ANTIGENIC ANALYSIS OF ENTEROBACTERIACEAE BY TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS WITH EMPHASIS ON ENTEROBACTERIAL COMMON ANTIGEN (ECA)

by

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DEDICATION

I could dedicate this dissertation to a number of people; friends, both professionally and personally, who have offered tremendous support, love, and understanding through some difficult times in pursuit of this degree. One friend stands out among the rest; my husband Phil, who for eleven years has encouraged me to try new challenges, has given me confidence in my ability, and then has had the courage and love to live with me when I have pursued these goals. Without him this dissertation would not have been possible. I would like to dedicate the achievements I have made in pursuit of this degree to him.

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STATEMENT OF THE PROBLEM

Since its discovery in 1962, several attempts have been made to extract and purify the enterobacterial common antigen (ECA). To date, no one has been successful in isolating a purified preparation of ECA which retains all of its native biologic properties. The isolation of ECA in a pure form is essential for its biochemical characterization, as well as the production of monospecific antisera.

Two dimensional crossed immunoelectrophoresis (XIE) has been used for evaluating the antigenic composition of several microorganisms, as well as isolating a variety of individual microbial antigens. This technique has not previously been applied to the isolation and characterization of antigens in Enterobacteriaceae.

The primary goals of this investigation are:

1. To evaluate the antigenic composition of ECA-positive and ECA-negative strains of Enterobacteriaceae.
2. To utilize XIE in identifying and characterizing ECA.
3. To determine whether other cross-reacting antigens exist among members of the family Enterobacteriaceae.

INTRODUCTION

A. DISCOVERY, DETECTION AND BIOLOGICAL ACTIVITY OF ECA

The enterobacterial common antigen was discovered in 1962 by Kunin and co-workers (1,2,3) in the course of an investigation aimed at elucidating the relationship between E. coli serotypes and E. coli antibodies in patients with urinary tract infections. They found that extracts from 136 distinct E. coli serotypes were capable of sensitizing erythrocytes when tested by an indirect hemagglutination assay (IHA) using certain heterologous antisera. Cross-reactivity was best demonstrated with antiserum to E. coli 014, but also occurred to a lesser extent with antisera to E. coli 056, 0124, and 0144. These antisera were also capable of reacting with their homologous antigens, not only by IHA, but also by bacterial agglutination and precipitin techniques.

Cross-reactivity of E. coli 014 antiserum was removed by adsorption with extracts of several heterologous E. coli strains. Reactivity to homologous E. coli 014 antigen remained following adsorption. Adsorption of this antiserum with E. coli 014 removed both cross-reacting and homologous IHA activity.

From these observations, Kunin concluded that antisera prepared against E. coli 014 possessed antibodies to at least two distinct antigens. One antigen, which represented the lipopolysaccharide (LPS) somatic O antigen, was serotype-specific for E. coli 014 and could be detected by bacterial agglutination, hemagglutination and precipitin techniques. A second

antigen was common to all E. coli strains, as well as other enteric bacteria, and could be detected only by indirect hemagglutination (IHA) and hemagglutination inhibition (HAI) (2,3). This antigen has been referred to as enterobacterial common antigen (ECA) (4). ECA is thus defined as a cross-reacting antigen present in most enteric bacteria, detectable by IHA using antiserum to E. coli 014 (4).

Kunin tested antisera prepared against E. coli serotypes other than 014 using IHA assay. These antisera did not possess activity to ECA. From these results Kunin postulated that the ECA of E. coli 014 was present in an immunogenic form, while in most other E. coli serotypes ECA existed in a non-immunogenic state (3).

Whang and Neter confirmed the existence of ECA in various enteric bacteria (5). The evidence they provided in support of the view that ECA was an entity distinct from LPS O antigen can be summarized as follows:

1. IHA cross-reactivity occurred only with crude enteric LPS O antigens; highly purified LPS generally did not cross-react.
2. Latex agglutination could be used to detect LPS O antigen, but not ECA.
3. Preincubation of E. coli 014 antiserum with crude LPS preparations from Shigella sonnei or E. coli 0111 removed heterologous IHA activity.

They further demonstrated that the IHA assay was specific

for the detection of ECA by showing that neither Salmonella Vi antigen nor staphylococcal antigens reacted with E. coli 014 antisera when tested by IHA.

ECA has also been detected using other immunochemical assays. Whang and Neter employed a hemolysis test, similar to IHA with the exception of added complement, and used erythrocyte lysis as a measure of ECA (5). ECA has been detected in tissue specimens by indirect immunofluorescence microscopy (IFA) (6). Finally, Johns and co-workers used double immunodiffusion and immunoelectrophoresis to detect ECA (7,8,9). ECA-negative mutants of enteric bacteria as well as non-enteric organisms were observed not to possess ECA when tested by double immunodiffusion (10).

Kunin's failure to demonstrate ECA by immunodiffusion (3) was attributed to his use of antisera preserved in 50% glycerol. This preservative renders antiserum hygroscopic, resulting in absorption of water and spillage from wells. ECA, due to its low molecular weight, diffuses rapidly in gel and may react with antibodies prior to diffusion, resulting in precipitation in the antiserum well (7). Later experiments by Whang and co-workers demonstrated that ECA-reactive antibodies (ECA-Ab), determined by IHA, must be of sufficiently high titer in order for gel precipitation to occur (10).

Bacterial agglutination was used by Marx and co-workers to demonstrate ECA using antisera prepared against four different ECA-positive bacterial strains. Organisms with rough LPS core types R-1 and R-4 were readily agglutinated.

In contrast, other LPS core mutants (Salmonella core type Ra, E. coli core types R-2 and R-3), as well as smooth forms of R-1 and R-4 core types demonstrated minimal agglutinating activity. Smooth strains of different enterobacterial species and genera failed to show any agglutinating ability. Adsorption of agglutinative antisera with an ECA-negative strain of E. coli did not remove agglutinating activity. Adsorption of antisera with the ECA-positive strain removed all agglutinating activity. ECA agglutinin formation in rabbits parallelled formation of ECA-Ab mediated IHA activity, indicating that the same antibody class might be involved in both bacterial agglutination and IHA activities (11).

E. coli 014 ECA antiserum was bactericidal only to homologous, but not heterologous E. coli strains, Shigella, or Salmonella species (2,12). ECA-Ab prepared against Salmonella typhi, E. coli 0111, and Salmonella typhimurium was bactericidal to E. coli 014 but not other enteric organisms, thus demonstrating a difference in ECA possessed by the E. coli 014 strain (12).

ECA-Ab was shown to opsonize ECA-positive E. coli and Salmonella for polymorphonuclear leukocyte (PMN) phagocytosis, but not Pseudomonas aeruginosa, an organism which does not possess ECA (12). Enhanced phagocytosis was also shown to occur when latex particles coated with ECA were incubated with PMN's in the presence of ECA-Ab (13).

Whang and Neter demonstrated that ethanol-soluble ECA isolated from non-immunogenic bacteria was selectively des-

troyed by supernatant fluid and filtrates from Pseudomonas aeruginosa. This activity (possibly an enzyme) was destroyed by heating to 100°C for 10 minutes (14). The ethanol-insoluble ECA from E. coli 014 was unaffected by this Pseudomonas factor (15).

In summary, a common antigen (ECA), distinct from LPS O antigen is present in enteric bacteria. ECA can be detected most reliably by IHA and HAI assays. Other methods for demonstrating ECA activity include complement-dependent, antibody-mediated erythrocyte hemolysis, immunoprecipitation, immunoelectrophoresis and IFA. ECA appears to exist in two forms. One form is found in immunogenic strains, such as E. coli 014. The other non-immunogenic form is found in most other enteric bacteria.

B. ISOLATION AND IMMUNOCHEMICAL CHARACTERIZATION OF ECA

Numerous studies aimed at characterizing ECA have been performed. Several investigators have analyzed different bacteria using different extraction and purification techniques. These studies have been hampered by the lack of purified preparations of ECA which retains their native immunologic and biochemical properties.

1. Isolation of ECA from E. coli 014

Isolation and characterization of ECA from E. coli 014 was first attempted by Kunin and co-workers in 1963 (16). They used the hot phenol-water method originally described by Westphal et al. (17) for isolation of LPS. After dialysis of the aqueous phase, nondialyzable material was concentrated,

and finally precipitated with ethanol. This precipitate was washed, redissolved, and then analyzed by DEAE cellulose anion-exchange column chromatography, eluted using a discontinuous NaCl gradient. Fractions were tested for ECA activity by HAI. Gel precipitin and HAI assays were used to detect LPS. The major peaks of ECA and LPS eluted with slightly different molarities of NaCl indicating that these two entities were at least partially separable.

ECA, isolated in this manner, contained 6.5% nitrogen as glucosamine and amino acids, and a small amount of nucleic acid contamination. The preparation contained small amounts of neutral sugars (2.3%), and a substantial amount of hexosamine (12.6%). The principal amino acids were identified as glutamic acid, alanine, glycine, aspartic acid, and probably diaminopimelic acid. These findings were consistent with the hypothesis that ECA was likely of cell wall origin (16), since the peptidoglycan, a cell wall component common to all bacteria, is known to contain n-acetyl glucosamine, alanine, glutamic acid, and meso-diaminopimelic acid (18).

Purified ECA was non-immunogenic for rabbits when inoculated intravenously (16). It retained HAI activity but was not adsorbed to erythrocytes. ECA-mediated HAI activity was resistant to periodate oxidation and trypsin digestion; it was not dialyzable through cellophane membranes and was passed through a Sephadex G-100 column. The molecular weight was estimated to exceed 40,000 (16).

Hammarström and co-workers (19) also employed the hot

phenol-water method of Westphal, et. al. for the isolation of ECA from E. coli 014. The extracted LPS was first treated with ribonuclease and then purified by Sepharose 4-B column chromatography. The resultant material was shown to contain 3.8% nitrogen, 3.2% O-acetyl residues and large amounts of sugars known to be present in the basal core of E. coli and Salmonella LPS, i.e., galactose (6.8%), glucose (14.4%), and heptose (3.7%). Glucosamine was present in small amounts (3.5%). This material possessed both IHA and HAI activity when tested using antisera reactive with either E. coli 014 O antigen or ECA.

