rapid detection test for the foodborne pathogens Campylobacter jejuni and Campylobacter coli: 1) the enrichment of Campylobacter cells into the detection limits of the tests and 2) the preparation of immunoreagents (antigens and antibodies) to these bacteria.

Campylobacter has been considered to be microaerophilic in nature. This has necessitated the use of an expensive microaerobic gas mixture (5% O_2, 10% CO_2 and 85% N_2) for its growth. This atmosphere is maintained in anaerobic jars and the process of culturing Campylobacter is therefore laborious and expensive. In this study, I have shown that Campylobacter can be grown efficiently in a simple atmosphere of 10% CO_2 in moist air. This greatly reduces the cost and labor of culturing campylobacters in liquid and on solid media. Eighteen strains of the most commonly isolated Campylobacter serotypes grew as good as or better in the 10% CO_2 in moist air atmosphere than in the microaerobic atmosphere.

For the preparation of antigens, Campylobacter cells were heated in the presence of ethylenediamine tetraacetate (EDTA) to dissociate their antigens into non-sedimentable forms. The recovery of lipopolysaccharide (LPS) was greater in EDTA extracts than that obtained from a standard hot phenol method. The extracted antigens were used in cloth based enzyme immunoassays and to produce anti-Campylobacter antibodies in egg yolks.

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Chicken eggs are an excellent source of obtaining large volume of high titre antibodies. The volume and titre of antibodies obtained from eggs is better than that obtained from the serum of animals or birds (e.g. rabbits, guinea pigs, chicken, etc.). In order to develop a method for producing and extracting antibodies, egg laying hens were immunized with EDTA heat extracted antigens to obtain antibodies from their egg yolks. The anti-Campylobacter antibodies produced were specific to Campylobacter antigens. The titre of antibodies extracted from the egg yolks was measured by using direct cloth based enzyme immunoassay. These antibodies could also detect Campylobacter antigens on immunoblots.

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TABLE OF CONTENTS

ABSTRACT i

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS iv

LIST OF FIGURES vi

LIST OF TABLES ix

ABBREVIATIONS x

GENERAL INTRODUCTION 1

CHAPTER 1. SIMPLIFIED GROWTH OF CAMPYLOBACTER JEJUNI AND CAMPYLOBACTER COLI 19

ABSTRACT 20

INTRODUCTION 20

MATERIALS AND METHODS 22

Campylobacter strains used 22

SDS-polyacrylamide gel electrophresis 25

RESULTS 26

Growth of Campylobacter in solid media 26

Growth of Campylobacter in liquid media 27

DISCUSSION 31

CHAPTER 2. SIMPLIFIED EXTRACTION OF CAMPYLOBACTER JEJUNI AND CAMPYLOBACTER COLI ANTIGENS 40

ABSTRACT 41

INTRODUCTION 41

MATERIALS AND METHODS 42

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Campylobacter strains used and growth conditions 42
Aqueous hot phenol method 43
EDTA heat extraction method 44
Protein assay 44
Assay of 2-keto-3-deoxyoctanoic acid (KDO) 45
SDS-polyacrylamide gel electrophoresis 45
RESULTS 46
DISCUSSION 54

CHAPTER 3. PRODUCTION, EXTRACTION AND CHARACTERIZATION OF ANTI-CAMPYLOBACTER ANTIBODIES FROM EGG YOLKS OF CHICKEN 56
ABSTRACT 57
INTRODUCTION 57
MATERIALS AND METHODS 59
Campylobacter strains used 59
Preparation of Campylobacter antigens 59
Immunization of chicken 60
Extraction and purification of antibodies from egg yolks 60
Slide-agglutination test 61
Immunodiffusion 61
SDS-PAGE 62
Western-blotting 62
Cloth based enzyme immunosorbent assay (CEIA) 63
RESULTS 64
DISCUSSION 90

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CONCLUSIONS.. 92
REFERENCES.. 95

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LIST OF FIGURES

Figure 1 Non-competitive solid phase EIA 8

Figure 2 Competitive solid phase EIA 10

Figure 3 Structure of Salmonella typhimurium LPS 14

Figure 1.1 SDS-PAGE of Campylobacter whole cells stained with Coomassie Brilliant Blue to visualize protein 29

Figure 1.2 Growth of Campylobacter coli LIO20 in Brucella Broth and Mueller-Hinton Broth in both the microaerobic and 10% CO_2 in moist air atmospheres, with or without 10% lysed sheep blood 32

Figure 1.3 Growth of Campylobacter jejuni LIO4 in Brucella Broth and Mueller-Hinton Broth in both the microaerobic and 10% CO_2 in moist air atmospheres, with or without 10% lysed sheep blood 34

Figure 1.4 Growth of 6 strains of Campylobacter in Brucella Broth (BBB) and Mueller-Hinton Broth (MHB) with 10% lysed sheep blood in 10% CO_2 in moist air atmosphere 36

Figure 2.1 Coomassie Brilliant Blue SDS-PAGE pattern of Campylobacter extracts from EDTA heat and phenol extraction methods and their comparison with whole cell extracts 50

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Figure 2.2 Silver stain SDS-PAGE pattern of _Campylobacter_ LPS extracts (proteinase K digested) from the EDTA heat and phenol extraction methods and their comparison with whole cell extracts. 52

Figure 3.1 Immunodiffusion of egg yolk extracts for isotyping antibodies present 67

Figure 3.2 Immunodiffusion of chicken serum for isotyping of antibodies present 69

Figure 3.3 Changes in antibody titre in egg yolk extracts using cloth based enzyme immunoassay after inoculation with EDTA heat extracted antigens of _Campylobacter_. 72

Figure 3.4 Application of egg yolk anti-_Campylobacter_ antibodies in western-blotting of _Campylobacter_ antigens (proteins and LPS predominantly) separated on SDS-PAGE. 74

Figure 3.5 Application of serum anti-_Campylobacter_ antibodies, in western-blotting of _Campylobacter_ antigens (proteins and LPS predominantly) separated on SDS-PAGE. 77

Figure 3.6 Application of egg yolk anti-_Campylobacter_ antibodies in immuno-blotting of _Campylobacter_ LPS separated on SDS-PAGE. 80

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Figure 3.7 Application of serum anti-Campylobacter antibodies, in immuno-blotting of Campylobacter LPS separated on SDS-PAGE.................. 82

Figure 3.8 Application of egg yolk anti-Campylobacter antibodies to immuno-blotting of protein antigens from 18 serotypes of C. jejuni and C. coli.. 84

Figure 3.9 Application of egg yolk anti-Campylobacter antibodies to immuno-blotting of LPS antigens from 9 serotypes of C. jejuni and C. coli.......................... 86

Figure 3.10 Immunoblots to show cross-reactivity of egg yolk extractions with other bacterial species.............. 88

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LIST OF TABLES

Table 1.1 List of *Campylobacter* Strains used in this study........... 23

Table 1.2 Growth of *Campylobacter* Strains on Mueller-Hinton Agar with or without sheep blood in a Microaerobic Atmosphere or 10% CO_2 (in Moist Air)........ 28

Table 2.1 Comparison of the recovery of lipopolysaccharide between two extraction methods as measured by KDO concentration........ 47

Table 2.2 Comparison of the presence of proteins in lipopolysaccharide samples extracted by two different LPS extraction methods........ 48

Table 3.1 Slide-agglutination test for semi-quantitation of anti-*Campylobacter* antibodies produced in egg yolks of immunized chicken over a period of 16 weeks from the day of first inoculation................ 65

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ABBREVIATIONS

A_{548}, A_{600}	absorbance at 548 and 600 nm
BA	Brucella agar
BAB	Brucella agar with 10% unlysed sheep blood
BB	Brucella broth
BBB	Brucella broth with 10% lysed sheep blood
BHI	brain heart infusion broth
^{0}C	degree Celsius
CBFA	Campylobacter blood free selective agar
CEIA	cloth-based enzyme immunoassay
CFU	colony forming units
cm	centimeter
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
EIA	enzyme immunoassay
FBP	ferrous sulphate, sodium metabisulfite and sodium pyruvate
g	gram
g	one unit of gravitational force on a unit mass
H+L	high and low
kDa	kilo Daltons
KDO	2-keto-3-deoxyoctanoic acid
l	liter

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Lane #	Lane number
LPS	lipopolysaccharide
M	molar
mA	microampere
Mab	monoclonal antibody
mg	milligram (10^{-3} g)
MH	Mueller-Hinton broth
MHA	Mueller-Hinton agar
MHAB	Mueller-Hinton agar with 10% unlysed sheep blood
MHB	Mueller-Hinton broth with 10% lysed sheep blood
min	minute
ml	milliliter (10^{-3} l)
µg	microgram (10^{-6} g)
µl	microliter (10^{-6} l)
MM	molecular mass
nm	nanometer
p	probability
PBS	0.01 M phosphate-buffered (pH 7.2) in saline (0.85% NaCl)
PBST	PBS containing 0.3% Tween 20
PEG	polyethylene glycol
RNA	ribonucleic acid
rpm	revolutions per min
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

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SOD	superoxide dimutase
TMB	3, 3', 5, 5', - tetramethylbenzidine
TSB	trypticase soy broth
w/v	weight/volume

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GENERAL INTRODUCTION

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Several Gram-negative bacteria have been recognized as agents of foodborne diseases. These include, among others, Salmonella sp., Campylobacter sp., Yersenia enterocolitica, Escherchia coli, Vibrio parahaemolyticus and Vibrio cholerae (Doyle, 1986). The ingestion of untreated water, consumption of undercooked meat, unpasteurized milk and contact with infected animals or humans have been major sources of human enterocolitis.

Campylobacter jejuni and Campylobacter coli have been referred to as "the next Salmonella", since the frequency of their isolation from diarrhea in patients is increasing to the level of Salmonella isolations (Stern and Kazmi, 1989). In many instances, the campylobacteriosis, caused by these bacteria is more severe and longer in duration than salmonellosis. Patients suffer symptoms of fever, malaise, headache and abdominal cramps followed by profuse diarrhea often with blood, pus or mucus (Cunningham, 1987). The cost of this disease to society is very high since the patients are absent from work for 10 to 14 days, often requiring hospitalization and treatment with antibiotics (Blaser et al., 1979).

Campylobacters have also been implicated as the cause of many other conditions such as abortion in sheep, dysentery in cattle, hepatitis in poultry and urinary infection in man. The main reservoir of these pathogens is the intestinal tract of animals with an almost 100% occurrence in poultry. They have also been frequently isolated from pork, beef and veal (Lammerding et al., 1988). Therefore Campylobacter contamination in meat is considerable (e.g. the carcass of a chicken may contain as high as 10^6 campylobacters). These bacteria are particularly important as foodborne pathogens in

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Ontario where the human population suffers the highest number of reported cases of campylobacteriosis in Canada (Lior, 1982). Ontario is a major meat producing province. Unlike salmonellae, campylobacters may not grow very well when released from their natural reservoir but they can survive for several weeks, both at room temperature and under refrigeration (4^0C), although freezing may reduce their viability.

These Gram-negative bacteria are curved to spiral rods of 1.5 to 3.5 µm that have a single flagellum and a cork-screw type of motility. Campylobacter jejuni and C.coli have an optimal growth temperature of about 42^0C which is higher than that of other species of Campylobacter (King, 1962). Thus, they are termed thermophilic campylobacters.

A unique characteristic of campylobacters is that they are considered microaerophilic (Smibert, 1978). That is, they require low levels of oxygen for growth. They also require elevated levels of carbon dioxide. Usually they are poisoned by high levels of oxygen in the air (21%). According to Leutchfeld et al. (1982), the optimal growth conditions for recovery of C.jejuni included an atmosphere of 5% oxygen with 3 to 10% carbon dioxide at 42^0C. Hoffman et al. (1979a), reported that the toxicity of oxygen was due to the organisms hypersensitivity to superoxide anion and free radicals.

Several observations have, however, contradicted the reported microaerophilicity of C. jejuni and C. coli. Although they are considered microaerophilic they possess superoxide dimutase (SOD) and catalase at concentrations typical of aerotolerant bacteria (Hoffman et al., 1979b). The chemical reduction of oxygen during

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cellular metabolism generates a variety of toxic derivatives of oxygen: superoxide anion, hydroxyl radical, single oxygen and hydrogen peroxide (Hoffman et al., 1979a). Hydrogen peroxide and superoxide anion are extremely toxic to cells because they attack the unsaturated fatty acid component of membrane lipids, thus damaging membrane structure (Lenninger, 1979). Aerobic cells protect themselves against these radicals by the action of SOD, a metal containing enzyme that converts superoxide radical into hydrogen peroxide. Catalase converts hydrogen peroxide into water and oxygen in the reaction. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being Streptococcus. The presence of high catalase activity in C. jejuni and C. coli suggests the possibility that biochemically they can meet the high oxygen concentration (21%) in air.

Currently, the presence of campylobacters is confirmed by a time-consuming cultural diagnosis (Skirrow et al., 1982), which consists of enrichment in rich media containing some supplements (e.g. blood, hematin, etc.) for 48 to 72 hours, followed by streaking on selective plates containing antibiotics (e.g. trimethoprim, vancomycin, polymyxin B, cephlothinamphotricin B etc.) (Doyle, 1986) and incubation for an additional 48 hours. Isolated colonies are further characterized biochemically and serologically. For epidemiological investigations, electrophoretic analysis of proteins and endonuclease digests of DNA are used in addition to the above. Some of the serological tests used in cultural diagnosis are bacterial and slide agglutinations, complement fixation,

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immunofluorescence, etc. (Griffiths and Park, 1990). All these methods are specific for the detection of campylobacters.

Nucleic acid probes and enzyme immunoassays (EIA) have also been used for the detection of Campylobacter infection (Doyle, 1986). Nucleic acid probes require specialized equipment and complicated protocols which are time consuming (Griffiths and Park, 1990).

It has been demonstrated that EIA can detect Salmonella in foods more rapidly than cultural methods, while the accuracy in both methods is similar (Flowers, 1985). Application of EIA to pathogen detection requires, however, sufficient growth of the organism from a sample because the number of bacterial cells must be within the detection limits of the EIA. Currently around 10^5 to 10^6 cells per ml can be detected by EIAs developed for Campylobacter detection (Flowers, 1985). Due to its microaerophilic nature, liquid culture of campylobacters requires the use of a microaerobic gas mixture (5% O_2, 10% CO_2 and 85% N_2) maintained in a gas jar or bubbled into the broth. Gassing jars are expensive and limited in size. Bubbling the microaerobic gas into many tubes is not only inconvenient and costly, but may also generate contaminated aerosols. There is a need for a simple and cost-effective atmosphere to grow Campylobacter. Chapter 1 of this thesis describes the development of less expensive and more convenient growth conditions for Campylobacter jejuni and C.coli on solid and in liquid media employing a simple 10% carbon dioxide in moist air atmosphere.

EIAs for the detection of antigens are dependent on two important biological molecules 1) specific antibodies and 2)

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enzymes with high catalytic power. EIAs consist of a two-pronged strategy: reaction between the immunoreactants (antibodies with corresponding antigens) and the detection of that reaction using enzymes attached to the reactants as indicators. Several other types of labels have been employed, such as radioactive tracers in radioimmunoassay, chemiluminescent and fluorescent labels, metal atoms, etc. Use of radioactive tracers requires expensive counters to measure radioactivity, special disposal and poses a health hazard.

EIA was developed in the mid sixties for identification and localization of antigen in histological preparations, analogous to immunofluorescence methods and for the identification of precipitation lines obtained by immunodiffusion and immunoelectrophoresis. EIA has been successfully applied to the detection of a wide variety of substances that are antigenic (e.g. proteins) or can be rendered immunogenic (e.g. haptens) from specific cell surface components of bacterial pathogens (Perera et al., 1983) to environmental pollutants (VanEmon et al., 1986). They are also useful for immunodiagnosis of infectious diseases in animals and humans by detecting antigen specific antibodies in sera or other secretions.

There are many different types of EIA procedures but the most popular and convenient are the non-competitive solid phase assays which employ an immunoreactant (antibody or antigen) immobilized on a solid phase for the immunospecific capture of its corresponding antigen or antibody from a sample (Engvall, 1980). The captured antigen or antibody is then detected using a specific antibody enzyme conjugate system and the bound enzyme activity

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(quantitative function of the amount of antigen or antibody captured on the solid phase) is measured by incubation with a suitable substrate yielding a detectable product (Figure 1).

In a competitive solid phase EIA for antigen, antibody is immobilized on the solid phase and the standard antigen is labeled with the enzyme (Figure 2A). In the test, binding of the antigen-enzyme conjugate by the immobilized antibody is inhibited by the addition of free sample antigen. Since a restricted number of antibodies are available the enzyme activity is lowered (Figure 2A). Figure 2B shows that, alternatively, the antigen may be immobilized and the antibody labeled with enzyme. In this case, sample antigens and immobilized antigens compete for limited amount of enzyme-labelled antibodies added to the sample. In either case (Figures 2A and 2B), the amount of enzyme-labeled immunoreagent bound to the solid phase is inversely related to the concentration of free antigen or hapten in the test sample (Engvall and Perlmann, 1972).

One important aspect of solid phase EIA is the immobilization of immunoreactants on a solid phase. The selection of a proper type of solid phase plays an important role towards the efficiency of an assay. Different types of solid phases which are used for immobilization of antigens and antibodies are: glutaraldehyde-treated HCl-activated nylon beads (Perera et al., 1983), Sephadex beads (Yolken, 1982) or a periodate-oxidized polyester film (Sarkar and Mandal, 1985). The above mentioned solid phases covalently bind to immunoreactants. Other commonly used solid phases are non-porous hydrophobic plastic surfaces, such as multiwell polystyrene microtitre plates, microporous surfaces like nitrocellulose

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Figure 1. Non-competitive solid phase EIA.

(A) Detection of antigens ("sandwich" format). Antibodies specific for the antigen of interest are immobilized on a solid phase. Antibody coated solid phase is then incubated with the test sample (e.g. a bacterial culture) containing antigen. After washing the solid phase with a buffer to remove the unbound sample components, the captured antigen is detected by incubation with an antigen-specific antibody-enzyme conjugate (the antigen becomes "sandwiched" between the capture antibody and the conjugate). Washing of substrate and its incubation with enzyme substrate follows. The presence of at least two antigen epitopes is important for both antigen capture and its detection using the conjugate.

(B) Detection of specific antibodies. In this case the solid phase is coated with antigen and then incubated with a test sample (e.g. serum) containing the antibodies to be detected. With proper washing antigen-specific antibodies are left behind. An anti-globulin antibody-enzyme conjugate detects the bound antibodies. After final wash, the solid phase is incubated with enzyme substrate.

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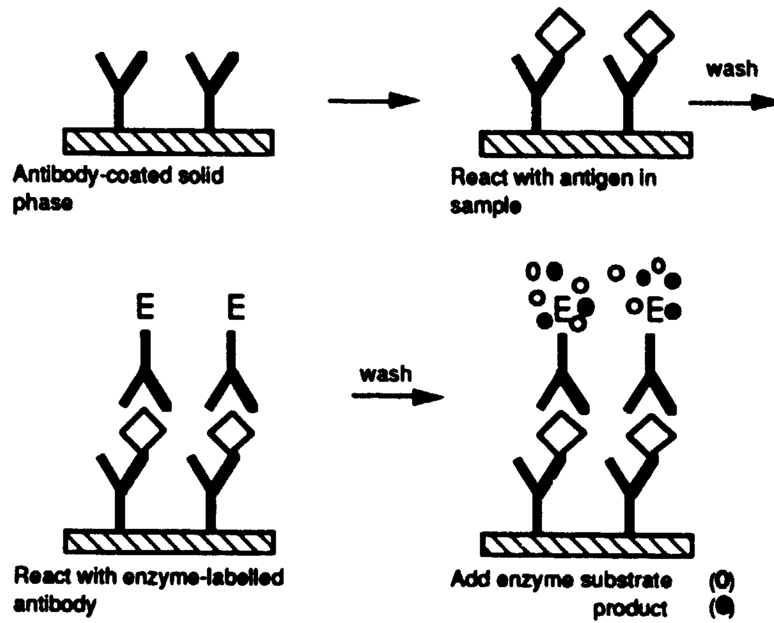
(A)



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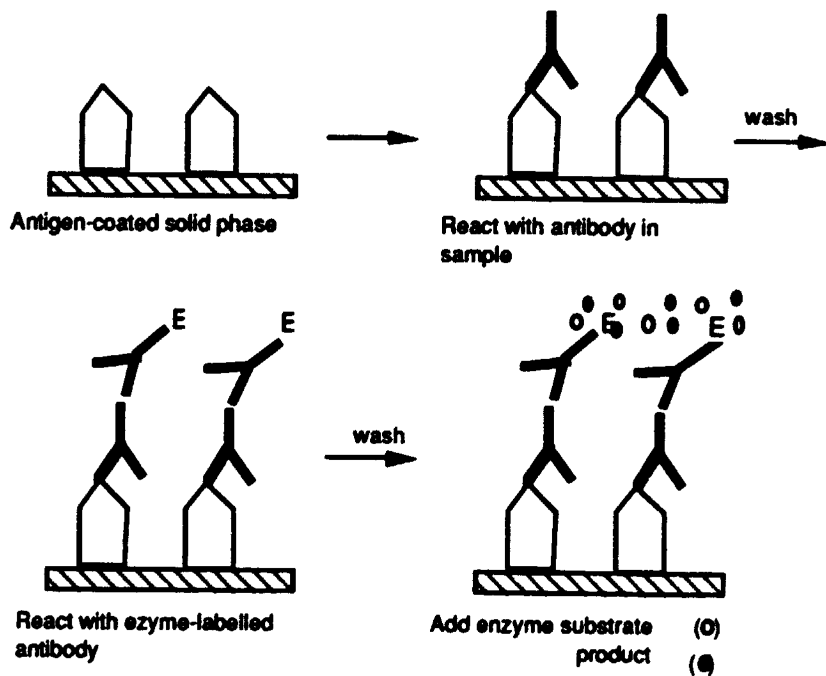

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Figure 2. Competitive solid phase EIA.

(A) Detection of antigen using enzyme-labeled antigen. An antibody specific for the antigen of interest is immobilized on the solid phase, and then incubated with the test sample containing free antigen, followed by a fixed amount of enzyme-labeled antigen. The solid phase is then washed and assayed for bound conjugate. In both cases, the amount of conjugate bound to the solid phase is inversely related to the concentration of antigen in the test sample.

(B) Detection of antigen using enzyme-labeled antibody. Antigen is immobilized on the solid phase, which is then incubated with a mixture of the test samples containing a mixture of the free antigen and a fixed amount of enzyme-labeled antibody. In both cases, the amount of conjugate bound to the solid phase is inversely related to the concentration of antigen in the test sample as above. The solid phase is then washed with buffer and assayed for bound conjugate by incubation with enzyme substrate.

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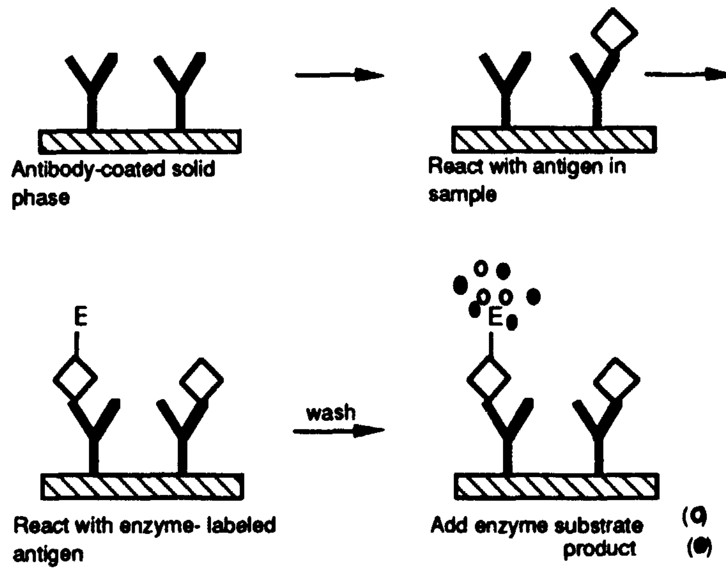
(A)



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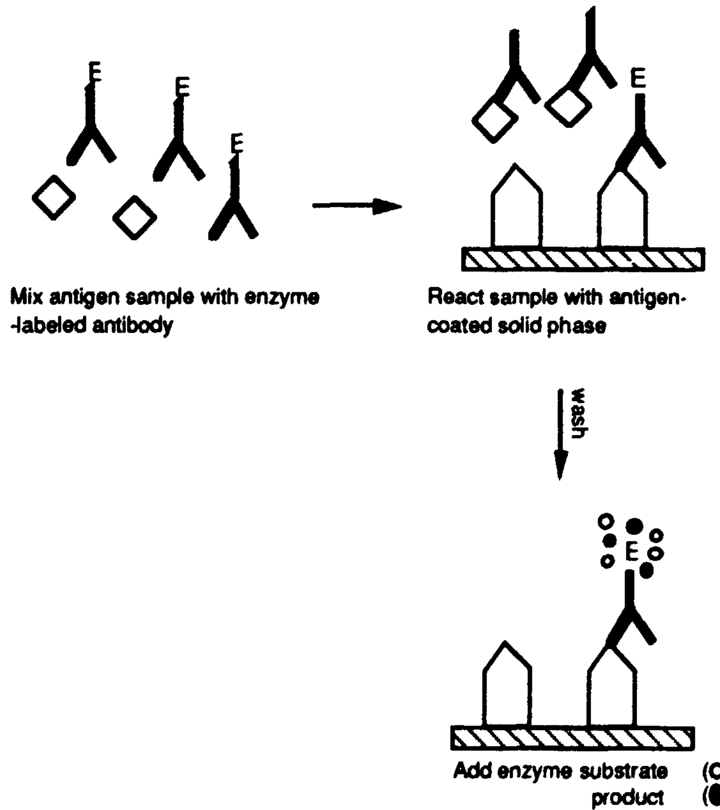

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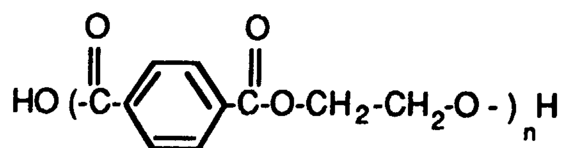
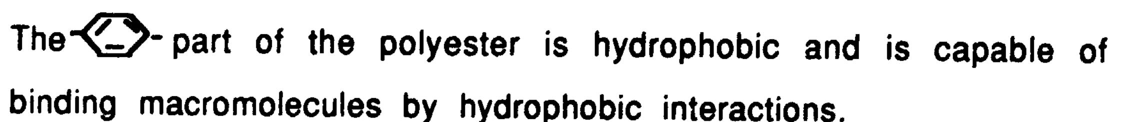
(Brower et al., 1985), and nylon membranes used by Tijssen (1987). The non-porous solid phases require longer incubation times for immobilization of immunoreactants. To overcome this problem microporous membranes such as nitrocellulose membrane can be used, as they provide a large surface area. However, these membranes are brittle and hard to handle. Only a limited quantity of sample can be applied per unit area. They usually require longer incubations with blocker (e.g. serum, bovine serum albumin, milk etc.) in order to avoid background signals. Blais (1990) has demonstrated the use of macroporous polyester hydrophobic cloths as immunoadsorbents for EIA for the detection of anti-*Salmonella* antibodies. The macroporous nature of polyester cloths provides the following advantages: 1) a large surface area and short diffusional distance for immunoreagent immobilization, 2) the ability to accommodate a relatively large volume of sample (per unit area) which is held in intimate contact with the immunoadsorbent surface, and 3) excellent filtration characteristics (which makes washing simple and efficient). In addition the polyester cloths are readily available, inexpensive and environmently friendly. Polyester has the following structure:




The -⌬- part of the polyester is hydrophobic and is capable of binding macromolecules by hydrophobic interactions.

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In order to develop an EIA for antigen detection, it is very important to select the antigen preparation to be used in or detected by EIA and one that can be used as an immunogen. Two fundamental requirements must be met by a molecule to be immunogenic: 1) it should be foreign to activate the defense mechanism, and 2) it must be of a certain complexity to react with the different components of the immune system necessary to induce the immune response (Tijssen, 1987). In general conjugated haptens and polysaccharides are strongly immunogenic.

In the case of Gram-negative pathogens the outer membrane plays an important role in the outcome of host-parasite relationships. Lipopolysaccharide (LPS) is the immunodominant surface antigen in the outer membrane and can contribute directly towards toxicity. Surface exposure of LPS and certain proteins allows contact with the immune system and as a result they can serve as immunogens (DiRienzo et al., 1978). Figure 3 shows the LPS structure of Salmonella typhimurium. As shown in the figure the basic structure of the LPS molecule consists of three regions designated as lipid A, core region and O-polysaccharide side chain (Hitchcock et al., 1986). According to Logan and Trust (1984), C. jejuni and C. coli are typical of bacteria having an LPS lacking O-polysaccharide chain. In the case of Salmonella sp., E. coli, Shigella sp. and Vibrio cholerae loss of O-polysaccharide chain normally results in a loss of virulence but this does not seem to be the case for C. jejuni and C. coli, since these cells analyzed directly from a primary isolation plate produce an LPS lacking O-polysaccharide chains.

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Figure 3. Structure of Salmonella typhimurium LPS.

Three well-defined regions have been described: 1) the O-specific antigen, also called O-antigen or somatic antigen, which is a long-chain polysaccharide consisting of repeating units containing one to seven sugars, i.e., monosaccharides, 2) the oligosaccharide core, composed of approximately 10 monosaccharides (including 3-keto-2-deoxyoctonate (KDO), heptose (Hep), glucose (Glu), galactose (Gal), and N-acetylglucosamine (GlcNac)), and 3) lipid A, a unique lipid backbone which is highly hydrophobic and serves to anchor the molecule in the outer membrane of the bacterium. This region consists of monosaccharides and a lipid. Other sugars present in this structure are rhamnose (Rha), mannose (Man) and abequose (Abe).

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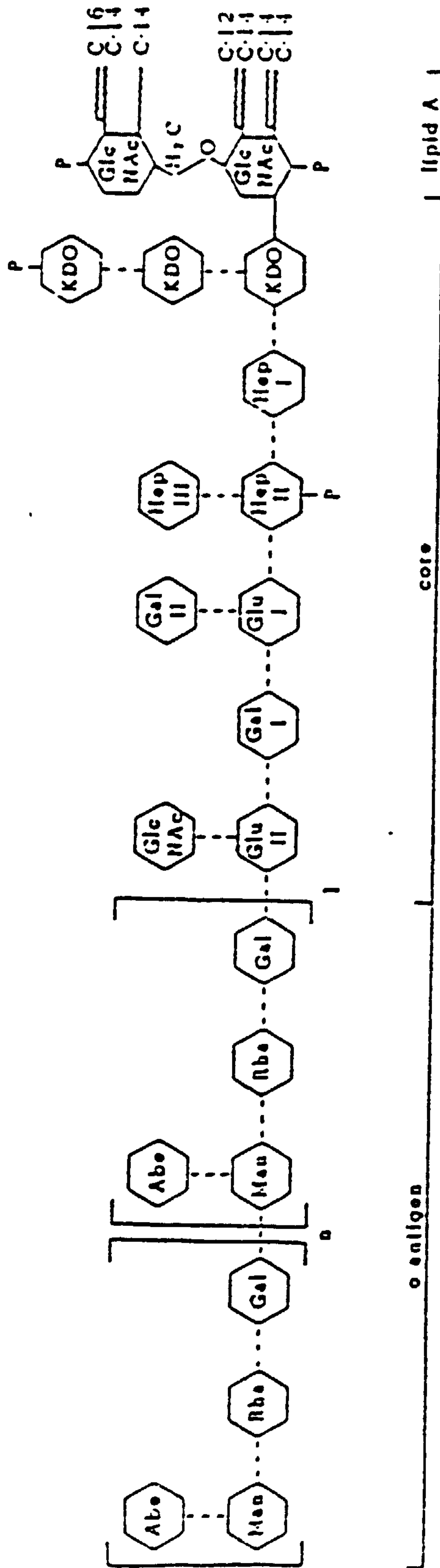

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There are a number of different methods which have been used to extract LPS from Gram-negative bacteria including cold and hot phenol extraction, EDTA extraction methods etc. In Chapter 2, I have shown a simpler, safer and more economical method for the efficient recovery of antigens containing LPS from C.jejuni and C.coli than the conventional hot phenol extraction method. The same antigen preparation can be used to produce antibodies when inoculated into appropriate hosts.