Alkali treatment of this material reduced its O-acetyl content and destroyed all non-ECA antigenic determinants. ECA activity, demonstrable by HAI, remained. When this material was further subjected to mild acid hydrolysis, a procedure known to cleave the acid-labile 2-keto-3-deoxyoctonate (KDO)-lipid A bond in LPS, and the acid soluble fraction applied to a Sephadex G-50 column eluted with pyridine acetate, 36% of the material eluted in a single major peak. The estimated molecular weight of this material was $2\text{-}3 \times 10^3$. This fraction possessed ECA-HAI activity but was not tested for its ability to coat erythrocytes or stimulate antibody production in rabbits. Chemical analysis of this material showed that it contained galactose, glucose, heptose and large amounts of KDO, glycosidically bound to other sugars. All of these constituents are part of the basal LPS core of E. coli 014 (19).

Kiss and co-workers subsequently demonstrated that the

immunogenic form of ECA was physically associated with the LPS core fraction, unlike ECA from non-immunogenic bacteria. These findings are consistent with the results of chemical analyses conducted by Hammarström on ECA derived from E. coli 014 (20). The precise contribution of LPS core sugars to the antigenic character of ECA remains unresolved. Bacteria with LPS R-1 core mutations which are deficient in ECA, have been demonstrated (20). Also, adsorption of ECA-reactive antiserum with ECA-negative R-1 mutant bacteria did not lower ECA-Ab titers (21). These observations support the notion that LPS core sugars are not an integral part of ECA.

2. Isolation of ECA from Non-Immunogenic Strains

Johns and co-workers (7) selected for ECA extraction a strain of Salmonella typhi (0901) which lacked Vi, H and K antigens. A water extract prepared from acetone-killed organisms was treated with picric acid to remove several protein antigens. The precipitated material was discarded and acetone added to the supernatant to precipitate residual antigenic material. This precipitate was then dissolved in buffer, dialyzed, and finally subjected to column chromatography using a Sephadex G-200 column. Four fractions were isolated and tested for the presence of LPS and ECA activity by immunoelectrophoresis and immunoprecipitation. The fraction identified as having ECA activity was further purified by gel electrophoresis. The final product was analyzed chemically and found to contain primarily hexose (42%), with lesser amounts of hexosamine (1.26%). The protein content was 4.45%, with 1.66% nitrogen. KDO, heptose, and phosphate were absent. The resulting material was

non-immunogenic and incapable of coating erythrocytes for hemagglutination. It did, however, exhibit ECA HAI activity.

Suzuki and co-workers (22) were able to separate ECA from LPS by ethanol fractionation. Bacterial suspensions were boiled for 1 hour and the supernatant treated with 95% ethanol. Ethanol-soluble and insoluble fractions were separated by centrifugation. ECA activity from non-immunogenic strains was concentrated in the ethanol-soluble fraction. ECA activity from E. coli 014, an immunogenic strain, was found in both fractions, with the major portion residing in the ethanol-insoluble fraction.

McLaughlin and Domingue (23) used the ethanol fractionation procedure to obtain a partially purified ECA preparation from three non-immunogenic ECA-positive bacteria. Chemical characterization of dried ethanol-soluble material, revealed protein, nucleic acids, a small amount of carbohydrate, hexose and lipid. Ethanol-soluble preparations, extracted by the hot phenol-water method of Westphal and Jann (24), were immunogenic, demonstrated ECA HAI activity, and were capable of coating erythrocytes.

Marx and Petcovici extracted ECA from Salmonella typhimurium TV 149 using heated ethanol. A bacterial suspension was heated to 60°C in 95% ethanol, centrifuged, cooled, and the supernatant evaporated to partial dryness. This material was resuspended in 85% ethanol, precipitated with acetone, centrifuged, and the sediment redissolved in water. The resultant material was purified by passage over a Sephadex G-75 column. "Purified"

ECA obtained in this manner was thought to possess a high molecular weight, since it eluted in the void volume of a Sepharose 2-B column. Chemical analyses revealed protein, glucosamine, glucose, and some constituents characteristic of a cephaline-type phosphoglyceride, i.e., glycerol, ethanolamine, phosphorus, and fatty acids (primarily palmitic acid). The isolated product was highly acidic in nature. It was immunogenic in rabbits and was capable of coating erythrocytes. The preparation lost its ability to sensitize erythrocytes upon incubation with phospholipase A. In addition, its HAI capacity was removed, suggesting that an essential immunodeterminant of ECA was destroyed by this enzyme (25).

Männel and Mayer (26) extracted ECA from Salmonella montevideo. The method they used is based on the principle that LPS which contains the ECA determinant can be extracted in phenol-water, while LPS which does not contain the ECA determinant can be removed by a phenol-chloroform-ether solution (27,9).

Bacteria, killed with phenol, were first subjected to phenol-water extraction. Phenol-petroleum-ether was then added to the aqueous phase. The resultant aqueous phase was collected and finally analyzed by DEAE cellulose chromatography. ECA activity, as determined by HAI and gel precipitation, eluted in fractions consistent with a molecular weight of <10,000. Chemical analyses of this material revealed alternating residues of N-acetyl-D-glucosamine (GlcN) and N-acetyl-D-mannosaminouronic acid (ManNUA), partly esterified by palmitic

acid. Amino sugars comprised approximately 50% of the product; palmitic acid approximately 2.5%. 11.7% of the material consisted of n-acetyl groups; 1.3%, o-acetyl groups. Neutral sugars were not detected. Palmitic acid, while apparently not an antigenic determinant of ECA, was thought to be essential for its erythrocyte-coating capacity and at least in part responsible for the hydrophobic character of ECA. Removal of palmitic acid by alkali treatment abolished erythrocyte-sensitizing ability (26).

Further studies by Männel and Mayer indicated that ManNUA was essential to the antigenicity of ECA (21). The following observations support this hypothesis:

a. Esterification of ManNUA resulted in loss of antigenic reactivity. Reactivity was restored by saponification (21).

b. Reduction of the carboxylic acid residue of ManNUA also diminished antigenic reactivity (26).

c. ManNUA was not present in three LPS mutants (rfe, rff, and rfb) which did not produce ECA (4).

d. ManNUA polymers were found in ECA-positive enteric strains of different genera (4).

The purified product of Männel et al., was capable of coating erythrocytes, possessed IHA and HAI activity and was precipitated in agarose gel by ECA-Ab (21). It did not induce antibodies in rabbits when injected intravenously. However, when the material was adsorbed onto an acetic acid-treated ECA-negative mutant of Salmonella minnesota, high titers of

ECA-Ab resulted after intravenous inoculation (21).

In summary, the available evidence supports the view that ECA is of low molecular weight (7,19,26). Discrepancies in molecular weight determinations might be explained by the observation that ECA in aqueous solutions forms micelles of varying molecular size due to its insolubility in water (26). In addition, ECA is probably a carbohydrate composed, at least in part, of the amino sugars D-GlcN and D-ManNUA. Evidence in support of this hypothesis is as follows:

a. ECA is not destroyed by heating to 120°C (7), trypsin (16,14), or pronase digestion (14).

b. ECA is resistant to periodate oxidation (16), and is present in bacteria which do not produce common neutral sugars (26), suggesting that ECA is probably not composed of the common hexoses.

c. D-GlcN and D-ManNUA have been isolated in significant amounts in purified preparations of ECA (26).

C. GENETICS OF ECA

Genetic studies of ECA have focused chiefly on the analyses of mutants deficient in ECA. Such mutant strains have been discovered fortuitously.

Johns et al., found that rough (R) mutants of S. minnesota with complete LPS cores (Ra and Rb) produced ECA, as determined by HAI. In contrast, strains with the defective core LPS (Rc, Rd, and Re mutants) did not produce ECA. Mäkelä and Mayer confirmed John's findings; namely only strains with

complete LPS core types produced ECA in large amounts (28). Thus, a relationship seemed to exist between ECA production and LPS core type (4). Several genes which control ECA production have been identified. They are discussed below.

Rfe genes

The rfe gene cluster has been shown to play a role in the synthesis of LPS O antigen side chain polysaccharides in certain Salmonella species. Mutations in this gene cluster result in an intact LPS core lacking only O antigen side chains (chemotype Ra) (29). Since the presence of ECA in S. minnesota R mutants is always associated with the presence of a functional rfe gene region and since rfe-negative mutants of S. minnesota and S. montevideo are either void of ECA or produce trace amounts, the rfe gene cluster seems to be related to production of ECA (28,29,30).

Rfe mutations in Salmonella typhimurium have not been described. However, hybrid strains of S. typhimurium have been produced by introducing the rfe-negative gene from S. montevideo. The hybrids were smooth strains which possessed S. typhimurium-specific LPS but did not produce ECA (30). This suggests that S. typhimurium contains a gene allelic to the rfe gene region of S. montevideo which is necessary for ECA synthesis but not LPS production. Studies by Mäkelä and co-workers provide additional support for this hypothesis. When the rfe-negative gene cluster of an ECA-negative hybrid of S. montevideo was replaced by the rfe-positive genes of either S. montevideo or S. typhimurium, the recombinants were ECA-

positive (30). The transfer of rfe-negative genes from S. typhimurium or S. montevideo to certain E. coli strains resulted in recombinants which were ECA-negative, rough, and had complete LPS cores. The rfe genes, therefore, seem to be required for ECA production in E. coli as well as Salmonella (4).

The gene products of the rfe gene have not been isolated. The rfe gene cluster is possibly involved in a single step common to the synthesis of ECA as well as LPS O antigen side chains of many different O antigen groups. One attractive hypothesis is that the rfe gene codes for the synthesis and modification of a carrier molecule used in the assembly of ECA and various LPS O antigen side chains (4).

Rfb genes

Mäkelä tested S. typhimurium R mutants in which varying segments of the rfb gene were deleted. Strains with major deletions were found to be ECA-negative, while those with minor deletions still produced ECA. These data suggest that at least one of the gene products regulated by the rfb gene cluster may be required for ECA synthesis. However, recombinant experiments in which the rfb gene region in a major deletion strain was replaced with a wild type allele from a S. typhimurium donor, resulted in progeny which were still ECA-negative. Mäkelä concluded therefore, that the ECA-negative phenotype must be due to a second mutation outside the rfb gene cluster (4).