After the selection of methods for antigen extraction and the type of antigens to be used in EIA and for antibody production, one has a choice between producing conventional polyclonal antisera or monoclonal antibodies (Mabs). The choice between them depends to a large degree upon matching the special properties of the antibodies to the assay requirement. Some salient differences between the properties of polyclonal antisera and Mabs include:

1) Cross-reaction due to structural relatedness between antigens - Cross-reaction may be defined as the reaction of an antiserum against an antigen molecule not present in the immunizing preparation. It defines the specificity of an antibody preparation. It is usually due to structural similarities between the immunizing and the cross-reacting antigen. In the case of polyclonal antisera there is high level of cross-reactivity but the cross-reacting antibodies may be removed (to some extent) from the antiserum. Mabs, on the other hand experience less cross-reaction due to structural relatedness between antigens but if it is present it is nearly impossible to remove.

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2) Cross-reactions due to multiple specificity of individual clones - Sometimes there may be cross-reaction due to multiple specificity of individual clones in the case of Mabs. These types of cross-reactions are seldom, if ever, seen in polyclonal sera. The specificity of a conventional polyclonal antiserum is the result of a combination of thousands of different clones, and chances of cross-reactions due to multispecificity are very slim (Goding, 1986).

3) A monoclonal antibody may sometimes be too specific - As mentioned above the specificity of a polyclonal antisera depends on the consensus of thousands of clone products, which bind to antigenic determinants covering most or all of the external surface of the antigen. Thus, a small change in the structure of the antigen will have little or no effect on polyclonal antibody binding. On the other hand, Mabs bind to a single unique site on the antigen molecule and if this site is altered, the antibody may not continue to bind. In addition the probability of finding a Mab which recognizes all species in one genus is small.

4) Affinity of polyclonal and monoclonal antibodies - The homogeneity of Mabs means that each clonal product will have a well-defined affinity whereas most polyclonal sera contain antibodies of a wide range of affinities. Both types of antibodies afford their own advantages and disadvantages. In the case of Mabs, it is possible to select the affinity but it is difficult to find high affinity Mabs. In the case of low affinity antibodies excessive washing may dissociate the antigen-antibody complexes. In addition minor changes in the structure of antigen may completely abolish the binding (Godings, 1986).

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5) The time and expense (~$50K) of producing Mabs is initially very high as compared to the time and expense (~$250/rabbit or $600/goat) of polyclonal sera (Tijssen, 1987).

Thus, in general considering the low cost, ease and short time in the production of polyclonal antisera becomes attractive. In Chapter 3 of this thesis, I have shown the production, extraction and characterization of polyclonal antibodies in the egg yolks of chickens. By using the eggs of inoculated chickens a cheap and continuous supply of heat stable, high titre antibodies can be obtained without sacrificing animals. The amount and concentration of antibodies is much higher in egg yolks than in the serum of birds and animals. In addition to that, I have shown that polyester cloth can be used as a solid phase in a cloth based enzyme immunoadsorbent assay (CEIA) for the quantitative analysis of anti-*Campylobacter* antibodies produced in the egg yolks of chickens.

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CHAPTER 1

SIMPLIFIED GROWTH OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI*

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ABSTRACT

Two different atmospheres: 10% carbon dioxide in moist air and a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) were compared for their ability to support the growth of 18 different serotypes of Campylobacter jejuni and C. coli, in liquid and on solid cultures. The growth of these bacteria in 10% CO_2 in moist air was the same as or better (in some cases) than in the microaerobic atmosphere. Addition of 10% sheep blood to the growth media dramatically improved growth in either atmosphere. High humidity was essential for the growth in an atmosphere of 10% CO_2 in air. It was labour intensive and costly to use a microaerobic atmosphere in an anaerobic jar. But use of a standard CO_2 incubator for 10% CO_2 in air greatly simplified and economized culturing of campylobacters.

INTRODUCTION

The most important characteristic of the thermophilic campylobacters in relation to their isolation for diagnosis, is their microaerophilicity. It is believed that microaerophilic bacteria require oxygen for their growth but they are poisoned by the level of oxygen (21%) present in air (Smibert, 1984). High levels of carbon dioxide in the growing environment have also been shown to help the growth of Campylobacter (Stern and Kazmi, 1989).

Studies on the microaerophilic nature of C. jejuni and C. coli have shown that these organisms are sensitive to superoxide anions

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and hydrogen peroxide despite the presence of superoxide dimutases and catalase at concentrations typical of aerotolerant bacterial species (Garcia et al., 1983).

Several types of media have been developed to enhance the aerotolerance of C. jejuni and C. coli. These media contain different supplements to neutralize toxic oxygen derivatives and free radicals. There are many other substances which are known to have this effect including blood, haemin, haematin, dihydroxyphenyl compounds (i.e. norepinephrine) and iron salts (Skirrow et al., 1982).

According to Hoffman et al. (1979a), the use of a supplement consisting of 0.05% each of ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP), to enhance the oxygen tolerance of campylobacters, did not cause any physiological change or affect any physiological process in C. jejuni. Increase in the oxygen tolerance of Campylobacter is attributed to the ability of FBP to quench superoxide anions and hydrogen peroxide present in the medium (Hoffman et al.,1979b).

Leuchtefeld et al. (1982) found that C. jejuni and C. coli grew optimally in a microaerobic atmosphere consisting of 5% oxygen, 10% carbon dioxide and 85% nitrogen. Therefore such an expensive gas mixture is commonly used to grow C. jejuni and C. coli. Furthermore expensive gassing jars are required to contain such an atmosphere. These jars hold only a limited number of samples at a time and gas-exchange in them is a time-consuming process.

In this chapter we show that C. jejuni and C. coli grow well in an atmosphere of 10% carbon dioxide in moist air. Eighteen different serogroups (Lior, 1984) of C. jejuni and C. coli were grown

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in an atmosphere of 10% carbon dioxide in moist air and the growth in this atmosphere was compared with the growth in a microaerobic atmosphere in different types of culture media.

MATERIALS AND METHODS

Campylobacter strains used:

C. jejuni and C. coli strains used in this study are listed in Table 1.1. These strains represent the 18 serogroups (Lior's scheme) most commonly isolated from humans and animals in Canada. Campylobacter strains were grown on Mueller-Hinton agar with 10% sheep blood in a mixture of 5% O_2, 10% CO_2, 85% N_2 (microaerobic atmosphere). After 48 hours, colonies were harvested and suspended in saline (0.85% NaCl) before inoculation into the final media. The cell concentration was determined spectrophotometerically at 600 nm in 1 cm cuvettes. Cell suspensions (in saline) with an absorbance 1.0 at 600 nm (A_{600}) contained approximately 1 X 10^{10} cells per ml. A 0.1 ml of the inoculum containing a known number of cells was cultured in either solid or liquid media.

In the case of solid culture, a 0.1 ml inoculum of each Campylobacter strain was plated on: 1) Mueller-Hinton agar with (MHAB) or without (MHA) 10% unlysed sheep blood, 2) Brucella agar with (BAB) or without (BA) 10% unlysed sheep blood, and 3) Campylobacter blood free selective agar (CBFA).

All media were products of Oxoid Limited. Plates were incubated for 24, 48 or 72 hours at 37^{0}C in the following

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Table 1.1. List of Campylobacter Strains Used In This Study[a]

Serogroup[b]	Species	Biotype[c]	Source
LIO 1	C.jejuni	I	Human
LIO 2	C.jejuni	II	Human
LIO 4	C.jejuni	I	Human
LIO 5	C.jejuni	I	Human
LIO 6	C.jejuni	III	Human
LIO 7	C.jejuni	I	Human
LIO 8	C.coli	I	Human
LIO 9	C.jejuni	I	Human
LIO 11	C.jejuni	I	Human
LIO 17	C.jejuni	I	Chicken
LIO 18	C.jejuni	III	Chicken
LIO 20	C.coli	I	Swine
LIO 21	C.coli	I	Chicken
LIO 28	C.jejuni	I	Human
LIO 29	C.coli	II	Human
LIO 36	C.jejuni	II	Human
LIO 44	C.coli	II	Human
LIO 53	C.jejuni	I	Human

a: Strains provided by H. Lior, National Reference Center for Campylobacters, L.C.D.C., Ottawa, Ontario, Canada.

b: Lior et al. (1982)

c: Lior (1984)

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environments:

1) Anaerobic gassing jars containing 5% O_2, 10% CO_2 and 85% N_2 (microaerobic atmosphere) (Leuchtefed et al., 1982) were used. In order to fill the jars with this special gas mixture, the jars were evacuated three times and refilled with the microaerobic atmosphere. As recommended for optimal growth the jars were only half filled with plates (Skirrow et al., 1982). The jars were kept at 37^0C in an upright position in an incubator large enough to accommodate the jars.

2) Ten percent CO_2 in moist air atmosphere incubators was used. Ten percent CO_2 in air was bubbled through water held at the bottom of incubator to keep a moisture level of 95-99%. The temperature was kept at 37^0C. The flow of the gas mixture was maintained at 5 to 10 liters per minute. The CO_2 incubators were National CO_2 incubators with inner capacity of 165 litres, Weinicke Co. In order to avoid contamination plates were kept in large autoclave bags with one side open towards the door. These acted as a shield against the moist incubator walls.

The extent of growth was determined by colony forming units (CFU) per plate (averaged over 5 plates) and by colony size in mm (averaged over 10 colonies per plate). A student's T test was used to determine any significant difference in growth between the two atmospheres. Combined data from 18 strains for colony size or colony count was compared using $p \leq 0.05$ and also colony number and colony size of all 18 strains were compared between media with and without 10% unlysed sheep blood.

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In the case of liquid culture, 0.1 ml of cell suspension of each of C. juni strains LIO4, LIO29, LIO54 and C.coli strains LIO8, LIO20, LIO28 was inoculated into 5 ml of each of these media: 1) Mueller-Hinton broth with (MHB) or without (MH) 10% lysed sheep blood, 2) Brucella broth with (BB) or without (BBB) 10% lysed sheep blood, 3) Trypticase Soy broth (TSB), 4) Brain Heart Infusion broth (BHI), and 5) Rosef's broth (without resazurin and antibiotics). In order to prepare the broths with blood, 1 volume of sheep blood was added to 4 volumes of warm (40^0C to 50^0C) sterlized distilled water to lyse the blood cells. The mixture of lysed cells and water (5 volumes) was added to 5 volumes of a twice concentrated broth solution. A supplement of 0.05% FBP was added to the above mentioned broths without blood. Cultures were grown in 16 mm X 125 mm autoclaved capped culture tubes. Again all media, sheep blood and FBP were products of Oxoid Limited.

The liquid cultures were grown in triplicate samples. The cultures were incubated in the same way in the microaerobic and 10% CO_2 in moist air atmospheres, as for solid cultures. After 0, 6, 12, 24, 48 and 72 hours, aliquots (0.1 ml) of culture were removed from each tube and diluted appropriately in saline and plated on MHAB plates. The plates were incubated for 48 hours at 37^0C in the same atmosphere as the respective liquid cultures were grown. The number of CFU (average of 5 plates) was determined for extent of growth.

SDS-polyacrylamide gel electrophoresis

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The method described by Brooks et al. (1986) was followed. Briefly *Campylobacter* cells were harvested into 0.01 M Tris buffer from MHAB plates after 48 hours incubation at 37^0C in either the microaerobic or 10% CO_2 in moist air atmosphere. The cells were washed in 0.01 M Tris buffer three times. The protein concentration was determined and adjusted as described (Brooks et al., 1986). Proteins were stacked in 6% acrylamide and separated in 12% acrylamide. Two hundred µg to 1.2 mg of proteins were applied per lane. Gels were run at a constant current of 20 mA per gel. After electrophoresis, protein profiles were visualized with Coomassie Brilliant Blue R250. Electrophoresis chemicals and protein molecular mass standards (lysozyme, subunit molecular mass (MM) 14,400; soybean trypsin inhibitor, MM 21,500; carbonic anhydrase, MM 31,000; ovalbumin, MM 45,000; bovine serum albumin, MM 66,200; and, phosphorylase B, MM 97,400) were purchased from BioRad Laboratories Ltd., Mississauga, Ontario.

RESULTS

Growth of Campylobacter in solid media

In the case of solid media each of the 18 strains of *Campylobacter* were grown individually on CBFA, MHA and MHAB in both the microaerobic and 10% CO_2 in moist air atmospheres. CFU and colony size were determined after 12, 24, 48 and 72 hours. Table 1.2 represents the data of CFU and colony size on MHA and MHAB media in both atmospheres after 48 hours. MHA and MHAB gave

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better growth of Campylobacter among the media tested. The data was statistically compared using the student's T test. There was no significant difference ($p \leq 0.05$) in the number of CFU in both the microaerobic and 10% CO_2 in moist air atmospheres. The colony sizes in 10% CO_2 in moist air were larger however than the colony sizes in the microaerobic atmosphere. The addition of blood to MHA significantly increased the number of CFU and colony sizes.

Observations were made during the course of experimentation that the presence of moisture was important for the growth of Campylobacter in the 10% CO_2 in moist air atmosphere. Campylobacter did not grow in the absence of moisture. Campylobacter did not grow either in air alone in the presence of moisture.

Furthermore, growing Campylobacter in 10% CO_2 in moist air did not affect the serotyping (Lior et, al., 1982)) of the strains. The serotyping was done by Lior's serotyping method based on slide agglutination. Presence or absence of agglutination on glass slide was determined after a small loopful of Campylobacter was mixed with a drop of rabbit anti-Campylobacter antiserum (crude or absorbed with homologous heat-stable and heterologous, unheated cross-reactive antigen suspensions). The electrophoretic protein profiles of 6 strains in 10% CO_2 in moist air and microaerobic atmosphere were similar (Figure 1.1).

Growth of Campylobacter in liquid media

Attempts were made to grow strains of Campylobacter in liquid media with or without sheep blood. Several different growth

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Table 1.2. Growth of Campylobacter Strains on Mueller Hinton Agar with (MHAB) or without (MHA) Sheep blood in a Microaerobic Atmosphere or 10% CO_2 (in Moist Air) after 48 hours.

	Atmosphere							
	Microaerobic				10% CO_2 in moist air			
	MHA		MHAB		MHA		MHAB	
Strain	CFU[a]	Colony size[b]	CFU	Colony size	CFU	Colony size	CFU	Colony size
LIO1	44(3)[c]	pp	126(3)[c]	pp	49(3)[c]	pp	118(3)[c]	2.0(3)[c]
LIO2	ng	ng	74(6)	1.0(10)[c]	ng	ng	60(3)	2.0(2)
LIO4	60(4)	1.0(1)[c]	150(2)	1.0(3)	73(3)	2.0(1)[c]	200(4)	3.0(1)
LIO5	30(2)	pp	60(10)	1.5(5)	24(2)	pp	50(4)	1.5(1)
LIO6	34(7)	pp	30(6)	2.0(2)	12(2)	pp	34(4)	2.0(4)
LIO7	30(3)	pp	120(8)	1.0(2)	21(4)	pp	170(4)	1.0(10)
LIO8	39(8)	pp	51(6)	1.0(1)	24(5)	pp	30(3)	3.0(2)
LIO9	36(6)	pp	40(5)	1.5(6)	20(5)	pp	28(2)	2.5(2)
LIO11	ng	ng	90(4)	pp	ng	ng	90(4)	1.0(3)
LIO17	ng	ng	40(3)	pp	ng	ng	20(2)	pp
LIO18	156(3)	pp	198(1)	pp	141(8)	1.0(8)	183(2)	2.0(2)
LIO20	57(1)	pp	120(5)	1.0(6)	59(7)	1.0(8)	66(2)	2.5(4)
LIO21	40(4)	pp	56(5)	1.0(10)	40(7)	1.0(7)	64(4)	2.0(5)
LIO28	36(5)	pp	290(4)	1.0(5)	29(6)	1.0(1)	260(3)	2.0(3)
LIO29	50(5)	0.5(10)	64(4)	1.0(1)	36(4)	1.0(2)	120(3)	1.0(3)
LIO36	37(2)	1.0(1)	56(3)	1.0(2)	39(4)	1.0(3)	56(6)	2.0(2)
LIO44	57(4)	pp	147(6)	0.5(1)	14(4)	pp	127(5)	1.5(1)
LIO53	27(4)	1.0(1)	50(6)	1.0(1)	10(2)	1.5(3)	30(3)	2.5(6)

a: Colony forming units (CFU)
b: Colony size in mm
c: Mean (% standard error), n=5 for CFU and n=10 for colony size
pp: Pinpoint (for calculating sample mean pp was assigned a value of 0.1 mm)
ng: No growth (for calculating sample mean ng was assigned a value of 0)

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Figure 1.1. SDS-PAGE of Campylobacter whole cells stained with Coomassie Brilliant Blue to visualize protein. One mg of proteins was applied per lane.

Lane #	Description
1	Protein molecular weight standards (in kDa)
2	Protein profiles of LIO4 (C.jejuni) grown in 10% CO_2 in moist air atmosphere
3	Protein profiles of LIO4 (C.jejuni) grown in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2)
4	Protein profiles of LIO8 (C.coli) grown in 10% CO_2 in moist air atmosphere
5	Protein profiles of LIO8 (C.coli) grown in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2)
6	Protein profiles of LIO11 (C.jejuni) grown in 10% CO_2 in moist air atmosphere
7	Protein profiles of LIO11 (C.jejuni) grown in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2)
8	Protein profiles of LIO20 (C.coli) grown in 10% CO_2 in moist air atmosphere
9	Protein profiles of LIO20 (C.coli) grown in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2)
10	Protein profiles of LIO29 (C.coli) grown in 10% CO_2 in moist air atmosphere
11	Protein profiles of LIO29 (C.coli) grown in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2)
12	Protein profiles of LIO53 (C.jejuni) grown in 10% CO_2 in moist air atmosphere
13	Protein profiles of LIO53 (C.jejuni) grown in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2)

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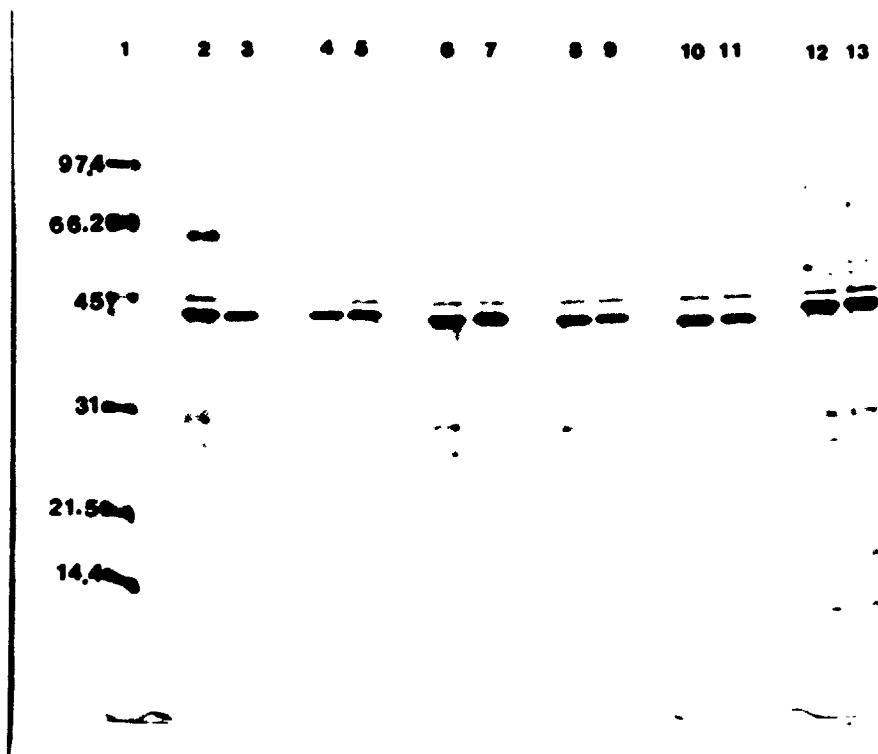

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media were compared for the growth of *Campylobacter* in the two atmospheres, microaerobic and 10% CO_2 in moist air. These included BHI, Rosef's, TSB, MH and BB all with and without 0.05% FBP. FBP did not significantly affect the growth of *Campylobacter* in either atmosphere (data not shown). BB and MH gave a doubling time of approximately 18 hours and BHI, TSB and Rosef's gave a growth rate of more than 22 hours in both atmospheres.

When BB and MH were supplemented with 10% lysed sheep blood, the growth rate of *Campylobacter* significantly increased. Figures 1.2 and 1.3 show the growth of *C.coli* (LIO20) and *C.jejuni* (LIO4) respectively in the microaerobic and 10% CO_2 in moist air atmospheres. An average doubling time of 120 min. was achieved. All 18 strains grew very well in these media in both atmospheres. Figure 1.4 shows the growth of 6 strains of *Campylobacter* in BBB and MHB with 10% lysed sheep blood in 10% CO_2 in moist air atmosphere. An inoculum of 10 cells per ml was enough to initiate the growth of *Campylobacter* in MHAB and BBB in either atmosphere (data not shown).

DISCUSSION

For liquid culture the growth rates of the *Campylobacter* strains in both the microaerobic and 10% CO_2 in moist air atmospheres were similar (Figure 1.2, 1.3 and 1.4). For solid culture, the growth was either same as (CFU) or better (colony size) than in the 10% CO_2 in moist air than in the microaerobic atmosphere (Table 1.2). Good

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Figure 1.2. Growth of Campylobacter coli in Brucella Broth and Mueller-Hinton Broth in both the microaerobic and 10% CO_2 in moist air atmospheres, with or without 10% lysed sheep blood. This figure represents the growth of C.coli LIO20 in Brucella Broth (BB) (Figure 1.2A) and Mueller-Hinton Broth (MH) (Figure 1.2B) in 5% O_2, 10% CO_2 and 85% N_2 (□,■) or in 10% CO_2 in moist air (Δ,▲) with (■, ▲) or without (□ , Δ) 10% sheep blood. The procedure for growth is described in Materials and Methods. In order to monitor the growth, aliquotes of culture were removed at timed intervals and the CFU per ml was determined by plating these aliquots on MHAB plates.

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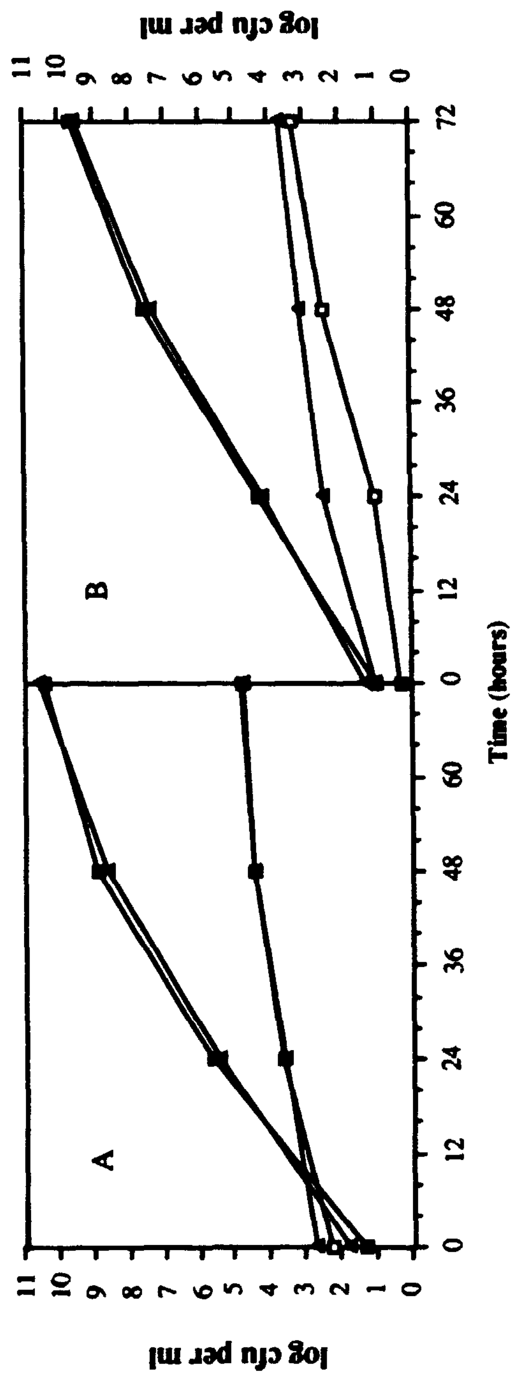

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Figure 1.3. Growth of Campylobacter jejuni in Brucella Broth and Mueller-Hinton Broth in both the microaerobic and 10% CO_2 in moist air atmospheres, with or without 10% lysed sheep blood. This figure represents the growth of C.jejuni LIO4 in Brucella Broth (BB) (Figure 1.3A) and Mueller-Hinton Broth (MH) (Figure 1.3B) in 5% O_2, 10% CO_2 and 85% N_2 (□,■) or in 10% CO_2 in moist air (Δ,▲) with (■, ▲) or without (□ , Δ)10% sheep blood. The procedure for growth is described in Materials and Methods. In order to monitor growth, aliquots of culture were removed at timed intervals and the CFU per ml was determined by plating these aliquots on MHAB plates.

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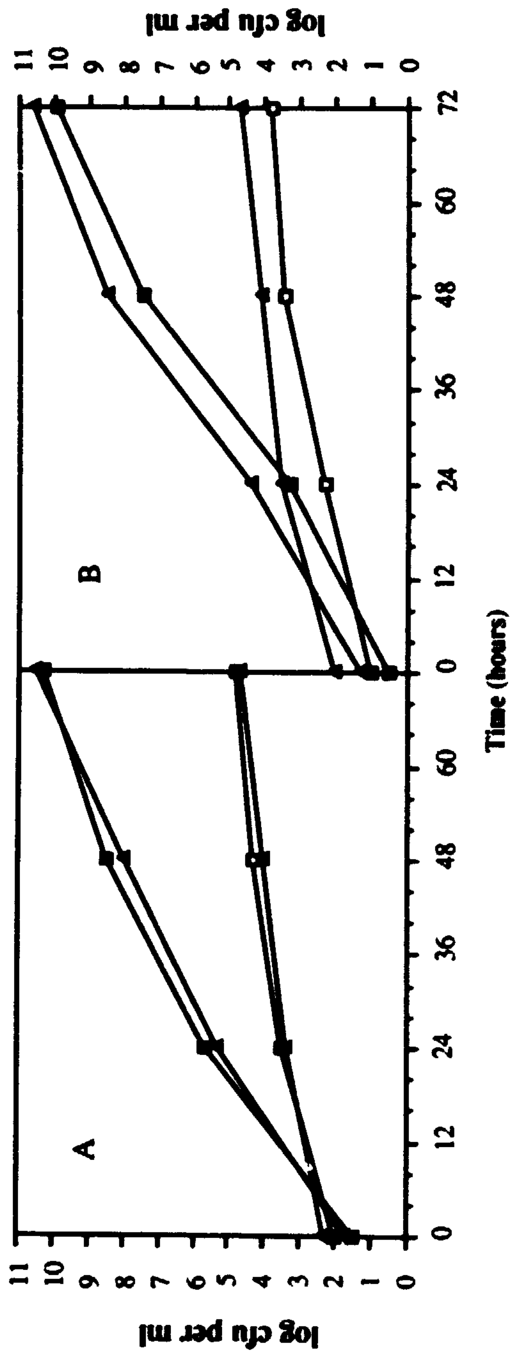

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Figure 1.4. Growth of 6 strains of Campylobacter in Brucella Broth (BBB) and Mueller-Hinton Broth (MHB) with 10% lysed sheep blood in 10% CO_2 in moist air atmosphere. Figure 1.4A represents the growth curves of 6 strains LIO8 (□), LIO11 (Δ), LIO21 (o), LIO28 (■), LIO29 (●), and LIO53 (▲) in BBB in 10% CO_2 (in moist air). Figure 1.4B shows the growth curves of the same 6 strains of Campylobacter in MHB in 10% CO_2 in moist air. The growth conditions are described in Materials and Methods.

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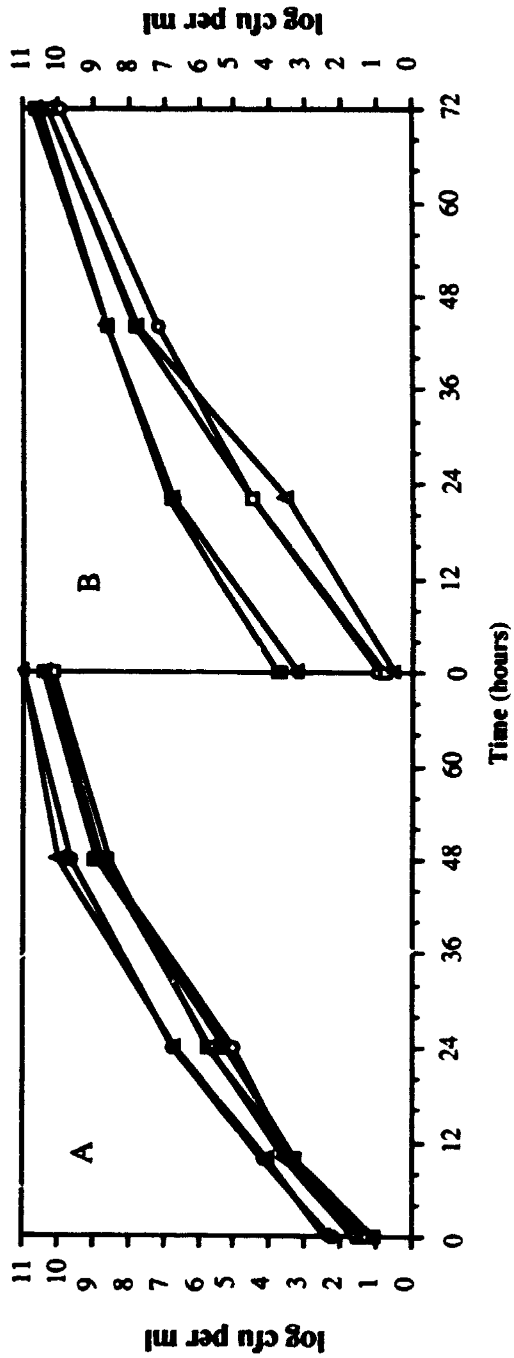

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growth was obtained with as little as 1% to 2% CO_2. Further studies are necessary, however, to determine the minimum CO_2 requirement for growth of campylobacters using incubators where CO_2 can be more accurately regulated at the lower levels. This is an important consideration in further reducing the cost of culturing.

For both liquid and solid cultures the use of 10% CO_2 in moist air greatly reduced the expense and difficulty of culturing Campylobacter. The benefits of using CO_2 incubators have already been demonstrated by Bolton and Coates (1983) and Thompson et al., (1990). However they used an expensive microaerobic gas mixture in their incubators. In the case of gassing jars it is recommended to only half fill them to obtain optimum gas exchange but in case of CO_2 incubators (which can already accommodate much larger sample sizes) the growth of bacteria was not affected by filling the incubators to capacity. For anaerobic jars, the gas mixture had to be replenished every time the jar was opened but the CO_2 incubators could be opened and closed any time because there was a continuous supply of 10% CO_2 in the incubators. The presence of moisture was an important factor for the growth of Campylobacter. There was no growth on MHAB plates in a dry incubator.

It is important to emphasize that growth in the CO_2 atmosphere did not affect the serotyping and biotyping characteristics (Lior scheme) of the strains. This is an important consideration since these are well established schemes currently used in Campylobacter identification. Protein profiles which are often used in epidemiological investigations and taxonomy of campylobacters were also unaffected by growth in this atmosphere.