The activity of the rfb gene appears to vary in different

Salmonella species. When the rfb gene region of an ECA-positive strain of S. typhimurium was replaced with an rfb-positive gene region from S. montevideo, the resulting recombinant produced only trace amounts of ECA (30). In contrast, deletions in the rfb gene region of S. montevideo result in strains which still produce ECA (4).

The function of the rfb gene cluster is not clearly understood. Mutant and hybrid strains of S. typhimurium lacking only the rfb gene still produce ECA in trace amounts. Mäkelä and co-workers postulated that the rfb gene may have a regulatory function in ECA production (4).

Rff genes

A mutant strain of S. minnesota which produced smooth colonies yet lacked ECA was first described by Galanos et al. (31). The existence of such a mutant demonstrated that mutations which result in the loss of ECA production were not necessarily associated with alteration in LPS synthesis (4). The mutation in this strain was shown to involve the rff gene region, a region closely linked to the rfe gene locus. The existence of this mutant with normal LPS phenotype, clearly demonstrated that the rff and rfe genes were distinct (4).

The precise function of the rff gene product(s) are not known. It is possible that rff genes which do not participate in LPS synthesis could be involved in the synthesis and assembly of such ECA-specific compounds as ManNUA (4).

Rfa genes

Rfa genes play a role in determining LPS chemotype (32).

Mäkelä demonstrated that loss of the rfa gene cluster did not directly result in loss of ECA production (28).

Rfa mutations do, however, influence the immunogenicity of ECA. Marx described an rfa mutant of E. coli 014 which was characterized by incomplete synthesis of LPS core and loss of ECA immunogenicity. Chemical analyses revealed the absence of one glucose and one galactose molecule in the core region of LPS. ECA was present exclusively in the free form, i.e., not bound to the LPS core. Free ECA was soluble in ethanol, susceptible to alkali treatment,and immunogenic only after removal of LPS. Based on these observations, the LPS appeared to suppress ECA immunogenicity. ECA in the wild type E. coli 014, on the other hand, existed in a free form as well as bound to the LPS core. Bound ECA was ethanol-insoluble and resisted alkali treatment (33). Marx's observations suggest that synthesis of the complete core of LPS is a prerequisite for ECA binding in E. coli 014, and that binding of ECA to the core is required for immunogenicity (33).

Stocker and co-workers demonstrated that the gene rfaL codes for a translocase which is required for the attachment of the O antigen side chains to the LPS core (32). Schmidt et al., have also provided evidence that rfaL mutants of immunogenic R-1 and R-4 core types of E. coli and Shigella produced ECA, but were not immunogenic. The rfa gene therefore appears to participate in the enzymatic transfer of ECA to the LPS core in immunogenic strains (34).

A summary of the genes involved in ECA synthesis, regulation and immunogenicity is presented in Table 1.

Table 1

PROPOSED GENE CLUSTERS INVOLVED IN ECA SYNTHESIS

Gene	Proposed Function	Proposed Role in ECA Production
rfe	Possibly involved in synthesis and modification of carrier molecule used in assembly of several polymers; eg., several types of LPS O antigen side chains	Required for synthesis of ECA; may be involved in synthesis or modification of a carrier molecule used in the assembly of a number of polymers such as ECA and certain LPS O antigen side chains
rfb	Possibly involved in synthesis and assembly of LPS O antigen side chains	Known to participate in ECA production in S. typhimurium. May produce enzyme which synthesizes or regulates ECA production
rff	Function unknown	Required for synthesis of ECA; may be involved in synthesis and assembly of ECA-specific compounds such as ManNUA
rfa	Affects a specific transferase of an LPS core constituent, thus affecting LPS chemotype	May be involved in translocation of ECA to certain LPS core types thus rendering ECA immunogenic

Adapted from: Mäkelä, P. H., and H. Mayer. 1976. Enterobacterial Common Antigen. Bacteriological Reviews. 40:591-632.

D. IMMUNOGENICITY OF ECA

Immunogenic Versus Non-immunogenic Strains

Kunin et al., originally reported that antibodies to ECA were found only in sera of rabbits immunized with E. coli 014, 056, 0124 and 0144 (2). Other E. coli serotypes did not elicit ECA-Ab formation (2). These results have been corroborated by others (33,35,36).

Experiments by Whang and co-workers demonstrated that rough mutants of E. coli core type R-1 were highly immunogenic for ECA, in contrast to rough mutant E. coli core types R-2, R-3, and Salmonella core types Ra, Rd and Re (35). In addition, few strains of bacteria (eg., E. coli 014, Shigella boydii, E. coli 08:K27 rough mutant and two rough mutants of E. coli 09) elicited an antibody response to ECA when heat-killed bacteria were administered intravenously (9,35,36). Heat-killed suspensions of smooth strains of Enterobacteriaceae produced a minimal response to ECA.

Subsequently, Suzuki and co-workers demonstrated that ethanol-soluble ECA from non-immunogenic strains of E. coli was immunogenic in rabbits following removal of LPS. Immunogenic strains, such as E. coli 014, differed from non-immunogenic strains in that they possess ECA both in a free and LPS-bound form. Free ECA was soluble in ethanol, LPS-bound ECA was not (22).

Mayer et al. (9,37,38), noted that certain rough mutants of E. coli and Shigella were as immunogenic for ECA as was E. coli 014. These rough mutants were similar to E. coli 014

strains which possess terminal or subterminal core glucose arranged in beta linkage, whereas in non-immunogenic strains, alpha linkage is observed (39). It is thought that these constituents are important determinants in ECA immunogenicity (4).

The apparent requirements for ECA immunogenicity are summarized below:

a. The LPS core must be complete (33,35).

b. The LPS core must be free with no attached O antigen side chains (38).

c. The LPS core must possess a configuration compatible with linkage of ECA (4,20,40,41,42).

d. The LPS core must possess suitable acceptor properties for ECA, i.e., the absence of a terminal glucosamine residue (42,43,44,45).

e. The anomeric linkage of the terminal and subterminal LPS core glucose residue contributes to the immunogenic properties of ECA (39).

E. OTHER FACTORS AFFECTING IMMUNOGENICITY

1. LPS-Associated Immunosuppression

Suzuki and co-workers were the first to demonstrate that the interaction between free ECA and LPS in non-immunogenic bacterial strains renders those strains non-immunogenic. Following separation of ECA from the LPS by ethanol fractionation, the ethanol-soluble ECA is capable of eliciting antibodies in rabbits upon intravenous injection. In contrast,

injections of whole cell extracts, or the ethanol-insoluble fraction derived from non-immunogenic strains did not result in ECA-antibody production. Furthermore, when the ethanol-soluble fraction was mixed with the ethanol-insoluble fraction prior to injection, the immunogenic capacity of ECA was markedly reduced (22). This phenomenon was termed LPS-associated immunosuppression (46) and occurred only when ECA and LPS were in intimate contact and injected simultaneously at the same site (47).

The mechanism of immunosuppression is not clearly understood. It appears that ECA in non-immunogenic strains aggregates nonspecifically with LPS. This can be effected by prior mixing of ECA and LPS in a test tube, by co-extracting LPS and ECA from bacteria, or by injecting heat-killed bacteria in which both components are present. Each of these techniques results in minimal antibody response to ECA as determined by IHA, when the components are injected intravenously. However, subsequent single intravenous injection of free ECA (separated from LPS) results in an anamnestic antibody response to ECA characterized by rapid rise and high peak titers (eg., IHA titers as high as 1:6000) (48,49).

Whang and Neter studied the quantitative and kinetic aspects of LPS-associated immunosuppression. At least 2 days were required after the priming injection for the booster dose to be effective. Immunologic priming persisted for at least 8 weeks. A 1000-fold dilution of ECA-LPS aggregates derived by ethanol fractionation from E. coli 0111 could be

administered and still affect priming. Similarly, booster injections of ethanol-soluble ECA diluted 1:1000 resulted in substantial anamnestic responses of ECA-Ab. The specificity of this phenomenon was demonstrated by showing that organisms which did not contain ECA, such as Pseudomonas aeruginosa and Staphylococcus aureus did not induce immunologic priming (50).

Several different LPS preparations have been tested for their immunosuppressive effect on ECA. LPS present in crude extracts or ethanol-insoluble sediments, as well as several purified LPS preparations were effective immunosuppressants (47,50,51). However, LPS obtained from strains which did not produce ECA due to mutations in the rfe gene region, did not suppress ECA-antibody formation (52). The lipid A portion of the LPS molecule was also found to have an immunosuppressive effect on ECA, although in some experiments it was less active than native LPS (53).

Other substances have been shown to suppress ECA antibody production. Many of these immunosuppressants are membrane-active compounds, i.e., lipids or ampholytes. Examples include cardiolipin (54), whole serum from certain animal species (46), Mycoplasma membranes (55), gangliosides, methyl palmitate, and detergents such as Triton X and Tween 20 (56).

Whang and Neter reported that cholesterol reversed the immunosuppressive effect of LPS on ECA (57). Agarwall et al., showed that cholesterol also prevented the immunosuppressive effect of Triton X and methyl palmitate (56).

2. Particle Size and its Effect on Immunogenicity

Whang et al., studied the importance of particle size in ECA-antibody formation. They subjected the ethanol-soluble fraction of ECA isolated from S. typhimurium to high speed centrifugation. While both the pellet and supernatant possessed the same ECA-HAI activity, only the pellet elicited a primary ECA antibody response when administered intravenously into rabbits using multiple injections. Rabbits immunized with the supernatant did however, respond with enhanced production of ECA-Ab following secondary injections (58).

Ethanol-soluble ECA was passed through millipore filters of varying pore sizes, and the filtrates used for immunization. Millipore filtration through a pore size smaller than 0.45 μm eliminated the immunogenic properties of ECA (58).

3. Effects of Heat, Freezing and Alkali Treatment on ECA-Ab Production

Whang et al., found that heating for 1 hour at 100°C or treatment with 0.25 N NaOH reduced the immunogenicity of ethanol-soluble ECA derived from S. typhimurium, without affecting its antibody neutralizing or immunologic priming capacity. The immunogenicity of heated antigen preparations, as well as membrane filtrates of ethanol-soluble ECA, were restored upon repeated freezing and thawing of the preparation. Freezing and thawing also partially restored immunogenicity to alkali-treated antigen. They postulated that heat and alkali treatment altered the state of aggregation of ECA, thus diminishing its immunogenicity (58,59,60).