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Robinson (1981) has shown that as few as 10 cells per ml of Campylobacter can lead to symptoms of gastroenteritis in human adults. Considering this observation, MH and BB supplemented with 10% sheep blood provide excellent media for the liquid culture of Campylobacter since an inoculum of less than 10 cells per ml of Campylobacter was enough to initiate growth.

The culturing methods described here can facilitate the diagnosis of Campylobacter by standard culture techniques and by rapid detection techniques like enzyme immunoassay and nucleic acid hybridization. Enumeration techniques will also benefit from this new simplified method for Campylobacter culture.

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CHAPTER 2

SIMPLIFIED EXTRACTION OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* ANTIGENS

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ABSTRACT

Heating _C. jejuni_ and _C. coli_ in an ethylenediamine tetraacetate (EDTA) solution was found to extract lipopolysaccharide (LPS) in a non-sedimentable fraction. The method permitted the extraction of _Campylobacter_ antigens from solid rich samples and the preparation of solid free samples by means of centrifugation. The LPS recovery by this simple method was greater than that by the hot phenol method, a method which is time-consuming and hazardous. LPS extracted by both methods was visualized with silver stain on SDS-polyacrylamide gels. The EDTA heat method did extract some proteins. The EDTA heat extraction method provided a simple technique for preparing antigens for enzyme immunoassay as well as immunogens for antibody production.

INTRODUCTION

Enzyme immunoassay (EIA) for pathogenic bacteria requires a simple, fast, economic and safe method for extracting sample antigens. In addition, EIA often requires the growth of organisms into the detectable range of the EIA (usually greater than 10^5 cells per ml). Safety has to be considered while working with such a high cell number of any foodborne pathogen. It would be beneficial to kill the _Campylobacter_ cells before they are applied to an EIA.

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Lipopolysaccharides (LPS), an immunodominant cell surface antigen, and some outer membrane proteins are heat stable antigens of Gram-negative bacteria and contribute directly towards the toxicity of Gram-negative cells (Shands, 1975). For EIA, the antigens of Campylobacter should be in their soluble form. Many food samples as well as faeces are rich in solids (e.g. meats) which would interfere with EIA. If the antigens can be extracted in non-sedimentable forms they can readily be separated from solids by a simple centrifugation step. The same antigen preparation can also be used as an immunogen for producing antibodies. Blais (1990) has shown that heating Salmonella cells with an ethylenediamine-tetracetate (EDTA) solution, extracts LPS and some heat stable surface antigens in non-sedimentable forms.

In this chapter it will be shown that the lipopolysaccharide (LPS) of Campylobacter can be effectively extracted by heating Campylobacter cells at an elevated temperature in the presence of EDTA. EDTA heat extractions of the LPS of C. jejuni and C. coli were compared with the traditional hot phenol method for LPS extraction.

MATERIALS AND METHODS

Campylobacter strains used and growth conditions

For the present study, two strains of Campylobacter, C. jejuni LIO4 and C. coli LIO20 (Lior, 1989) were used.

Both strains of Campylobacter were grown in a 10% CO_2 in moist air atmosphere on Mueller-Hinton Agar with 10% sheep blood

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at 37^{0}C (Chapter 1). The cells were harvested from the plates after 48 hours and washed with 0.01 M phosphate buffered (pH 7.2) - 0.85% NaCl (PBS) for extraction by the EDTA method and with saline (0.85% NaCl) (4^{0}C) for extraction by the hot phenol method. The optical density of the cell suspension in PBS or saline was measured at 600 nm in a 1 cm cuvette. PBS or saline cell suspensions at an absorbance of 1.0 at 600 nm contained approximately 1 X 10^{10} CFU per ml. A cell suspension of 10 ml containing approximately 2 to 4 X 10^{11} CFU per ml is equal to about 0.1 g dry weight of Campylobacter cells. Cell suspensions with approximately the same number of CFU were treated by the following procedures for LPS extraction.

Aqueous hot phenol method

The procedure of Luderitz et al. (1966) was followed. Ten millilitres of the cell suspension (2 to 4 X 10^{11} CFU per ml) at 65^{0}C to 68^{0}C was mixed with an equal volume (10 ml) of 90% phenol at 65^{0}C to 68^{0}C. The mixture was stirred vigorously and incubated for 15 min. at the same temperature. After centrifugation @ 10,000 X g for 15 min. the upper aqueous layer was carefully separated. An equal volume of hot water was again added to the rest of phenol layer and mixed thoroughly and treated as above. The aqueous layers were then combined and were dialyzed in Spectrapor membrane tubing #132720, molecular weight cutoff 3,500 against distilled water at 4^{0}C for 2 to 3 days, with water changes every 24 hrs. The dialysates were either stored at 4^{0}C or lyophilized.

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EDTA heat extraction method

Ten millilitres of the cell suspension (2 to 4 X 10^{11} CFU per ml) was mixed with one tenth volume of 0.5 M EDTA (in PBS) pH 7.2. The cell suspension with 0.5 M ETDA was autoclaved at 121^{0}C for 10 minutes (lids open), cooled down to room temperature and centrifuged for 10 minutes at 10,000 rpm. The pellet was discarded. The supernatant was dialyzed against distilled water as described for the hot phenol method.

Protein assay

The amount of protein (mg per ml) was determined by the Bradford method (1976). All chemicals and the protein standards were obtained from BioRad Laboratories Ltd., Mississauga, Ontario. Fifty microliter of the samples (EDTA heat extracts and hot phenol extracts) and several dilutions of protein standard containing from 0.2 to 1.2 mg protein per ml were prepared directly in 1 cm spectrophotometer cuvettes. To each cuvette 2.5 ml of diluted dye reagent (1:4 in distilled water) was added. After mixing the standard and dye reagent, the cuvettes were incubated for 5 min. to 1 hr. After incubation absorbance was measured at an optical density of 595 nm (A_{595}) versus reagent blank (containing saline or PBS with dye reagent). The protein concentration in the samples was determined from a linear optical density and protein standard curve.

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Assay of 2-Keto 3-Deoxyoctanoic Acid (KDO)

KDO of LPS in the extracts was determined by the method of Weisbach and Hurwitz (1959) as modified by Osborn (1963). Lyophilized samples from two different extraction methods were suspended in equal volumes (2 ml) of 0.02 N sulphuric acid and heated at $100^{0}C$ for 20 min. to release KDO from the polymer. 0.025 N of periodate reagent was added, mixed thoroughly and incubated at room temperature for 20 min. Addition of 0.2 N sodium arsenite solution was followed by the addition of the thiobarbituric acid reagent. The reagent was mixed and heated at $100^{0}C$ for 20 min. The optical density was determined in 1 cm cuvette at 548 nm (A_{548}). Ten microgram per ml of KDO gives an absorbance of 0.75 at 548 nm.

SDS-polyacrylamide gel electrophoresis

Proteins and LPS in the EDTA and the hot phenol extracts were fractionated by the SDS-polyacrylamide gel electrophoresis system described by Brooks et al. (1986). For comparison whole cells, washed and suspended in 0.01 M Tris buffer, were similarly analyzed. Two hundred microgram to 1.2 mg of proteins were stacked in 6% acrylamide and separated in 12% acrylamide. Gels were run at a constant current of 20 mA per gel. After electrophoresis the protein profiles were visualized with Coomassie blue R250 and the LPS profiles were visualized by staining with the silver staining technique of Hitchcock and Brown (1983). Electrophoresis chemicals and protein molecular mass standards were purchased from BioRad Laboratories Ltd., Mississauga, Ontario.

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LPS of Salmonella typhimurium was obtained from a collection at ADRI, Nepean.

RESULTS

To determine the efficiency of LPS recovery by the two extraction methods, approximately the same number of C. jejuni and C. coli cells were treated by the hot phenol and EDTA heat extraction procedures. The extracts was lyophilized and the lyophilized samples from both extractions were suspended in equal volumes (2 ml) of 0.02 N sulphuric acid (not equal weights). Aliquots were tested for KDO concentration. Since 3 molecules of KDO are present per LPS chain, the amount of KDO was taken as a measure of LPS. Table 2.1 shows that the LPS content of KDO in the EDTA extract was 1.9 times greater than that in the hot phenol extract.

The EDTA heat extract contained a considerable amount of protein compared to the phenol extracted samples (Table 2.2). The proteins extracted by the EDTA heat method were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. In Figure 2.1 lanes 4 and 6 represent the proteins present in EDTA heat extracted samples of C. jejuni and C. coli, respectively. Lanes 3 and 5 represent the proteins present in whole cell heat extracted samples of C. jejuni and C. coli, respectively. The EDTA heat method extracted a limited number of proteins. Since some membrane proteins are known to be heat stable, there is a possibility that some of the EDTA heat extracted proteins represent outer

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Table 2.1. Comparison of the recovery of lipopolysaccharide between two extraction methods as measured by KDO concentration.

Extraction method	*Campylobacter* Strains	Amount of KDO[a] (µg per ml)
EDTA heat	*C. jejuni* LIO4	34.7[b], 33.3[b]
	C. coli LIO20	30.0, 30.0
Hot phenol	*C. jejuni* LIO4	18.0, 18.0
	C. coli LIO20	21.4, 21.4

a: Osborn (1963)

b: Two separate determinations.

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Table 2.2. Comparison of the presence of proteins in lipopolysaccharide samples extracted by two different LPS extraction methods.

Extraction method	*Campylobacter* Strains	Amount of protein[a] (mg per ml)
EDTA Heat	*C. jejuni* LIO4	0.21[b], 0.21[b]
	C. coli LIO20	0.31, 0.30
Hot phenol	*C. jejuni* LIO4	0.05, 0.05
	C. coli LIO20	0.05, 0.05

a: Bradford (1976)

b: Two separate determinations.

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membrane proteins. Outer membrane proteins of have molecular weights 62,000, 44,000, 31,000, 27,000, 24,000 and 19,000 Daltons (Mills et al., 1986). These proteins can be used as antigens in EIA and also as immunogens for the production of antibodies. Lanes 8 and 10 in Figure 2.1 represent the phenol extracts and it is evident that there were no protein bands detectable with Coomassie Blue stain.

To analyze the LPS present in C. jejuni and C. coli the EDTA and phenol extracts along with whole cell heat extracted samples were digested with proteinase K, separated on SDS-polyacrylamide gels and the gels silver stained. Proteinase K digests the proteins and leaves behind the LPS and other non-proteinaceous components. Figure 2.2 shows the silver stained LPS from the extracted samples. The LPS of C. jejuni and C. coli in the EDTA and phenol extracts lack O-side chain, which is present in S. typhimurium (Figure 2.2, lane 1). The LPS patterns of C. jejuni and C. coli are different as evidenced by two distinct bands and some lesser bands (as determined by intensity of stain) seen in C. coli (Figure 2.2, lanes 3 and 4) whole cell versus one distinct band and several others seen with C. jejuni. EDTA extraction appears to have extracted the same prominent bands from C. jejuni and C. coli (Figure 2.2, lanes 3 and 5) as the whole cell extracts though the C. coli bands seem to be of lower molecular weight in the EDTA heat extracts. The LPS from the phenol extracts appears similar for both C. jejuni and C. coli and seems to involve a broader molecular weight range of LPS.

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Figure 2.1. Coomassie Brilliant Blue SDS-PAGE pattern of *Campylobacter* extracts from EDTA heat and phenol extraction methods and their comparison with whole cell extracts. One mg of proteins was loaded per lane in case of whole cell extracts and the EDTA extracts were loaded enough so that the proteins extracted were stained and visualized.

Lane #	Description
1	Low molecular weight standards (BioRad) (in kDa)
2	High molecular weight standards (BioRad) (in kDa)
3,7	Protein profiles of whole cell extracts of *C.jejuni* (LIO4)
5,9	Protein profiles of whole cell extracts of *C.coli* (LIO20)
4	Proteins present in the EDTA heat extract of *C.jejuni* (LIO4)
6	Proteins present in the EDTA heat extract of *C.coli* (LIO20)
8	Proteins present in the hot phenol extract of *C.jejuni* (LIO4)
10	Proteins present in the hot phenol extracts of *C.coli* (LIO20)

The SDS-polyacrylamide gel electrophoresis procedure is described in Materials and Methods.

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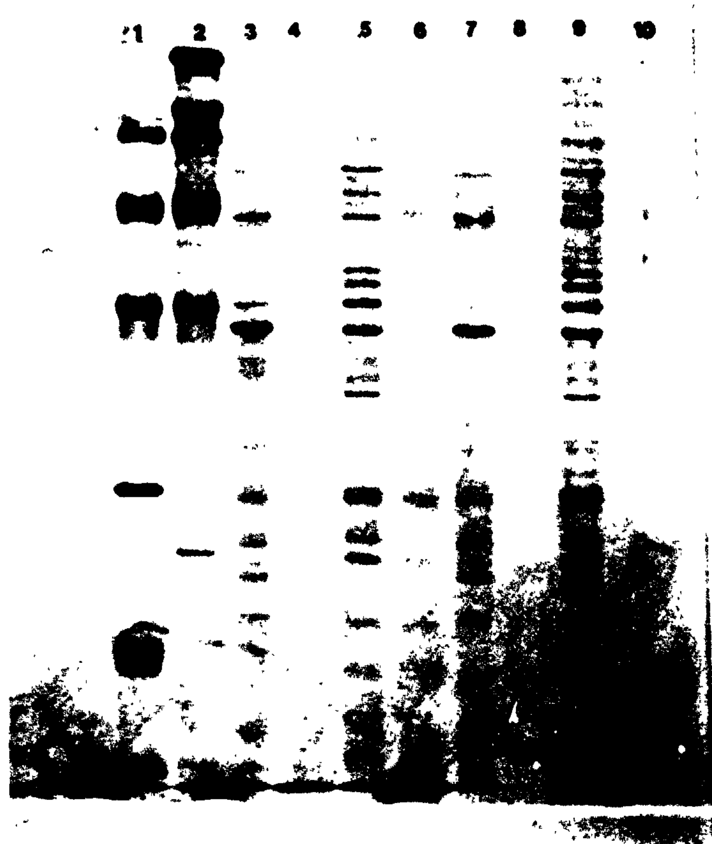

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Figure 2.2. Silver stained SDS-PAGE patterns of Campylobacter LPS extracts (proteinase K digested) from the EDTA heat and hot phenol extractions methods and their comparison with whole cell extracts.

Lane #	Description
1	LPS of Salmonella typhimurium, (although difficult to see in this photograph the LPS ladder of S. typhimurium extends to the arrow)
2,6	LPS bands from whole cell extracts of C.jejuni (LIO4)
4,8	LPS bands from whole cell extracts of C.coli (LIO20)
3	LPS band in the EDTA heat extract of C.jejuni (LIO4)
5	LPS band in the EDTA heat extract of C.coli (LIO20)
7	LPS band in the hot phenol extract of C.jejuni (LIO4)
9	LPS band in the EDTA heat extract of C.coli (LIO20)

The SDS-polyacrylamide gel electrophoresis procedure is described in Materials and Methods.

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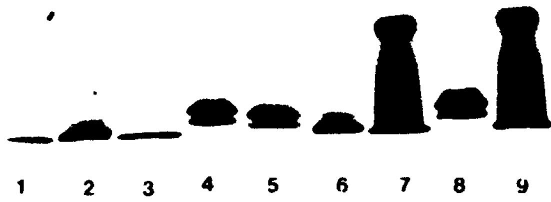

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DISCUSSION

In this chapter, I have demonstrated that the EDTA heat extraction provided a simple and efficient method for extraction of LPS as well as some heat stable proteins from thermophilic campylobacters. The hot phenol extraction method is time-consuming and it is not safe to work with hot phenol fumes. Phenol requires special disposal procedures which increase the cost of this extraction method. With the EDTA heat method no special disposal is required and the samples were very safe to handle as bacteria were killed at the elevated temperature used. Thus, the EDTA heat method provided a simple, rapid and safe method for preparing soluble antigen samples free from solid since heating *Campylobacter* cells in EDTA leads to the dissociation of their antigens into non-sedimentable form. This permits the preparation of solid free liquid samples from solid rich samples such as poultry, meat, faeces etc. As the LPS extracted from the EDTA heat extraction method appeared to be same as the LPS present in the whole cell extracts, the EDTA heat extracted extracts should be as immunogenic as the LPS of the whole cell extracts.

The phenol extracted LPS samples were different from that of the EDTA heat extracted and the whole cell extracted LPS samples. Phenol will also extract RNA. As no further purification of the phenol extracted LPS samples was carried out (i.e. RNAase treatment), RNA may silver stain as well as the LPS of *C. jejuni* and *C. coli* respectively.

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Ethylenediamine-tetraacetate has two effects on the cell surface of coliform bacteria. It causes an increase in permeability to substances that usually cannot enter and it causes the loss of a large fraction of the surface lipopolysaccharide (LPS) (Leive et al.,1968). LPS is commonly stabilized by divalent cations such as magnesium and calcium in the outer membranes of Gram-negative bacteria. When these cations are chelated by EDTA, the outer membrane is destabilized and LPS is shed from the cell surface (Hannecart-Pokorni et al., 1973). Heating facilitates the dissociation of LPS as well as heat stable proteins from the outer membrane into non-sedimentable forms.

The following chapter will focus on the use of LPS and heat stable outer membrane proteins extracted from Campylobacter by the EDTA heat extraction method as immunogens for producing antibodies and for use in cloth-based enzyme immunosorbent assay (CEIA).

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CHAPTER 3

PRODUCTION, EXTRACTION AND CHARACTERIZATION OF ANTI-*CAMPYLOBACTER* ANTIBODIES FROM EGG YOLKS OF CHICKEN

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ABSTRACT

Anti-*Campylobacter* antibodies were produced in chicken egg yolks. Chickens were inoculated at four intervals with a mixture of EDTA extracted antigens of *Campylobacter jejuni* and *C. coli*. Antibodies were extracted from the egg yolks of immunized chickens. Only the IgG type of immunoglobulin was found in egg yolk extracts while the chicken serum contained IgG, IgA and IgM. A semi-quantitative test (slide-agglutination) and a quantitative cloth based enzyme immunoassay (CEIA) were performed to follow the increase in antibody titre over a period of 16 weeks after the first inoculation. Immunoblots using yolk anti-*Campylobacter* antibodies were able to detect the EDTA extracted antigens as well as whole cell antigens, fractionated by SDS electrophoresis. The antibodies produced against the two strains of *Campylobacter* could detect 18 different *Campylobacter* serotypes used in a immunoblot. Thus the yolk antibodies should be useful as antigen capturing reagents as well as antigen-detecting reagents in enzyme immunoassays for *Campylobacter*.

INTRODUCTION

Enzyme immunoassay (EIA) consists of a two-pronged strategy: reaction between the immunoreactants (antibody with corresponding antigen) and detection of that reaction using enzymes, labeled to the reactants, as indicators. As discussed in the General Introduction

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polyclonal antisera take less time and less effort to prepare and are less expensive than monoclonal antibodies. The routine industrial use of EIA definitely benefits from a less expensive supply of antibodies. Polyclonal antibodies can serve this purpose. The cross-reactivity that can be present in polyclonal antisera, is easily removed with immunosorbents. Polyclonal antibodies are less sensitive to inactivation by freezing and thawing, changes in pH and other physical properties (Mosmann et al., 1980) than monoclonal antibodies. These superior properties of polyclonal antisera are helpful for their purification, longer shelf life and other applications.

Patterson et al., (1962) have shown that avian antibodies can be transferred from the serum of infected birds and concentrated in the egg yolks. The antibody content (IgY) of the egg yolks is higher than that of hen's serum (Rose et al., 1974) and it can be economically purified. The serum of rabbits and guinea pigs contain much lower concentrations of specific antibodies and are more difficult to purify. One rabbit under continuous immunization may give 10 to 15 ml of blood maximally per week with an antibody titre much lower than that which may be obtained in eggs from immunized hens. On average a chicken lays 4 to 5 eggs per week and a volume of 5 ml of purified antibodies can be obtained from the yolk of one egg. Thus, egg yolks can be a convenient source for the production of polyclonal antibodies. In addition the birds are not sacrificed to obtain the antibodies.

This chapter demonstrates the production and extraction of antibodies in chicken egg yolks. Chicken were inoculated with EDTA

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heat extracted antigens of C. jejuni and C. coli. The antibodies extracted from the egg yolks were characterized and tested for specificity. Cloth based enzyme immunoassay and western-blotting were used to follow changes in the titre of antibody production in eggs before and after inoculation of the birds.

MATERIALS AND METHODS

Campylobacter strains used

Table 1.1 shows a list of the 18 most common serotypes (according to the Lior serotyping scheme, 1982) of Campylobacter jejuni and C. coli, isolated from humans and animals in Canada. Strains were grown on Mueller-Hinton agar with 10% sheep blood (MHAB) plates in a 10% CO_2 in moist air (Chapter 1) at 37^0C for 48 hours.

Preparation of Campylobacter antigen

For antigen preparation, Campylobacter cells were harvested and extracted as described in Chapter 2 under "EDTA Extraction Method" by the EDTA heat extraction method. Sterility testing on extracted samples was done by plating 0.1 ml of the extracted samples on MHAB plates and incubating the plates at 37^0C for 48 hours in a 10% CO_2 in moist air atmosphere. The confirmed sterile extracted samples were stored at 4^0C until use.

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Immunization of chicken

Three white Leghorn laying hens of 18 to 22 weeks of age were selected for this project. One of the hens was kept as unimmunized control. One week from the day that the hens started laying eggs, the hens were inoculated intramusculary with a 0.75 ml mixture of antigen prepared from *C. jejuni* and *C. coli* (10^9 cells per ml) and 0.25 ml of an LES+STM adjuvant for chickens (RIBI, Immunochemical Research Inc., Montana, U.S.A). The birds were inoculated at four different sites (a 0.25 ml inoculum at each site). The birds were inoculated again at 7, 14, and 21 days after the first inoculation. Eggs were collected every day before and after the inoculations, labelled and stored at 4^0C. Serum was collected from the hens, before the inoculations were begun, as a control and stored at -20^0C. The hens were sacrificed 8 weeks after the final inoculation and serum was collected again and stored at -20^0C.

Extraction and purification of antibodies from egg yolk

To extract antibodies from the egg yolks, the method of Polson *et al.* (1985), was followed. Egg yolk was separated from albumen manually and washed in a stream of cold water to remove traces of albumen. One volume of washed egg yolk was suspended in 4 volumes of 0.1 M phosphate buffer, pH 7.6. Polyethylene glycol (PEG) molecular weight 8,000 (Sigma chemical Co. No. P-2139) was added to a final concentration of 3.5% (w/v) and centrifugated at 5,000 X *g* for 20 min. The pellet was discarded and PEG was added to the supernatant to a final concentration of 8.5% (w/v). The mixture was centrifuged at 5000 X *g* for 25 min. The supernatant was discarded

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and pellet was resuspended in 0.1 M phosphate buffer (2.5 X volume of egg yolk). PEG was added to a final concentration of 12% (w/v). The mixture was centrifuged at 5000 X g for 25 min. Finally the pellet was suspended in the buffer (0.25 X volume of egg yolk) (at $0^{0}C$) and 50% ethanol (same volume as the buffer, pre-cooled to -20 ^{0}C) was added to the final suspension. After centrifuging the mixture at 10,000 X g for 25 min., the precipitate was suspended in 0.25 volume of 0.1 M phosphate buffer and dialyzed in a Spectrapor membrane tubing (molecular weight cut off 3,500) against phosphate buffer for 24 hours. The extracts were stored at $4^{0}C$. The antibodies from each egg were extracted separately.

Slide-agglutination Test

For slide-agglutination *Campylobacter* cells were harvested from MHAB plates and suspended in saline. The cell suspension was further diluted in a ratio of 1:2 to 1:32 in saline and one volume of each dilution was placed on a clean slide. One volume of purified egg yolk extract (from one egg) was mixed with one volume of each different dilution of cells on the slide and immediately any precipitation reaction was noted by holding the slide up to a source of white light. The degree of precipitation was marked as a -, +, ++ and +++.

Immunodiffusion

To determine the type of immunoglobulin present in the egg yolk, isotyping was performed by immunodiffusion. One percent Noble agar (Difco Ltd.) was prepared in Tris buffer (0.1 M Tris-HCl, 1

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M NaCl, pH 7.2). Agar in the mixture was melted in a microwave oven and 15 ml of the mixture was poured on a 65 X 123 mm rectangle of Gel Bond film (LKB1850-101). After the gel solidified, wells were made using a template punch. Twenty µl of egg yolk extract were placed in the outer wells and 10 µl of either isotyping anti-chicken IgG (H+L), anti-chicken IgA or anti-chicken IgM serum (ICN ImmunoBiologicals, Mississuaga, Canada.) was placed in the central well. The gel was incubated overnight at room temperature in a humidified chamber and then washed in 0.15 M saline for 24 hours. To dry the films they were covered with Whatmann #1 filter paper and left at room temperature for 24 hours. Dried films were then stained with Coomassie Blue (3.6 g of Coomassie Brilliant BlueR (Sigma B-0630) in 450 ml methanol, 90 ml glacial acetic acid and 360 ml distilled water) and then destained in a mixture of 450 ml ethanol, 100 ml glacial acetic acid and 450 ml distilled water.

SDS-PAGE

The procedure for running SDS-polyacrylamide gel electrophoresis (SDS-PAGE) has been described in the Materials and Methods of Chapter 1 and Chapter 2.

Western-blotting

To follow the immunogenic response in the egg yolk extracts, the immuno-blotting technique described by Towbin et al., (1971) was used. After separating the various components of the EDTA heat and whole cell extracts of C. jejuni and C. coli by SDS-PAGE, these components were transferred from the gel to a nitrocellulose

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membrane by the methanol-Tris glycine system. Western electroblotting was carried out in a LKB Bromma 2005 transphor transblot apparatus for 1 hr. at 60 v. After transfer, the nitrocellulose membrane was washed with PBS and blocked with PBST (0.3% Tween 20 in PBS) for 1 hour. The membranes were incubated for 16 to 18 hours in antibody extracted from egg yolks (eggs obtained after final inoculation) (diluted 1:200 in PBST). The nitrocellulose membrane was washed 5 times by shaking (for 4 min. each time) in PBST. The membranes were incubated in goat anti-chicken IgG alkaline phosphatase labeled conjugate (ZYMED Lab. Inc., SanFransisco, CA.) (1:1000 in PBST) for 2 hours. Finally alkaline phosphatase colour reagent (containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in dimethylformamide) (BioRad laboratories Limited, Richmond, CA.) was diluted 1:100 in 0.1 M carbonate buffer pH 9.8 and was used to visualize the conjugated antibodies.

Cloth based enzyme immunosorbent assay (CEIA)

CEIA was performed to demonstrate the changes in antibody titre of antibodies in egg yolk extracts before and after inoculation of the hens. Segments (6 mm square) of non-woven polyester cloth (Dupont, Sonatara 8100) were soaked in PBS. Each cloth segment was coated with 50 µl of EDTA heat extracted antigens from 10^9 CFU per ml Campylobacter jejuni or C. coli and incubated at room temperature overnight. Each cloth segment was washed with a total of 5 ml of PBST on a filter under vacuum suction. Each cloth segment was coated with 25 µl of egg yolk extraction diluted 1:200

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in washing buffer for 15 min. at room temperature. Segments were washed as before with PBST and incubated with goat anti-chicken IgG peroxidase labeled conjugate diluted 1:3000 in PBST for 30 min. After a final wash, cloth segments were incubated with constant shaking at room temperature in 0.8 ml of a TMB substrate system (Kirkegaard and Perry Lab. Inc., Gaitherburg, U.S.A.) for 30 min. The reaction was stopped by adding 0.2 ml of 0.5 M sodium fluoride. The developed substrate solution was transferred to a 1 cm cuvette and its absorbance at 370 nm (A_{370}) was determined in a Bausch and Lomb Spectronic 21 spectrophotometer.

RESULTS

To produce antibodies, two laying hens were immunized with a mixture of the EDTA heat extracted C. jejuni (LIO4) and C. coli (LIO20) antigens and a third hen was kept as an unimmunized control. Antibodies were extracted from each egg yolk by the method of Polson et al., (1988). The method is based on the principle that polyethylene glycol (PEG) (m.w. 8000) precipitates proteins.

To characterize the egg yolk extracts a number of different tests were performed. A semi-quantitative slide agglutination test was carried out on each egg yolk extract to determine whether or not anti-Campylobacter antibodies were produced after immunization. Table 3.1 shows the results of changes in the antibody titre over time. The results confirmed that there was a positive increase

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Table 3.1. Slide-agglutination test for semi-quantitation of anti-*Campylobacter* antibodies produced in egg yolks of immunized chicken, over a period of 16 weeks from the day of first inoculation.

Quantitation of precipitation reaction between dilutions of egg yolk extracts and *Campylobacter* cells					
	Dilutions of egg yolk extracts				
No. of weeks	1:2	1:4	1:8	1.16	1:32
→ 0	+	-	-	-	-
→ 1	+	-	-	-	-
→ 2	++	+	-	-	-
→ 3	++	+	+	+	-
4	+++	+++	++	++	++
5	+++	+++	+	+	+
6	++	++	+	+	+
7	++	++	+	+	+
8	+	+	+	-	-
9	++	+	+	+	-
10	++	++	+	+	+
11	++	++	+	+	-
12	+	+	+	+	-
13	+	+	+	-	-
14	+	+	+	-	-
15	+	+	+	-	-
16	+	+	-	-	-

- : no reaction or ppt
+ : week reaction (+ve)
++ : positive reaction
+++: very positive reaction
-> : time of inoculation

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in antibody titre in the egg yolks after the immunization of the chickens. However without continued inoculation this titre started to drop after the final inoculation. To determine the type of immunoglobulin present, an immunodiffusion was performed. Figure 3.1A confirmed the presence of immunoglobulin IgG (called as IgY in egg) in egg yolk of chicken by the formation of precipitin bands. Figures 3.1B and 3.1C show the absence of band formation between egg yolk extracts and anti-chicken IgM and IgA serum respectively, demonstrating that immunoglobulins IgA and IgM were absent in the egg yolk extracts. Figure 3.2 shows that serum of chicken contained immunoglobulin IgG, IgA and IgM.

A cloth based enzyme immunoassay was developed to provide a more quantitative analysis of the anti-Campylobacter antibodies produced in the egg yolks after immunization. For this, polyester cloth was saturated with antigens of C. jejuni or C. coli (EDTA heat extracted from 10^9 CFU per ml). The antigens adsorbed on the cloth were allowed to interact with diluted chicken antibodies of the egg yolk extract for a fixed time (15 min.). The bound antibodies were assayed by goat anti-chicken IgG peroxidase conjugate. Absorbance of the colour developed by peroxidase was taken as an antibody titre.

According to Goodman et al., (1950) and Crawle (1961) addition of PEG and NaCl can facilitate antigen binding to purified chicken yolk antibodies however the addition of either component did not make any difference in the results of CEIA (data not shown). For CEIA cloth segments were either saturated with C. jejuni or C. coli but the results were basically the same. Figure 3.3 shows the change in the antibody titre in egg yolks for 15 weeks after the

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Figure 3.1. Immunodiffusion of egg yolk extracts for isotyping antibodies present. (A) This figure demonstrates the formation of a precipitin line (which forms a distinct hexagonal ring) between goat anti-chicken IgG serum and the egg yolk extracts (i.e. it demonstrates that IgG immunoglobulin was present in chicken eggs). No reaction or hexagonal ring or precipitin lines were seen between anti-chicken IgA (B) or IgM (C) serum and the egg yolk extracts, which confirmed the absence of IgA and IgM immunoglobulins in the egg yolk extracts. In this figure numbers 0 to 5 represent the number of weeks after inoculation of the chickens with *Campylobacter* antigens that the eggs were obtained for antibody extraction. The chickens were immunized at week 1, 2, 3 and 4.