Factors that influence immunogenicity of ECA may be summarized as follows:

a. The binding of ECA to LPS renders ECA immunogenic (20).

b. Free ECA (not associated with LPS) is also immunogenic (22).

c. The presence of substances such as LPS and other membrane-active compounds inhibit immunogenicity of free ECA (22,46,54,55,56). Though no antibody response occurs upon injection of ECA and LPS, immunologic priming does take place (48,49).

d. Particle size and molecular aggregation affect the immunogenicity of free ECA (58,59,60).

F. LOCATION OF ECA IN THE BACTERIAL CELL

The exact location of ECA in bacterial cells is not known. It appears that at least small amounts of ECA reside on the cell surface, since ECA was readily eluted from cells washed in buffer (3). Aoki and co-workers visualized ECA in the cell walls of Enterobacteraceae using IFA (6).

Domingue and Johnson offered the most direct evidence for the localization of ECA in bacterial cell walls (61). They utilized a fractionation procedure involving disruption of bacterial cells in a French pressure cell, followed by high speed centrifugation. Three fractions resulted: a) intact cell envelopes; b) membrane vesicles and envelope fragments; and c) solubilized cytoplasmic constituents. Several bacterial species were fractionated and tested for ECA by HAI. Results

of HAI demonstrated that ECA activity was generally found in all fractions. Additional fractionation using sucrose density gradient showed that ECA activity was associated with the membrane fraction of E. coli 014, while in Salmonella typhimurium ECA activity was predominantly in the solubilized fraction. It is possible that the stability of the ECA-cell wall association is greater in E. coli 014 than in Salmonella typhimurium. Alternately, ECA in S. typhimurium may not be exclusively associated with the bacterial cell envelope.

G. CLINICAL IMPORTANCE OF ECA

Biological Effects

Kunin tested ECA fractions isolated from E. coli 014 for pyrogenicity and lethal effects in rabbits. He found that 500 µg of their preparation produced fever but no other untoward effects (16). Johns et al., found that injection of 250 µg of presumably purified S. typhi ECA was pyrogenic, while 100 µg produced no effect (7). These results should be interpreted with caution, however, since neither of these ECA preparations were in their native state as evidenced by their inability to coat erythrocytes (7,16).

Kessel et al., performed a mouse toxicity test using S. typhimurium ECA in which mice were injected intraperitoneally with varying amounts of ethanol-soluble ECA. 1000 µg of ECA did not cause death. In contrast, 1 µg of LPS caused death in 20% of mice tested. Similarly, the ethanol-soluble fraction (containing ECA) was 100 to 1000-fold less active than

the LPS-containing ethanol-insoluble fraction when tested in the following additional endotoxin assays: promotion of Schwartzman-like reaction by epinephrine in rabbits, immediate production of nonspecific resistance to Salmonella infection in mice, and direct cytotoxicity to monolayers of guinea pig peritoneal macrophages. They concluded that either ECA produced effects characteristic of endotoxin when present in large amounts, or that it lacked endotoxic activity altogether, and the minimal biological responses exhibited by ECA were due to contamination with small amounts of LPS (62).

Suzuki and co-workers reported that the ethanol-soluble fraction isolated from a variety of enteric organisms was capable of modifying sheep erythrocytes for lysis by ECA antibodies and guinea pig complement (22). There is no evidence indicating that this process occurs in human infections.

Complement-dependent bactericidal activity of ECA-Ab was demonstrated against E. coli 014 using antisera prepared against several enteric bacteria. Bactericidal activity was only demonstrable when E. coli 014 was used as a target organism; other organisms were not killed (2,12). The reason for this difference in susceptibility is not understood. Domingue et al., postulated that it may be due to the surface location of ECA in E. coli 014 (12).

Domingue and Neter, performing in vitro opsonization experiments, demonstrated that ECA-Ab enhanced phagocytosis of E. coli 014, S. typhimurium and E. coli K-12 (12). Van Oss and co-workers obtained serum specimens from ten human

volunteers who had been previously immunized with ethanol-soluble ECA derived from E. coli 0111. Sera were tested for opsonizing activity using E. coli 07. A significant increase in opsonizing activity was demonstrated in post-immune sera from five out of nine subjects (63).

In summary, ECA exhibits minimal endotoxic activity. ECA-reactive antibody is functional as an opsonin and as a mediator of complement-dependent bactericidal activity. The significance of these observations in human gram negative infections is unknown.

H. ECA AS A VIRULENCE FACTOR

Valtonen et al., examined the importance of Salmonella typhimurium ECA as a virulence factor for mice (64). Mice were injected intraperitoneally with isogenic Salmonella strains that varied only in their content of ECA and 50% lethal dose values (LD_{50}) were determined after 10 days. ECA-positive strains were ten-fold more virulent than ECA-negative strains. Growth rates of ECA-positive and ECA-negative strains were compared in vitro and found to be similar. In addition, no significant difference was noted in mouse clearance rates of ECA-positive and ECA-negative strains.

Carillo et al., analyzed the ECA content of E. coli strains isolated from infants with diarrhea and compared them to E. coli isolates from healthy control children (65). Those E. coli strains associated with infantile diarrhea possessed significantly less ECA. The amount of ECA present in E. coli

strains isolated during the convalescent state more closely resembled that found in healthy controls. Strains with high ECA content exhibited LD_{50}'s 3 to 4 times higher than those with low ECA content when tested using a chicken embryo toxicity assay.

I. PREVALENCE OF ANTIBODIES TO ECA IN SERA

Kunin reported the presence of low levels of ECA-Ab in normal human sera (1). Similarly, low levels of ECA-Ab were detected in cord blood, and in sera from children 2 to 6 months of age. ECA-Ab levels in cord blood were significantly lower than those found in maternal serum. Colostrum contained high levels of antibody reactive with both LPS and ECA. Whang and Neter also demonstrated ECA-Ab in cord blood (66). Eight out of 18 infants possessed high levels. Furthermore, they demonstrated that serum from healthy subjects as well as commercial gamma globulin, obtained from donors from different countries, contained ECA-Ab.

Kunin found that pooled sera from various animal species contained significant levels of ECA-Ab (2). Rabbits were an exception in which antibodies to ECA were not detected in whole sera or in pooled β or γ globulin fractions. Since rabbits possess small fecal populations of E. coli, it was reasoned that fecal flora may provide the antigenic stimulus for ECA-Ab production in other animal species, i.e., humans (4).

Whang et al., found that ECA-Ab induced in rabbits was largely present in the 19S fraction, with minimal amounts in

the 7S fraction, when determined by IHA. 90% of ECA-Ab obtained after a booster injection of ECA was found in the 19S fraction (67).

Immunization of pregnant rabbits with ECA produced high titers (1:1280) of ECA-Ab in fetal sera. Maternal titers were estimated at 1:10,240. Antibodies were predominantly of the 19S variety. When ECA-Ab was injected into pregnant rabbits one day prior to Caesarean section, approximately 6% of the maternal ECA-Ab titer was present in the fetal blood, indicating that passive transfer had occurred. No ECA-Ab was present in the amniotic fluid (67). In rabbits, both 7S and 19S antibodies are known to be passively transferred to the fetus through the yolk sac splanchnopleur (68,69,70,71).

Gorzynski et al., studied the response of four different mouse strains to intraperitoneal injection of ECA. C57BL/6Ha and CBA/st mice produced ECA-Ab titers as high as 1:128, while the DVA/2Jx and Swiss albino strains produced titers no higher than 1:16. Subsequent booster injections did not result in an anamnestic response to ECA (72). McCabe and Greely obtained significant ECA-Ab titers using heat-killed E. coli 014 in mouse strain CH1. Antibody activity was detected both in the 7S and 19S fractions (37).

Morgenstern et al., immunized guinea pigs subcutaneously with E. coli 014 extracts and ethanol-soluble ECA using Freund's adjuvant. Guinea pigs were found to respond poorly to immunization with ECA. Little or no antibody was detected on the 9th day after subcutaneous injection. A booster injec-

tion administered on the 20th day resulted in a minimal antibody response (73).

Van Oss et al., administered ECA from the ethanol-soluble fraction of E. coli 0111 intravenously to ten human volunteers. Nine of the ten immunized volunteers responded to the ECA-antigenic stimulus. ECA-Ab titers in preimmune sera were $\leq$1:40; post-immunization titers ranged from 1:160 to 1:1280 (63).

In summary, the rabbit is apparently the most useful laboratory animal for ECA-Ab production. Rabbit sera, unlike sera from other animal species, are generally void of ECA-Ab prior to immunization (2). Rabbits respond to intravenous injection by producing high titers of ECA-Ab. ECA is generally non-immunogenic in mice and guinea pigs (72,73).

J. ECA IN RELATION TO DISEASE

The role of ECA-Ab in a variety of enteric infections has been examined. In general, ECA-Ab levels are low and only few patients demonstrate rising titers during the course of infection. Low titer ECA-Ab responses have been occasionally observed in patients with Salmonella and E. coli enteritis (66,74,75,76), bacteremia (77,78) and acute urinary tract infections (74,79,80).

In contrast high ECA-Ab titers have consistently been observed in shigellosis (66,74), peritonitis (75,81) and chronic urinary tract infections (66,79,82). The high ECA-Ab titers in shigellosis may be due to the frequency of occur-

rence in Shigella of the immunogenic R-1 LPS core type rough mutant (38,83). Peritonitis and chronic urinary tract infections are persistent conditions. High ECA-Ab titers in these diseases may be the result of repeated, long-term antigenic stimulation (4).

Aoki and co-workers demonstrated ECA by IFA in kidney tissue of six patients with documented bacterial pyelonephritis, and in six out of seven cases of pyelonephritis in which bacteria had not been isolated. Patients with other non-bacterial chronic renal diseases gave negative results (84). Schwartz et al., also used IFA to detect ECA in kidney specimens from patients with pyelonephritis. ECA was found in one of nine cases of chronic pyelonephritis and three of five cases of acute pyelonephritis (85).