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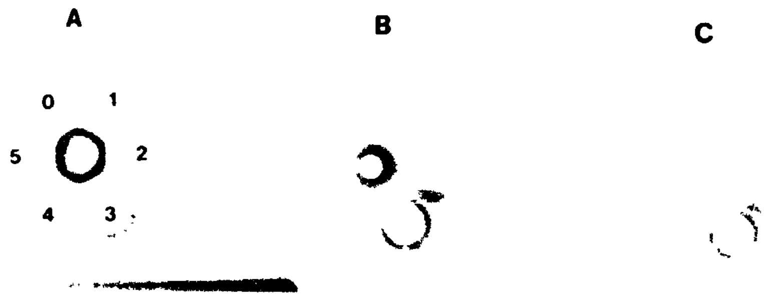

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Figure 3.2. Immunodiffusion of chicken serum for isotyping of antibodies present. This figure represents the formation of precipitin bands (distinct hexagonal rings) between goat anti-chicken IgG (A), IgA (B) and IgM (C) serum and chicken serum, which confirms that immunoglobulins, IgY, IgA and IgM were present in the chicken serum. Number 0 represent the serum obtained from chicken before immunization. Numbers 1, 2, 3, 4 and 5 represent the dilution ratios of 1:2, 1:4, 1:8, 1:16 and 1:32 respectively of serum (obtained 16 weeks after the first inoculation of chicken).

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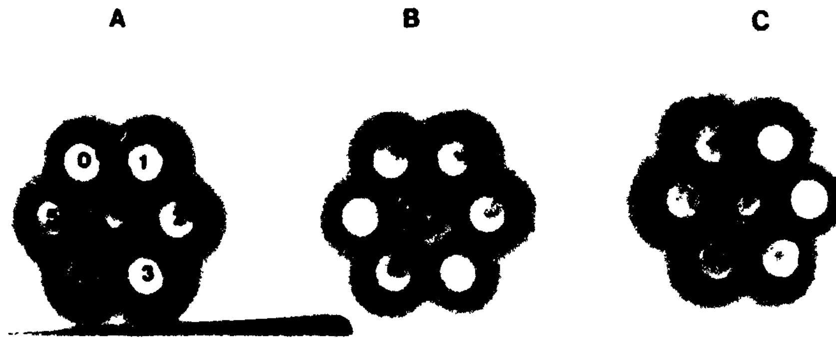

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inoculation. The antibody titre remained almost constant for the unimmunized control chicken. The antibody titre in the egg yolks of immunized chicken did not rise for two weeks. The titre went up after third inoculation and then began to decline after the 5th week. Also the titre of C. coli came up after the titre to C. jejuni although subsequent patterns remained similar.

In order to show whether the anti-Campylobacter antibodies produced in egg yolk could be used for detection of antigens, western-blotting was performed. Antigens from EDTA heat extracts along with whole cell extracts were separated on SDS-PAGE and were transferred to nitrocellulose membrane by electroblotting. The nitrocellulose membrane with Campylobacter antigens was incubated with egg yolk antibodies from unimmunized and immunized chickens. After incubating the antigen-antibody coated membrane in goat anti-chicken IgG alkaline phosphatase conjugate the membrane was soaked in alkaline phosphatase colour reagent. The intensity of the stain of the bands represented the concentration of anti-Campylobacter antibodies present in the purified egg yolk extracts. Figures 3.4A and 3.4B show the immunoblots of Campylobacter antigens from EDTA extracts and whole cell extracts with PEG purified egg yolk anti-Campylobacter antibodies from chicken before and after immunization, respectively.

The intensity of the stain was markedly greater in the purified egg yolk extracts after immunization than before although the egg yolks before immunization did contain a basal level of anti-Campylobacter antibodies. This confirms that inoculation of chickens with heat EDTA extracts of C.jejuni and C. coli increased

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Figure 3.3. Changes in antibody titre in egg yolk extracts using cloth based enzyme immunoassay after inoculation with EDTA extracted antigens of Campylobacter. Time 0 represents one week prior to the first immunization. Egg yolk antibodies were extracted, purified by PEG and assayed by cloth based enzyme immunoassay (CEIA) as described in Methods and Materials. The cloths were coated with either Campylobacter jejuni or C. coli antigens. (Δ) represents the antibody titre produced against antigens of C. jejuni and (□) represents the antibody titre produced against antigens of C. coli in the egg yolks of an immunized chicken over 16 weeks. (▲) and (■) shows the antibody titre produced in an unimmunized chicken, against antigens of C. jejuni and C. coli, respectively. The arrows represent the time of inoculations. The CEIA signals (A_{370} value) are plotted as mean value ± standard error (n=4).

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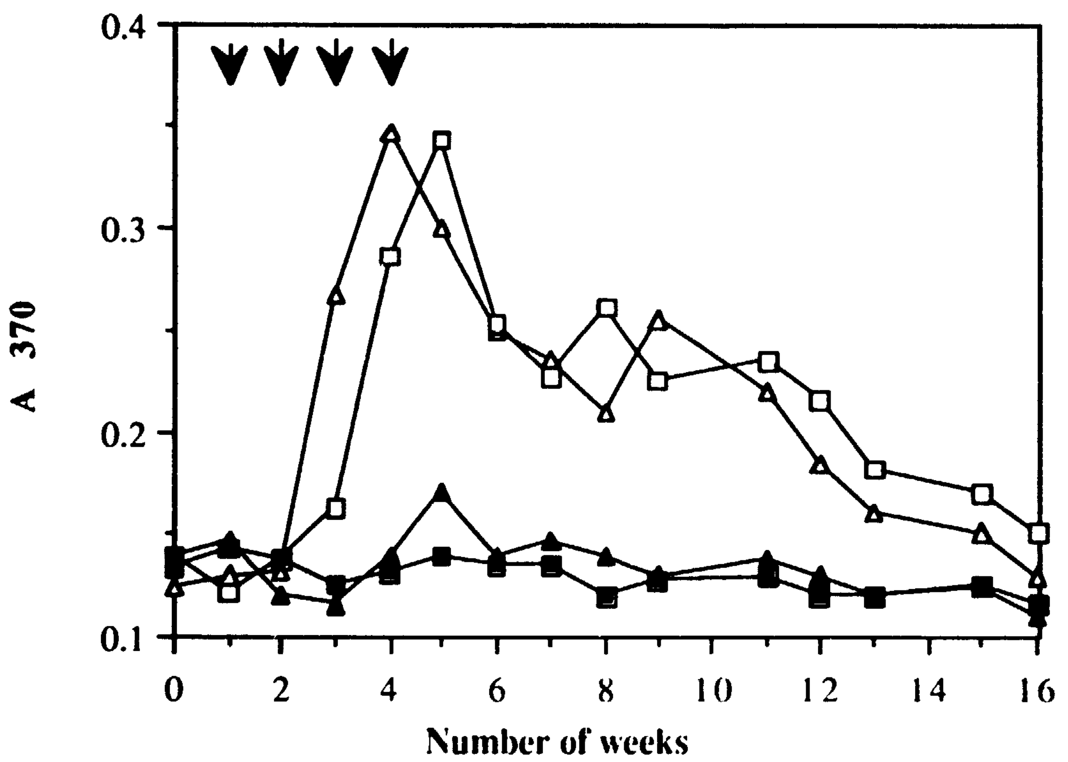

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Figure 3.4. Application of egg yolk anti-Campylobacter antibodies in western-blotting of ~~pylobacter antigens (proteins and LPS predominantly) sep... on SDS-PAGE. One mg of proteins was loaded per lane. The components in EDTA heat extracts and whole cell heat extracts were separated on SDS-PAGE and were transferred to nitrocellulose membrane by electroblotting. The antigens transferred on nitrocellulose membrane were detected by egg yolk antibodies (purified by polyethylene glycol and diluted 1 in 200 in PBST). The bound antibodies were detected by goat anti-chicken IgG alkaline phosphatase labeled conjugate. The conjugated antibodies were stained with BCIP-NBT substrate.

Lane #	Description
1	Immunoblots of the whole cell heat extracts of C. jejuni (LIO4)
2	Immunoblots of the EDTA heat extracts of C. jejuni (LIO4)
3	Immunoblots of the whole cell heat extracts of C. coli (LIO20)
4	Immunoblots of the EDTA heat extracts of C. jejuni (LIO4)

The above immunoblots were detected with egg yolk extracts of a chicken before (A) and after (4 weeks) (B) immunization.

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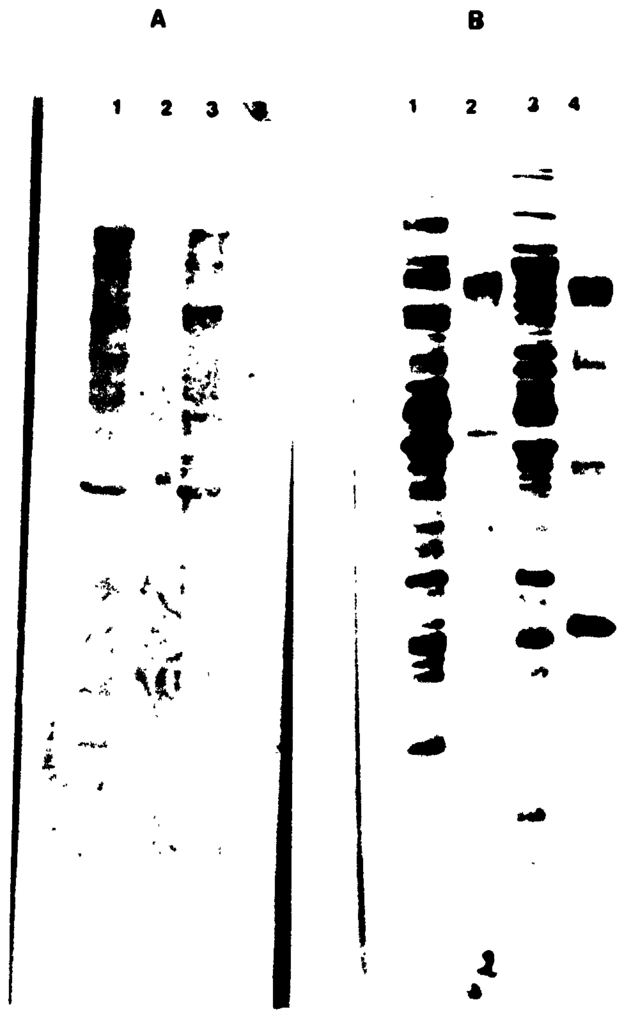

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the level of antibodies in the egg yolks and the antibodies produced were specific enough to interact with Campylobacter antigens. Figure 3.5 shows that the serum of the chicken before immunization also contained some basal level of anti-Campylobacter antibodies and that this level dramatically increased as a result of immunization. The protein patterns of the EDTA heat extracts and whole cell heat extracts on immunoblots (Figures 3.4 and 3.5) were similar to those of the Coomassie Blue stained gels (Chapter 2, Figure 2.1). Even though the chickens were inoculated with EDTA heat extracted antigens (LPS and some proteins - Figure 2.1) of the anti-Campylobacter antibodies produced in the egg yolks and serum revealed many other proteins in the whole cell heat extracts. It is possible that the EDTA heat extracts contained more proteins, immunogenic enough to produce an immune response in chicken, which the Coomassie Blue stain did not visualize on the polyacrylamide gel. Also some distinctly different bands were visualized in the EDTA heat extracts on immunoblots as compared to the whole cell extracts. In order to increase specificity of anti-Campylobacter antibodies towards a specific antigen a purified preparation of protein and LPS could be used to inoculate the chickens. In addition it is important to use birds which have minimal exposure to Campylobacter. This should reduce basal level of anti-Campylobacter antibodies.

To show if the yolk antibodies can be used to immunoblot the LPS of Campylobacter the EDTA heat extracted samples were treated with proteinase K to digest the proteins in the EDTA heat and whole cell extracts. The proteinase K treated samples were separated by

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Figure 3.5. Application of serum anti-*Campylobacter* antibodies, in western-blotting of *Campylobacter* antigens (proteins and LPS predominantly) separated on SDS-PAGE. One mg of proteins was loaded per lane. The components in EDTA heat extracts and whole cell heat extracts were separated on SDS-PAGE and were transferred to nitrocellulose membrane by electroblotting. The antigens transferred on nitrocellulose membrane were detected by chicken anti-*Campylobacter* antibodies present in serum. The bound antibodies were detected by goat anti-chicken IgG alkaline phosphatase labeled conjugate. The conjugated antibodies were stained with BCIP-NBT substrate.

Lane #	Description
1	Immunoblots of the whole cell heat extracts of *C. jejuni* (LIO4)
2	Immunoblots of the EDTA heat extracts of *C. jejuni* (LIO4)
3	Immunoblots of the whole cell heat extracts of *C. coli* (LIO20)
4	Immunoblots of the EDTA heat extracts of *C. jejuni* (LIO4)

The above immunoblots were detected with serum chicken before (A) and after (16 weeks) (B) immunization.

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SDS-PAGE and transferred to nitrocellulose membrane for western-blotting. Figures 3.6 and 3.7 show the immunoblots of LPS (proteinase K treated EDTA extracted and whole cell heat extracted samples). This confirms that antibodies specific to the LPS of Campylobacter were produced in the egg yolks by inoculating chicken with the EDTA heat extracts. The immunoblots of LPS (EDTA heat extracts and whole cell heat extracts) of all serogroups of C. jejuni and C. coli used for this study were different.

Figure 3.8 demonstrates that even though antibodies were produced against only two strains of Campylobacter, C.jejuni LIO4 and C. coli LIO20, these antibodies could also detect the protein antigens of all 18 strains of Campylobacter used in this study. The LPS was also detected for all 18 strains of Campylobacter (Figure 3.9). Whole cell extracts of each strain were loaded beside EDTA extracted cells.

In order to determine the degree of cross-reactivity of the antibodies, the egg yolk anti-Campylobacter antibodies were used in immuno-blotting of SDS-PAGE fractionated proteins in whole cell heat extracts from Salmonella typhimurium, Escherichia. coli, Proteus vulgaris and Citrobacter diversum. Figure 3.10B shows that the titre of antibodies to C. jejuni, C. coli and Salmonella increased (as evidenced by the intensity and number of bands stained) after inoculation of chicken with EDTA extracted samples of C. jejuni and C. coli. The increase in titre of Salmonella antibodies in the egg yolk extracts could be due to the presence of Salmonella proteinaceous fractions in the adjuvant used. Some cross-reactivity was also seen with Escherichia. coli and Proteus vulgaris. There

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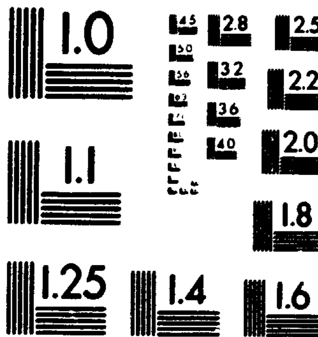

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Figure 3.6. Application of egg yolk anti-Campylobacter antibodies in immuno-blotting of Campylobacter LPS separated on SDS-PAGE. The components in EDTA heat extracts (proteinase K digested) and whole cell heat extracts (proteinase K digested) were separated on SDS-PAGE and were transferred to nitrocellulose membrane by electroblotting. The antigens transferred on nitrocellulose membrane were detected by egg yolk antibodies (purified by polyethylene glycol and diluted 1 in 200 in PBST). The bound antibodies were detected by goat anti-chicken IgG alkaline phosphatase labeled conjugate. The conjugated antibodies were stained with BCIP-NBT substrate.

Lane #	Description
1	Immunoblots of the proteinase K digested samples of whole cell heat extracts of C. jejuni (LIO4)
2	Immunoblots of the proteinase K digested samples of the EDTA heat extracts of C. jejuni (LIO4)
3	Immunoblots of the proteinase K digested samples of the whole cell heat extracts of C. coli (LIO20)
4	Immunoblots of the proteinase K digested samples of EDTA heat extracts of C. jejuni (LIO4)

The above immunoblots were detected with egg yolk extracts of a chicken before (A) and after (4 weeks) (B) immunization. Same amount of LPS was applied to the lanes in both the blots (A and B). The area between two arrows represent the blotted LPS extractions.

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A

B

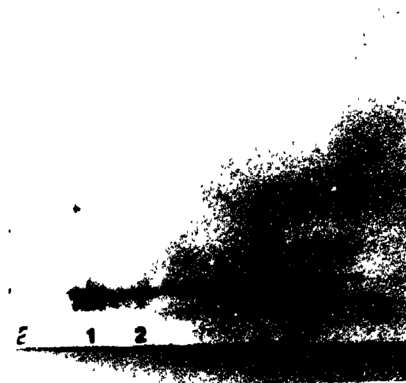

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Figure 3.7. Application of serum anti-Campylobacter antibodies in immuno-blotting of Campylobacter LPS separated on SDS-PAGE. The components in proteinase K digested samples of the EDTA heat extracts and proteinase K digested samples of the whole cell heat extracts were separated on SDS-PAGE and were transferred to nitrocellulose membrane by electroblotting. The antigens transferred on nitrocellulose membrane were detected by chicken anti-Campylobacter serum antibodies. The bound antibodies were detected by goat anti-chicken IgG alkaline phosphatase labeled conjugate. The conjugated antibodies were stained with BCIP-NBT substrate.

Lane #	Description
1	Immunoblots of the proteinase K digested samples of whole cell heat extracts of C. jejuni (LIO4)
2	Immunoblots of the proteinase K digested samples of the EDTA heat extracts of C. jejuni (LIO4)
3	Immunoblots of the proteinase K digested samples of the whole cell heat extracts of C. coli (LIO20)
4	Immunoblots of the proteinase K digested samples of EDTA heat extracts of C. jejuni (LIO4)

The above immunoblots were detected with serum of chicken before (A) and after (16 weeks) (B) immunization. Same amount of LPS was applied to the lanes in both the blots (A and B). The area between two arrows represent the immunoblots of LPS.

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Figure 3.8. Application of egg yolk anti-Campylobacter antibodies to immuno-blotting of protein antigens from 18 serotypes of Campylobacter. jejuni and C. coli. In the following list, EHE stands for EDTA heat extract and WCHE stands for whole cell heat extract.

Lane #	Description	Lane #	Description
1	EHE of C. jejuni (LIO1)	2	WCHE of C. jejuni (LIO1)
3	EHE of C. jejuni (LIO2)	4	WCHE of C. jejuni (LIO2)
5	EHE of C. jejuni (LIO4)	6	WCHE of C. jejuni (LIO4)
7	EHE of C. jejuni (LIO5)	8	WCHE of C. jejuni (LIO5)
9	EHE of C. jejuni (LIO6)	10	WCHE of C. jejuni (LIO6)
11	EHE of C. jejuni (LIO7)	12	WCHE of C. jejuni (LIO7)
13	EHE of C. coli (LIO8)	14	WCHE of C. coli (LIO8)
15	EHE of C. jejuni (LIO9)	16	WCHE of C. jejuni (LIO9)
17	EHE of C. jejuni (LIO11)	18	WCHE of C. jejuni (LIO11)
19	EHE of C. jejuni (LIO17)	20	WCHE of C. jejuni (LIO17)
21	EHE of C. jejuni (LIO18)	22	WCHE of C. jejuni (LIO18)
23	EHE of C. coli (LIO20)	24	WCHE of C. coli(LIO20)
25	EHE of C. coli (LIO21)	26	WCHE of C. coli (LIO21)
27	EHE of C. jejuni (LIO28)	28	WCHE of C. jejuni (LIO28)
29	EHE of C. coli (LIO29)	30	WCHE of C. coli (LIO29)
31	EHE of C. jejuni (LIO36)	32	WCHE of C. jejuni (LIO36)
33	EHE of C. coli (LIO44)	34	WCHE of C. coli (LIO44)
35	EHE of C. jejuni (LIO53)	36	WCHE of C. jejuni (LIO53)

The figure shows that the egg yolk antibodies produced against only two strains (LIO20 and LIO4) of Campylobacter can also detect other strains.

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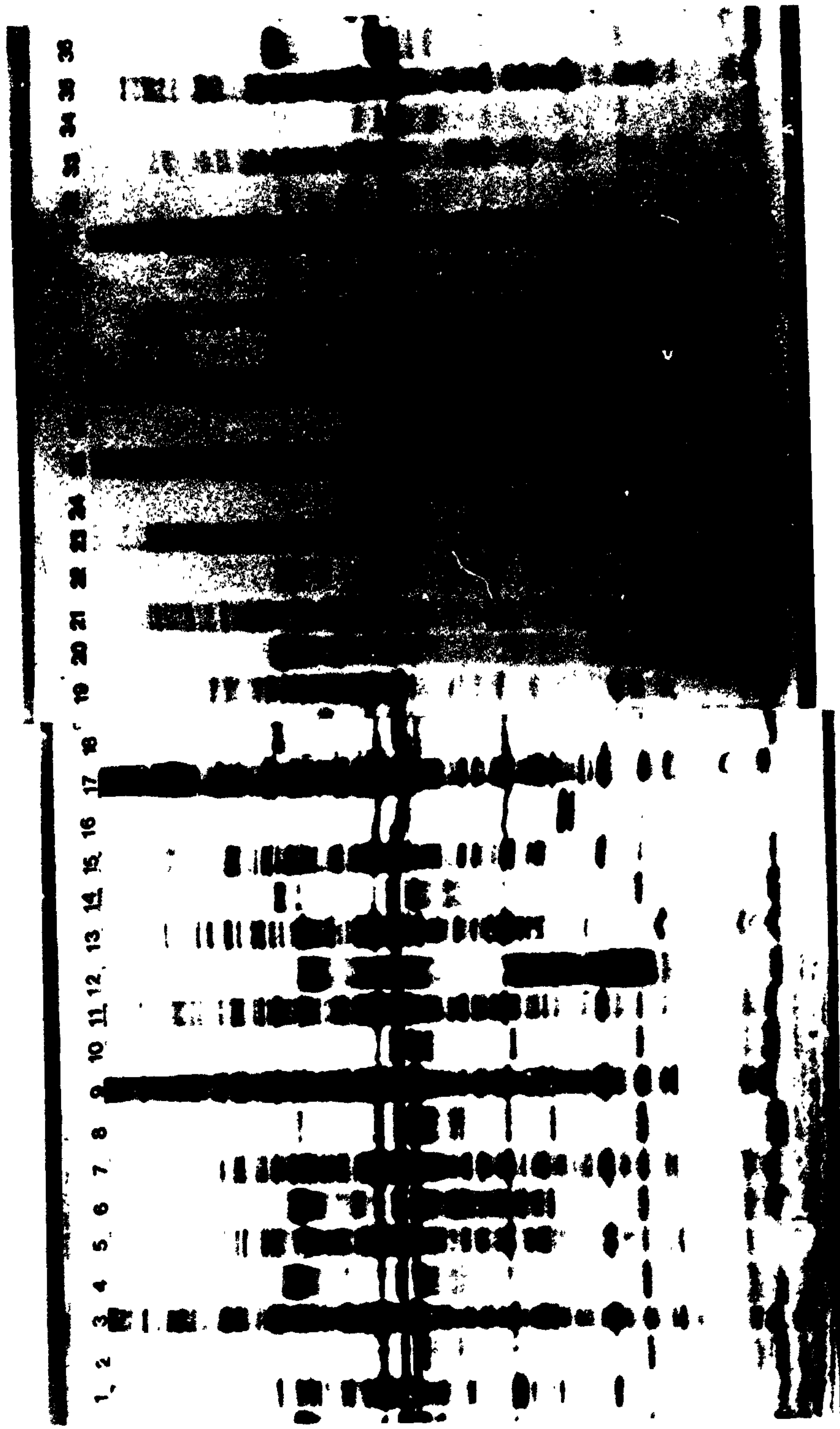

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Figure 3.9. Application of egg yolk anti-Campylobacter antibodies to immuno-blotting of LPS antigens from 9 serotypes of C. jejuni and C. coli. In the following list, EHE stands for proteinase K digested EDTA heat extract and WCHE stands for proteinase K digested whole cell heat extract.

Lane #	Description	Lane #	Description
1	EHE of C. jejuni (LIO1)	2	WCHE of C. jejuni (LIO1)
3	EHE of C. jejuni (LIO4)	4	WCHE of C. jejuni (LIO4)
5	EHE of C. jejuni (LIO4)	6	WCHE of C. jejuni (LIO4)
7	EHE of C. coli (LIO20)	8	WCHE of C. coli (LIO20)
9	EHE of C. jejuni (LIO28)	10	WCHE of C. jejuni (LIO28)
11	EHE of C. coli (LIO29)	12	WCHE of C. coli (LIO29)
13	EHE of C. jejuni (LIO36)	14	WCHE of C. jejuni (LIO36)
15	EHE of C. coli (LIO44)	16	WCHE of C. coli (LIO44)
17	EHE of C. jejuni (LIO53)	18	WCHE of C. jejuni (LIO53)

The figure shows that the antibodies produced against only two strains (LIO20 and LIO4) of Campylobacter can also detect other strains. The area between two arrows represent the immunoblots of LPS.

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Figure 3.10. Immunoblots to show cross-reactivity of egg yolk extractions with other bacterial species.

Lane #	Description
1	Immunoreaction of Campylobacter jejuni
2	Immunoreaction of Campylobacter coli
3	Immunoreaction of Salmonella typhimurium
4	Immunoreaction of E. coli
5	Immunoreaction of Proteus vulgaris
6	Immunoreaction of Citrobacter diversum

The above immunoblot were treated with egg yolk extracts, before (A) and after (B) immunization of chicken. Same amount of proteins (1 mg) was loaded into each well for both blots (A and B).

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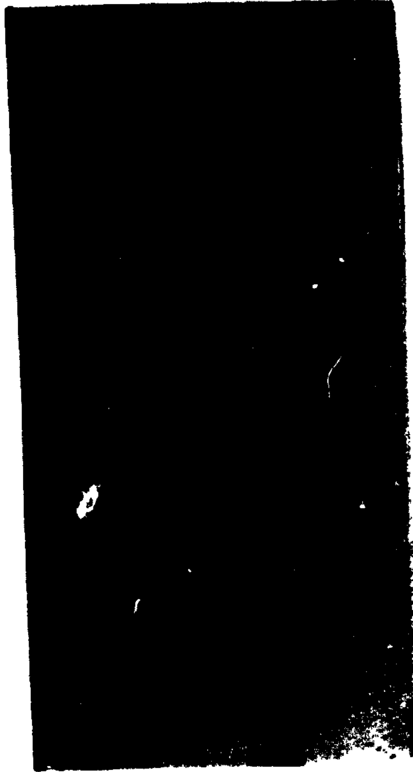
A



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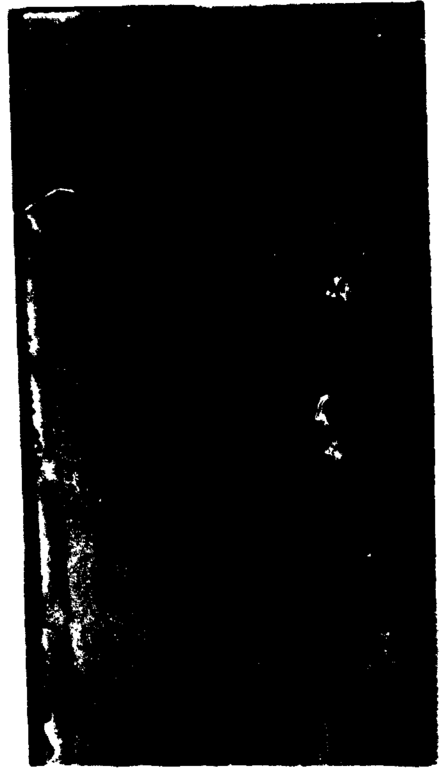

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was no cross-reactivity with _Citrobacter_. Further dilutions of the egg yolk antibody preparation could reduce this cross-reactivity relative to _Campylobacter_. In addition, the cross-reactivity can be reduced by inoculating chickens with selected antigens of _Campylobacter_ which are not shared by the other bacteria.

DISCUSSION

These experiments demonstrate that anti-_Campylobacter_ antibodies can be produced in the egg yolks of chickens, after immunization with EDTA heat extracted antigens of _C_. _jejuni_ and _C_. _coli_. The antibodies produced detected protein and LPS antigens from the 18 most commonly isolated strains of _Campylobacter_ selected for this study.

Aulisio and Shelokov, (1969) have shown that the inoculation of hens with antigens (e.g. bacterial or viral) generates high titres of antigen specific antibodies in the egg yolks. The results obtained from immunoblots, cloth based enzyme immunoassays and other experiments demonstrate that the anti-_Campylobacter_ antibodies produced in egg yolks were useful in the detection of _Campylobacter_ antigens. The type of antibody present in the egg yolk extracts was the IgG type (Figure 3.1A) even though the serum contained IgG, IgA and IgM types of antibodies (Figure 3.2). According to Malkinson (1965), the antibodies produced in egg yolk are IgG. The immunoglobulin IgM is not transferred to eggs from the circulation of the hen.

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The antibodies produced against Campylobacter (LPS and proteins) showed some cross-reactivity with some other bacteria. In order to decrease this cross-reactivity and increase in the specificity, a number of approaches are possible. Firstly, chickens can be inoculated with specific and much more highly purified antigens and secondly, anti-Campylobacter LPS antibodies can be purified after production (Blais, 1990). There were some basal Salmonella specific antibodies present in the egg yolk of the unimmunized chicken. It is possible that the protein fractions of Salmonella in the adjuvant raised the antibody titre against Salmonella antigens. This type of cross-reactivity can be avoided by using a different type of adjuvant. In order to reduce the cross-reactivity there is a need to use the birds with no previous exposure to Campylobacter or other pathogens.

A volume of 5 ml of extracted antibodies can be obtained from the yolk of one egg which is then further diluted (1:100 to 1:1000) for performing experiments like immuno-blotting, CEIA, etc. The IgG content of the yolk is actually higher than that of hen's serum (Rose et al., 1974). In addition, an average 5-6 eggs can be collected from a single hen per week. Eggs can be stored in a freezer until use.

Hens require less attention than most other animals and the collecting of eggs is a simple procedure. Bleeding of animals when using rabbits, guinea pigs, goats, etc. requires skilled personnel when techniques such as heart puncture are used. These animals are often sacrificed to collect blood. Bleeding is not necessary to obtain antibodies in the case of birds. Thus, yolks can be used as a humane alternative for antibody production.

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CONCLUSIONS

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This thesis has examined the development of simplified methods for culturing of Campylobacter jejuni and C. coli and for producing anti-Campylobacter antibodies for enzyme immunoassay. An atmosphere of 10% CO_2 in moist air was used for the growth of Campylobacter. This atmosphere provided simpler, less expensive and less laborious atmosphere to grow Campylobacter as compared to microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2). Brucella broth or agar as well as Mueller-Hinton broth or agar supplemented with 10% lysed or unlysed sheep blood supported excellent growth of all Campylobacter serotypes tested. The change of atmosphere did not affect the serotyping characteristics of the Campylobacter (Chapter 1).

A fast, economic and safe method for the extraction of Campylobacter antigens (proteins and lipopolysaccharide (LPS)) was developed. As compared to the standard hot phenol method, the EDTA heat method gave a better recovery of LPS (Chapter 2). The hot phenol method is time-consuming and hazardous and phenol requires special disposal procedures. The use of the EDTA heat extraction method eliminated the expense required for disposal of phenol and no hazard is involved. Further modifications to purify the LPS in the EDTA heat extraction method could provide a very promising method for LPS extraction.

Finally, a method for the production of polyclonal antibodies in the egg yolks of chickens was developed. In this method, laying chickens were inoculated with EDTA heat extracted antigens and the antibodies produced were extracted from the egg yolks. The antibodies produced were specific for Campylobacter antigens and

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could detect Campylobacter antigens on western-blot. Some cross-reactivity was seen but it can be reduced by inoculating birds with purified antigen samples. Eggs provide an excellent source of antibodies as hens require less attention than most other animals. The collecting of eggs is a simple procedure whereas skilled personnel are required for bleeding of animals. In order to get a continuous supply of antibodies, birds can be inoculated throughout the period of the experiment with antigens (booster shots).

Using the methods developed in this thesis for the growth of Campylobacter and extraction of immunoreagents (antigens and antibodies), it will be possible to develop an improved CEIA for the detection of Campylobacter infections.

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