Thomsen, using IFA, studied the temporal appearance of ECA in rat kidney following haematogenous induction of pyelonephritis. Early in the course of infection, ECA was readily demonstrated in the kidney. Renal tissue with chronic inflammatory changes, examined more than 4 weeks after bacterial injection, failed to reveal ECA. These results suggest that the IFA technique may be suitable for detecting ECA in acute renal infection, but not chronic disease (86).

McCabe and co-workers tested the sera of patients with gram negative bacillary bacteremia for the presence of ECA-Ab. A four-fold rise or greater in ECA-Ab was found in one-third of the bacteremic patients examined, regardless of their underlying disease or the species of enteric organism responsible

for their bacteremia. Human ECA-Ab was predominantly of the 19S variety. There was no correlation between the magnitude of ECA-Ab response and the occurrence of shock and death (78).

K. PROTECTIVE EFFECT OF ECA-ANTIBODY IN EXPERIMENTAL INFECTIONS

Gorzynski studied the protective effect of ECA-Ab in mice. Animals were injected intraperitoneally, first with rabbit ECA antiserum and 24 hours later with viable Salmonella typhimurium. Animals immunized with ECA antiserum survived longer than non-immunized controls; the protective effect of passive immunization dissipated after 4 days (87). Active immunization with ECA also prolonged survival (88).

Domingue et al., studied the protective effect of active ECA immunization against pyelonephritis in rabbits (89). Animals were first primed by intravenous injection of a heat-killed suspension of whole cells. A second intravenous injection of ethanol-soluble ECA was then given. Animals were subsequently challenged with viable P. mirabilis, either by direct retrograde ureteral implantation or by intravenous inoculation. Pyelonephritis developed less frequently in rabbits immunized with ECA than in control animals. Passive transfer of ECA-Ab was also found to protect against experimental pyelonephritis. These results have been confirmed by Frentz et al. (90).

L. ANTIGENIC SIMILARITIES BETWEEN ECA AND ANIMAL TISSUES

Antigenic cross-reactions between microorganisms and host tissue are thought to play a role in autoimmune disease. Patients with ulcerative colitis possess antibodies which react with colon antigens (91). Serum from patients with ulcerative colitis also reacts with colon tissue from germ free animals (76,92,93,94). Such sera have been found to react with ECA when tested by IHA or HAI (78). ECA-Ab have also been found in sera from patients with cirrhosis (76).

Gorzynski found that liver, and to a lesser extent, spleen and kidney of mice possess antigens that cross-react with ECA. Furthermore, tissue extracts from these organs were capable of priming rabbits for an anamnestic response to ECA immunization (95,96,97). The presence of such cross-reacting antigens might explain why ECA is poorly immunogenic in mice (72).

In summary, tissue components have been isolated from mice, and humans that cross-react antigenically with ECA. It is possible that this cross-reactivity may play a role in autoimmune diseases; however, definitive evidence in support of this view is lacking,

M. OTHER COMMON ANTIGENS OF GRAM NEGATIVE BACTERIA

Other common or cross-reacting antigens have been described among gram negative bacteria. Brodhage (98,99,100) demonstrated a common antigen in urea extracts of enteric bacteria using an indirect hemagglutination assay. The antiserum used for demonstrating cross-reacting antigen was

elicited in rabbits using Shigella sonnei, an organism which possesses an R-1 LPS core type (83) and which is immunogenic for ECA.

The outer membranes of all gram negative bacteria have remarkably similar chemical and structural compositions. They are composed of LPS, lipoproteins, proteins and phospholipids (101,102,103). The lipid A moeity of LPS, imbedded in the outer membrane, has, with few exceptions, similar structure in all gram negative bacteria and could possibly represent a common antigen.

The core oligosaccharide region of LPS is attached to lipid A. The LPS core region is identical in all Salmonella and similar in other Enterobacteriaceae. In Salmonella the deep core is represented by LPS chemotype Re and has been shown to contain KDO as the only saccharide moiety. Isolated Re-LPS is immunogenic. Antisera raised against Re-LPS have been shown to react with smooth strains of Enterobacteriaceae as well as several other non-enteric gram negative organisms by IFA. Like ECA, antisera to Re-LPS does not agglutinate bacteria or antigen-coated latex particles (104).

Braude and co-workers immunized rabbits with glycolipid obtained from the J-5 mutant of E. coli 0111 and found that antiserum protected mice against intraperitoneal challenge with smooth and rough strains of enteric bacteria, as well as heterologous organisms such as Pseudomonas aeruginosa. The antiserum possessed opsonizing activity and was capable of neutralizing the endotoxic properties of LPS (105).

Braun (106) reported that outer membrane proteins of several different gram negative bacteria have many similar properties. Rabbits immunized with whole bacteria of E. coli, Salmonella, or Shigella develop antibodies to a common cell wall lipoprotein antigen (107). Similarly, Seltman (108) described an acidic, thermolabile antigen, possibly a glycoprotein, which was common to all gram negative bacteria. Holmgren and Kaisjer screened gram negative bacteria for common antigens by immunodiffusion, immunoprecipitation, and immunoelectrophoresis (109,110,111). They found that different E. coli strains shared many cross-reacting antigens. One of these, an acidic antigen, was probably identical to that described by Seltman. This antigen is apparently common to E. coli, Proteus, Pseudomonas, and N. meningitidis, but not Staphylococcus.

Capsular antigens have been reported to cross-react among different bacterial species. For example, the capsular polysaccharide of Neisseria meningitidis group B is known to cross-react with E. coli K-1 capsular antigen. Antigenic cross-reactivity is thought to be related to their similar chemical composition (4,112,113,114).

MATERIALS AND METHODS

A. SOURCE OF BACTERIAL STRAINS

ECA-positive strains of *E. coli* 014 and *Salmonella typhimurium* were obtained from Dr. Emmett Johnson, Tulane University, School of Medicine, New Orleans, Louisiana. The ECA-negative strain of *E coli* 014 (F1327), an rfe-negative mutant, was provided by Dr. Günter Schmidt, Max-Planck-Institut für Immunbiologie, Germany. *E. coli* 0111 is a clinical isolate from the University of Oregon Health Sciences Center, Clinical Microbiology laboratory.

Bacterial stock cultures were maintained in trypticase soy broth (BBL) supplemented with 50% fetal calf serum at -70°C or -20°C.

B. REAGENTS AND EQUIPMENT

1. Saline--pH 7.20

0.15 M NaCl was added to distilled water and adjusted to pH 7.20 with 1.0 M KH_2PO_4.

2. Bacto Hemagglutination Buffer (Difco), pH 7.3

Total single-vial contents of dried buffer (8.6 g) were dissolved in distilled water and the volume adjusted to 1000 ml.

3. Sodium Barbital Buffer (Biorad), pH. 8.6

Sodium barbital buffer was prepared according to the manufacturer's recommendations, and further diluted 1:1 v/v with distilled water yielding a final 0.05 M solution. 1000 ml

of 0.05 M buffer was used per slide during electrophoresis.

4. Agarose Support Gel

Agarose (Biorad Laboratories, Richmond, California) -- 1.0 g agarose was dissolved in 100 ml 0.05 M sodium barbital buffer.

5. Wicks

Whatman grade 1 (95 mm x 85 mm), (3 wicks used per slide); or 3.25 x 4.0 inch, grade 470 electrophoresis wicks (Schleicher and Schuell, Inc., Keene, New Hampshire), 1 wick per slide.

6. Glass Plates

LKB 95 x 85 mm.

7. Power Source

LKB Model #2103, Bromma Sweden; voltage, current and power regulated power supply electrophoresis chamber.

8. Electrophoresis Chamber

LKB Multiphor Model #2117, plastic 2000 ml electrophoresis tray, Bromma, Sweden.

C. ISOLATION AND CONCENTRATION OF ENTEROBACTERIAL COMMON ANTIGEN

1. Fractionation by Differential Centrifugation

The method used for isolation of ECA was described by Domingue and Johnson (62). A single colony from an overnight trypticase soy agar culture was inoculated into 250 ml of trypticase soy broth and incubated overnight in a shaker water bath (90 oscillations per minute) at 37°C.

50 ml aliquots of broth culture were centrifuged at 2000 x g for 10 minutes and individual pellets, containing bacteria,

were combined and recentrifuged at 2000 x g for 10 minutes. The yield from 250 ml broth culture was approximately 2 g wet weight of bacteria.

A 40% w/v suspension of cells was prepared in sterile distilled water. The cells were ruptured in a French Pressure Cell, (American Instrument Company, Silver Springs, Maryland) at 6000 PSI, at 4°C. Breakage was assessed by phase contrast microscopy at 1000 X magnification using an oil immersion lens.

The suspension was centrifuged in a Sorvall RC-5 Superspeed refrigerated centrifuge at 2000 x g for 10 minutes and the supernatant collected. This was further centrifuged at 20,000 x g at 4°C for 30 minutes, and the resulting supernatant used to prepare the 144pl20 and 144sl20 fractions described below. The pellet was resuspended in distilled water and again centrifuged at 20,000 x g for 30 minutes at 4°C. The supernatant was discarded. The pellet, representing the washed cell envelope, was referred to as the 20p30 fraction.

The supernatant which was obtained following centrifugation of disrupted whole cells at 20,000 x g, was recentrifuged in a Beckman Ultracentrifuge, Model 65-55, using a SW 65L head, at 144,000 x g for 2 hours at 4°C. The pellet was washed in distilled water and recentrifuged at 144,000 x g for 2 hours, 4°C. This fraction, which represented fragmented cell envelope, membranous components, ribosomes, and other cellular inclusions was referred to as the 144pl20 fraction. The supernatant which represented soluble cytoplasmic constituents was referred to as the 144sl20 fraction. All fractions were

lyophilized and the dried material stored in sterile glass vials under desication at -70°C.

2. Separation by Ethanol Extraction

The method used for separation of ECA was a modification of that described by Suzuki, et al. (22). Overnight growth was harvested from the surface of 20 brain heart infusion agar plates and suspended in 20 ml of hemagglutination buffer. The bacterial suspension was mixed on a Vortex Mixer, and then heated in a boiling water bath for 1 hour. Viability was assessed by subculture. The suspension was centrifuged at 20,000 x g for 30 minutes at 4°C. The supernatant was collected, and finally adjusted with 95% ethanol to a final ethanol concentration of 85%. Following incubation in a shaker water bath for 18 hours at room temperature the mixture was centrifuged at 4°C at 20,000 x g for 30 minutes.

The resulting pellet represented an ethanol-insoluble fraction (LPS); the supernatant represented an ethanol-soluble fraction (free ECA). Both fractions were dried in open glass Petri dishes at 37°C. The dried material was scraped from the dish with a Bard-Parker blade and stored in sterile glass vials with desicant at -70°C.

D. INDIRECT HEMAGGLUTINATION

The method used was a modification of that described by Suzuki et al. (22). A 1% suspension of sheep erythrocytes was made by mixing 0.1 ml packed sheep red blood cells with 10 ml hemagglutination buffer. The suspension was washed

three times by centrifugation in hemagglutination buffer, reconstituted and finally sensitized with the addition of at least 1230 µg ECA. The ECA antigen used for sensitization was derived by ethanol fractionation from S. typhimurium (ECA-positive). The ECA-erythrocyte suspension was incubated on a rotating drum at 37°C for 30 minutes, washed three times in hemagglutination buffer, and restored to a final erythrocyte concentration of 1%.

Test antisera were inactivated at 56°C for 30 minutes in a water bath. Serum was diluted 1:4 in hemagglutination buffer. Diluted serum, 0.05 ml, was added to the first and second well of a microtiter plate. Phosphate buffer, 0.05 ml, was added to wells 2 through 12. Serial two-fold dilutions of serum in buffer were made in wells 2 through 12 using an Eppendorf pipette.

An equal volume of sensitized erythrocytes was added to each well. The plate was covered with a plastic adhesive strip and incubated at 37°C in a water bath for 90 minutes. Hemagglutination was ascertained visually.

E. ANTIGENS

Crude bacterial cell sonicates, for use in XIE and TXIE procedures, were prepared in the following manner: Approximately 1.0 ml of saline was placed on the surface of five trypticase soy agar plates (18 hour culture) and colonial growth dislodged by gently scraping with a sterile glass rod. The suspension was added to 10 ml chilled 0.15 M NaCl, pH 7.2

and washed by centrifugation at 20,000 x g for 10 minutes at 4°C. The pellet was resuspended in chilled saline, adjusted to an optical density of 10.0 at 600 nm, and sonicated using a Biosonik IV (Brownwell Scientific, Rochester, New York). Breakage was assessed by examining aliquots of a sonicated suspension under an oil immersion lens at 1000 X magnification using a phase contrast microscope. Total absence of intact whole cells in five consecutive fields was the criterion for complete breakage. This was usually achieved by five 10 minute bursts of sonication at an instrument setting of 40. Suspensions were cooled in an ice water bath during sonication. Sodium azide to a final concentration of 0.1% was added to all sonicates and they were stored at 4°C. The antigen preparations used in XIE and TXIE were dilutions of sonicated suspensions in sterile saline, adjusted to an optical density of 7.5 at 600 nm.

F. ANTISERA

Rabbits used for the preparation of antisera were New Zealand white males or nonpregnant females, 2-5 kg in weight.

1. Preimmune Sera

10-30 ml of venous blood was drawn from the ear vein of each rabbit 1 day prior to immunization. Serum was collected and stored at -70°C or -20°C until use.

2. Immunization with the 20p30 Fraction and the Crude Lyophilized Antigen

Immunization was performed according to the schema of

Domingue and Johnson (62). The lyophilized antigen (20p30 fraction or crude antigen) was weighed and dissolved in 1.0 ml sterile distilled water. The antigen was injected into the ear vein of rabbits using a tuberculin syringe, according to the following schedule:

Day 1	123 µg/ml
Day 3	123 µg/ml
Day 4	123 µg/ml
Day 5	615 µg/ml
Day 8	1230 µg/ml
Day 13	Final bleed

3. Immunization with the Ethanol-Soluble Fraction of ECA (free ECA)

Immunization was performed according to the method described by Suzuki et al. (22). Dried ethanol-soluble ECA was weighed and dissolved in sterile distilled water to a final concentration of 1000 µg/ml. One ml of this suspension (1000 µg ECA) was injected into the ear vein of rabbits using a tuberculin syringe, on days 1, 2, 3, 4, 5, 9, 10 and 11. Rabbits were bled on day 16.

4. Postimmune Sera

Rabbits were sacrificed by cardiac puncture. The total volume of blood obtained from a single rabbit ranged from 25-90 ml. Blood was allowed to clot and the sera removed and stored at -20°C or -70°C.

G. ADSORPTION OF ANTISERA

The bacterial strain to be used for adsorption was cultivated on trypticase soy agar plates. Growth from five plates was usually required for adsorption of 10 ml of antiserum. Approximately 1-2 ml of 0.15 M NaCl was added to the surface of an overnight culture, and growth dislodged by gently scraping with a sterile glass rod. Suspensions were washed three times in 0.15 M NaCl by centrifugation at 20,000 x g for 10 minutes at 4°C. The final pellet was added to 10 ml of antiserum and mixed by vortexing. The antiserum was incubated at 37°C for 2 hours and at 4°C for 18 hours. Mixtures were then centrifuged at 20,000 x g for 10 minutes at 4°C and the supernatant collected. This procedure was repeated five times. The final supernatant, containing adsorbed antiserum was filter-sterilized by passage through Millex 0.22 µm pore size membrane filters.

H. TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS (XIE) PROCEDURE

The methods used are similar to those described by Axelson, ed. (115). A 12 ml volume of liquified agarose support gel was spread evenly onto the surface of a clean glass plate resting on a level support. The agarose was allowed to congeal (3 to 5 minutes). One-half of the agarose was removed with a Bard-Parker blade, leaving a 50 mm x 80 mm strip. A 4 mm diameter well was cut into the left bottom corner of the gel exactly 1 cm from both the bottom and left hand edges of the slide.

The slide was positioned in the electrophoresis chamber such that the well was adjacent to the cathode, i.e., opposite the anode. Ten microliters of antigen preparation were inserted into the well. Following application of wicks, the first dimension electrophoresis was conducted at a constant current of 17.5 mA per slide for 1 hour and 45 minutes. Cooling was achieved using cold running tap water.

After completion of the first dimensional electrophoresis, the slides were removed from the electrophoresis chamber and the wicks discarded. Three-fifths of the upper portion of the remaining support gel was removed with a Bard-Parker blade leaving a 20 mm x 80 mm strip of agarose. A 10 ml aliquot of liquefied support gel containing 0.75 ml of rabbit antiserum was spread evenly across the uncoated portion of the slide. The interface formed between this layer and the remaining agarose gel demonstrated complete coalescence. The agarose was allowed to congeal for 3 to 5 minutes. The slide was positioned in the electrophoresis chamber so that the long axis of the slide was oriented perpendicular to the direction of the first dimensional run. Wicks were applied and the second dimension electrophoresis conducted at a constant current of 7.5 mA per slide for 17 hours. Cooling was achieved with cold running tap water. Figure 1 illustrates the method of two dimensional crossed immunoelectrophoresis.

I. TANDEM TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS (TXIE) PROCEDURE

This technique was performed exactly as in XIE with the

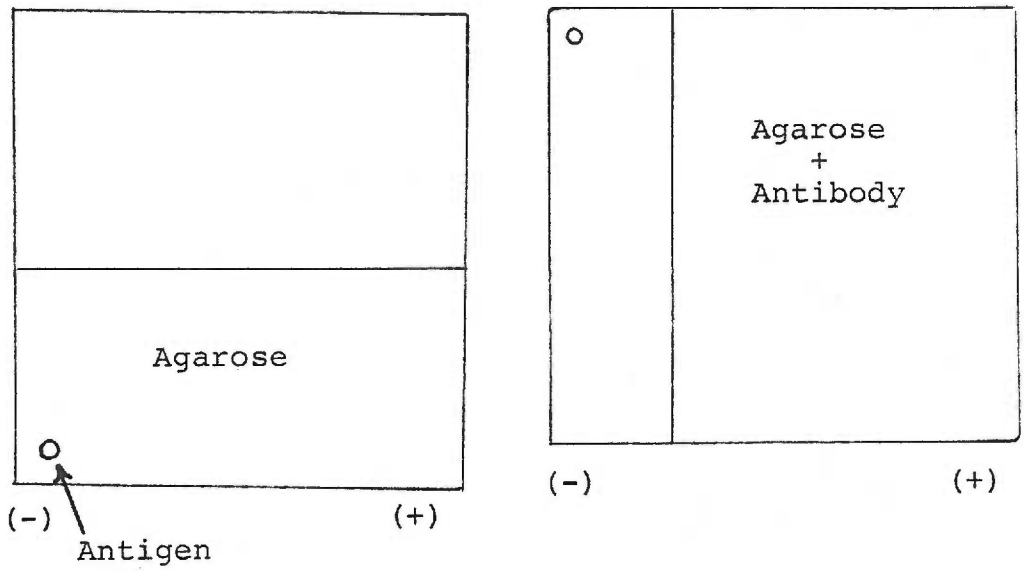
following exceptions: Prior to the first dimension electrophoresis, two 4 mm diameter wells were cut in the bottom of the first dimension support. The center of the first well was placed in the same location as in XIE. The center of the second well was placed 1 cm from the bottom and 2 cm from the left-hand edge of the plate, resulting in a center to center distance of 1 cm between the two wells. 10 µl aliquots of different antigen preparations were placed in each well. Figure 2 illustrates the method of tandem two dimensional crossed immunoelectrophoresis.

J. DEVELOPMENT OF PLATES

After electrophoresis the slides were placed in a humid chamber and the precipitin lines allowed to develop for 24 hours at 4°C. Slides were then covered with Whatman #1 filter paper and pressed between paper towels for 10 minutes. The slides were washed in two changes of 0.1 M saline for 15 minutes and allowed to dry in a Petri dish for 1 hour at 55°C. After drying, precipitin lines were stained by submerging slides for 20 minutes in a solution containing 0.2% Coumassie Brilliant Blue (Sigma Chemical Co.), 45% ethanol and 1.0% glacial acetic acid. Slides were subsequently destained in three changes of a 45% ethanol solution in 1.0% glacial acetic acid for a total of 30 minutes. After destaining, the slides were air-dried.

FIGURE 1

TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS

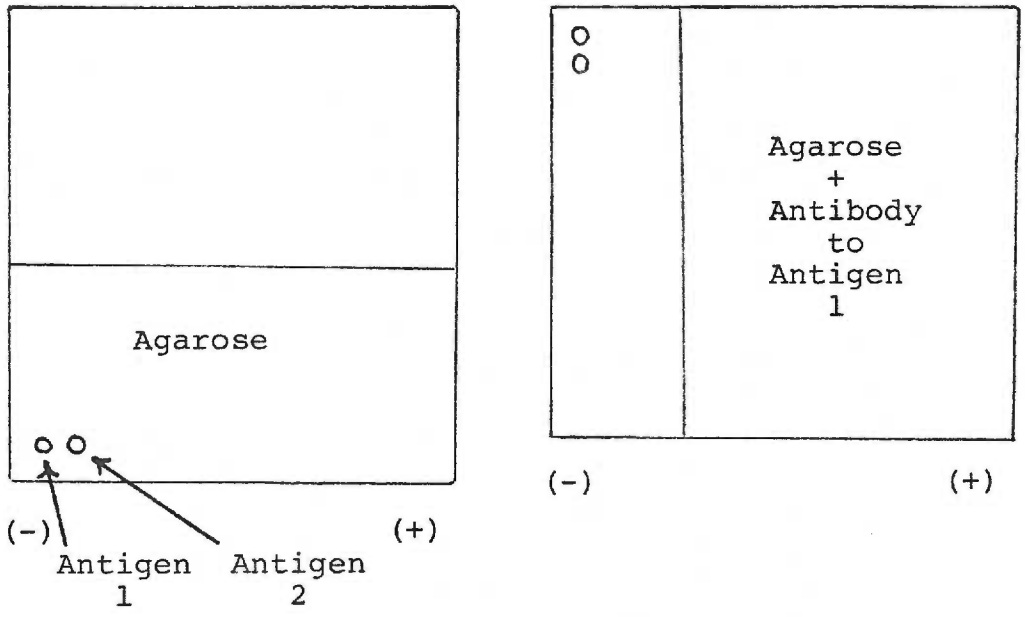


First Dimension Electrophoresis

Second Dimension Electrophoresis

FIGURE 2

TANDEM TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS



First Dimension Electrophoresis

Second Dimension Electrophoresis

RESULTS

All of the antigen preparations presented in the results section have been tested by two dimensional crossed immunoelectrophoresis (XIE) or tandem two dimensional crossed immunoelectrophoresis (TXIE) using antisera derived from at least two rabbits. Antisera which possessed antibodies reactive with the largest number of antigens were used for illustration, and as references for comparison of other antigen preparations. The comparison of antigens by XIE was based on two parameters of immunochemical identity: precipitin line morphology and location (116). The criterion used for immunochemical identity in TXIE was total fusion of two precipitin lines.

Bacterial strains used in this study were tested for ECA immunogenicity by indirect hemagglutination. Results are shown in Table 2. Antisera raised against ECA-positive strains demonstrated titers of 512 or greater; in some sera titers were greater than 8192. An ECA-negative strain elicited a titer of less than 8.

A. ANTIGENIC ANALYSIS OF *E. COLI* 014 (ECA-POSITIVE) BY TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS (XIE)

1. *Crude Antigen Sonicate of E. coli 014 (ECA-positive) Versus Antiserum to the 20p30 Fraction of the Same Organism.*

Figures 3, 4 and 5 illustrate two dimensional crossed

TABLE 2

DETECTION OF ECA BY INDIRECT HEMAGGLUTINATION (IHA) USING *SALMONELLA TYPHIMURIUM* AS SENSITIZING ANTIGEN[1]

Antiserum[2] Against	Rabbit No.	IHA Endpoint[3]
E. coli 014 (ECA-positive)	8-D	>8192
E. coli 014 (ECA-positive)	60-F	>8192
E. coli 014 (ECA-positive)	29-G	>8192
E. coli 014 (ECA-positive)	31-G	>8192
E. coli 014 (ECA-negative)	29-F	< 8
Salmonella typhimurium (ECA-positive)	27-F	> 512
E. coli 0111	31-L	>8192
E. coli 0111	32-L	>8192

[1] Ethanol-soluble fraction (free ECA).

[2] Antisera were prepared against the 20p30 fraction of all organisms listed except *E. coli* 0111. Antisera against *E. coli* 0111 were prepared against the ethanol-soluble (free ECA) fraction.

[3] The endpoint in the IHA test is the reciprocal titer of the highest dilution of serum which exhibits hemagglutination.

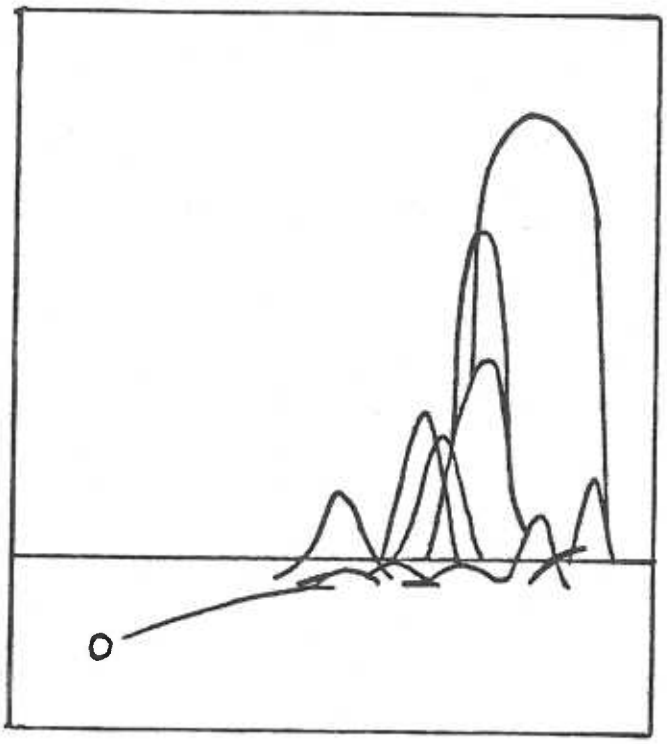


Figure 3

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 60-F) against the 20p30 fraction of E. coli 014 (ECA-positive).

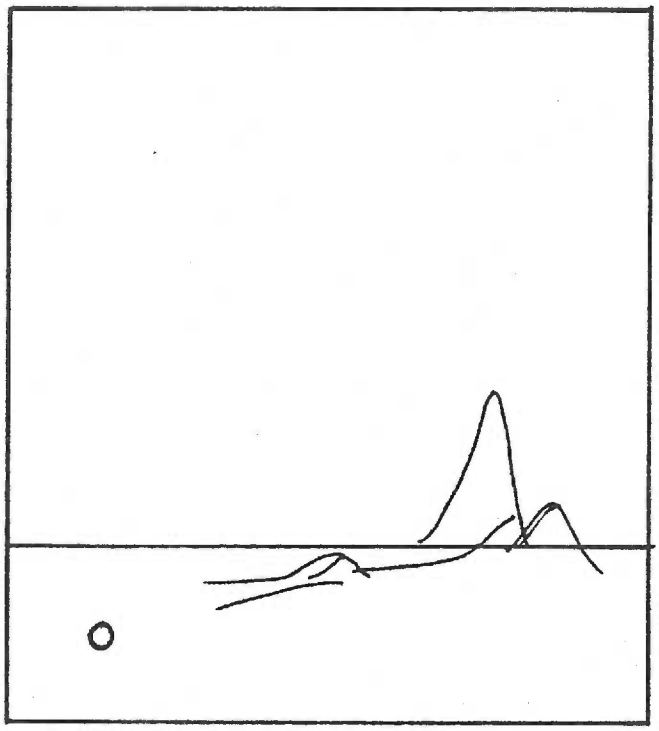


Figure 4

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 29-G) against the 20p30 fraction of E. coli 014 (ECA-positive).

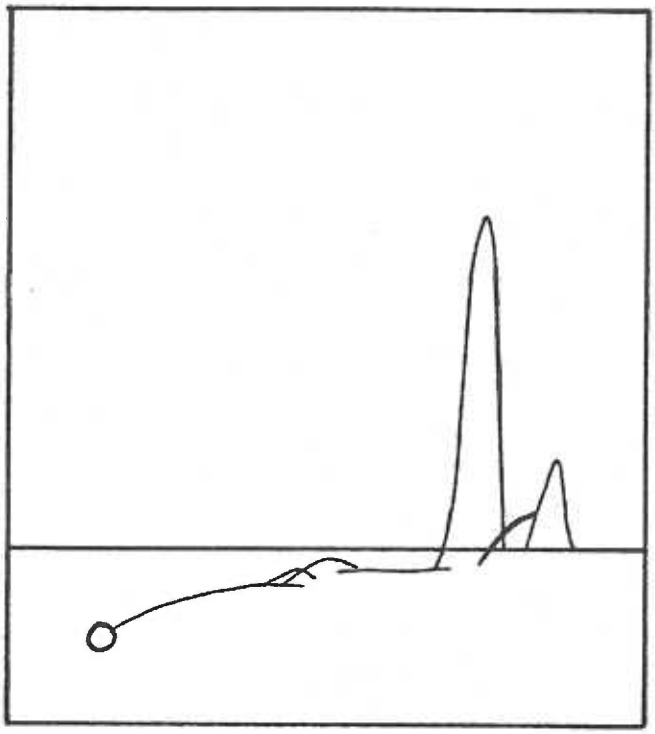


Figure 5

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 8-D) against the 20p30 fraction of E. coli 014 (ECA-positive).

immunoelectrophoresis (XIE) precipitin patterns obtained when a crude antigen sonicate of E. coli 014 (ECA-positive) was analyzed using antiserum produced against the 20p30 fraction of the same organism. Antiserum derived from rabbit 60-F was used in Figure 3. Thirteen different antigen peaks were observed. This antiserum exhibited the largest antibody response to the 20p30 fraction of E. coli 014. Fewer antigen peaks were seen with antisera derived from two other rabbits (29-G and 8-D). Figure 4 illustrates XIE results using antiserum from rabbit 29-G. Six antigens were demonstrable. Figure 5 illustrates results obtained with antiserum derived from rabbit 8-D. Again, six antigen peaks were demonstrable.

2. Crude Antigen Sonicate of E. coli 014 (ECA-positive) Versus Antiserum to the Crude Antigen of the Same Organism.

Figure 6 demonstrates results of XIE using a crude antigen sonicate of E. coli 014 (ECA-positive) and antiserum prepared against the crude antigen of the same organism. The antiserum was derived from one rabbit immunized with a reconstituted preparation of bacterial cells. Eight antigen peaks were observed. Five of the thirteen reference antigen peaks (Figure 3) were not visualized with this antiserum. Antiserum obtained from a second rabbit demonstrated only four peaks.

3. 20p30 Fraction of E. coli 014 (ECA-positive) Versus Antiserum to the 20p30 Fraction of the Same Organism.

The 20p30 fraction of E. coli 014 (ECA-positive) was reconstituted in phosphate buffer and analyzed by two dimensional

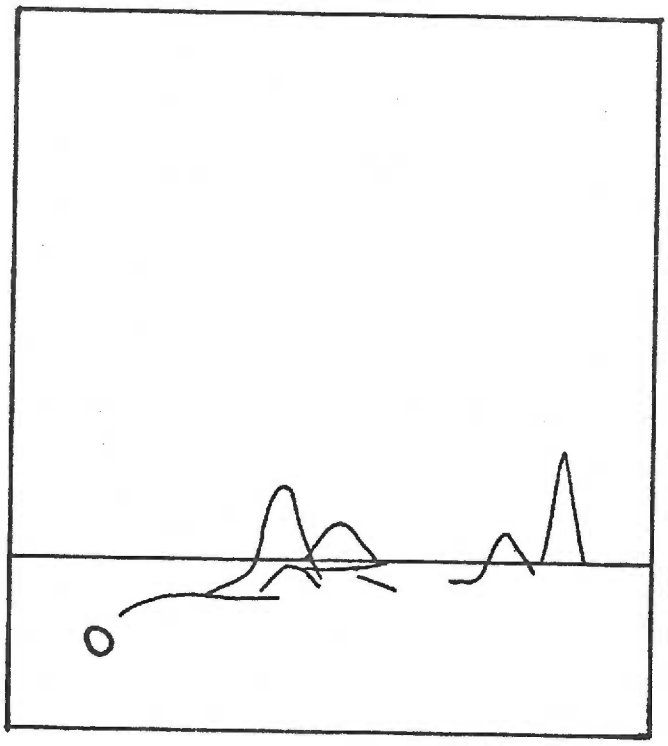


Figure 6

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 13-F) against a crude preparation of disrupted E. coli 014 (ECA-positive).

XIE using antiserum prepared against the same fraction. Figure 7 illustrates the results obtained when antiserum derived from one rabbit (8-D) was used. Six antigens were detected. Antiserum derived from a second rabbit demonstrated only two peaks.

4. Crude Antigen Sonicate of E. coli 014 (ECA-positive) Versus Antiserum to the 20p30 Fraction of E. coli 014 (ECA-negative).

A crude antigen sonicate of E. coli 014 (ECA-positive) was tested by XIE using antiserum (29-F) against the 20p30 fraction derived from a mutant E. coli 014 which is ECA-negative. As illustrated in Figure 8, three antigen peaks were obtained. When the same antigen preparation was tested against antiserum derived from a second animal immunized with the 20p30 fraction of E. coli 014 (ECA-negative), only two antigenic peaks were seen. Figure 9 represents an interpolation of Figures 3 and 8. In this case, three shared antigens (marked with an arrow) were apparent.

B. ANTIGENIC ANALYSIS OF E. COLI 014 (ECA-NEGATIVE) BY TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS

1. Crude Antigen Sonicate of E. coli 014 (ECA-negative) Versus Antiserum to the 20p30 Fraction of the Same Organism.

A crude sonicate of E. coli 014 (ECA-negative) was analyzed by XIE using antiserum prepared against the same organism. Antigen peaks were compared to the reference peaks illustrated

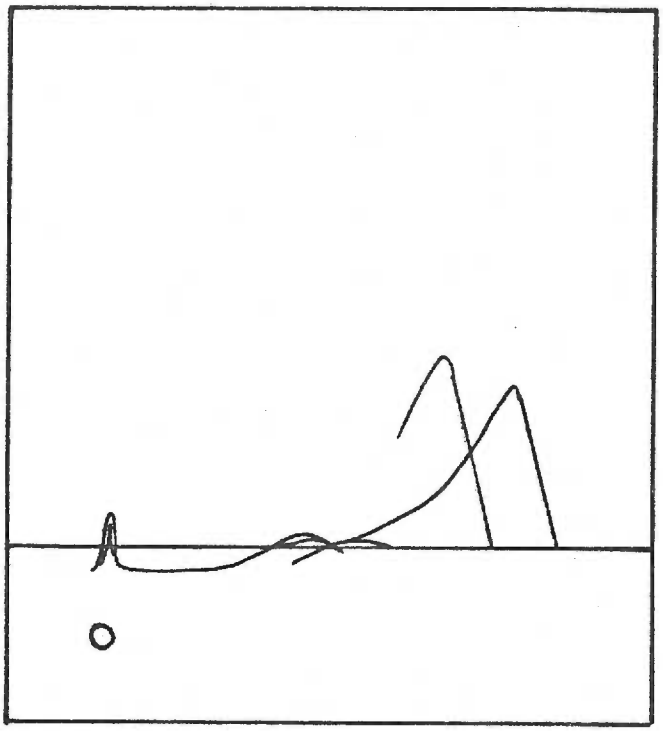


Figure 7

Two dimensional crossed immunoelectrophoresis analysis of the 20p30 fraction of E. coli 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 8-D) against the 20p30 fraction of E. coli 014 (ECA-positive).

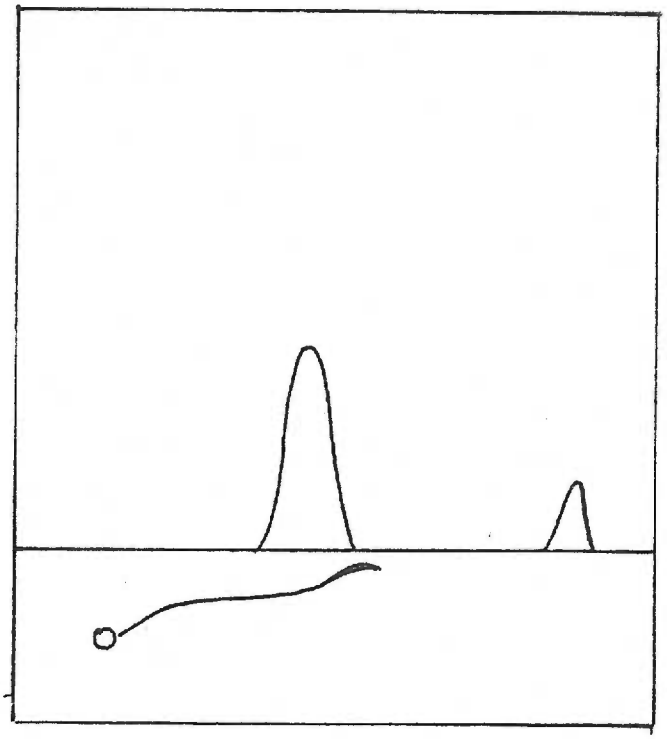


Figure 8

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 29-F) against the 20p30 fraction of E. coli 014 (ECA-negative).

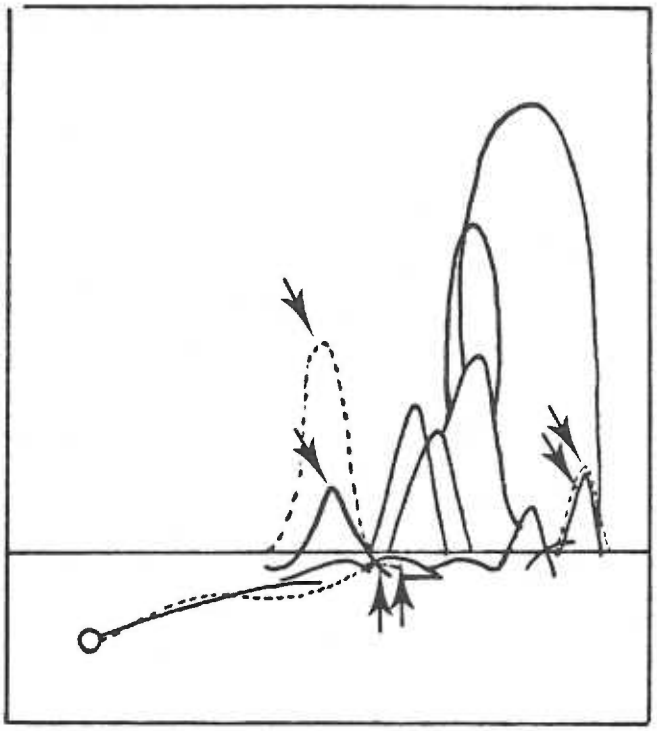


Figure 9

Interpolation of Figures 3 and 8.

(———): E. coli 014 (ECA-positive) versus antiserum prepared against the same organism (Figure 3).

(- - -): E. coli 014 (ECA-positive) versus antiserum prepared against E. coli 014 (ECA-negative) (Figure 8).

in Figure 3.

Figure 10 shows the results obtained with antiserum derived from rabbit 29-F. Four antigens were demonstrable. Antiserum derived from a second rabbit immunized with the 20p30 fraction of E. coli 014 (ECA-negative) demonstrated only one peak.

2. Crude Antigen Sonicate of E. coli 014 (ECA-negative) Versus Antiserum to the 20p30 Fraction of E. coli 014 (ECA-positive).

The crude antigen sonicate of E. coli 014 (ECA-negative) was tested against antiserum to the 20p30 fraction of E. coli 014 (ECA-positive). Figure 11 shows the results obtained with antiserum derived from rabbit 8-D. Only two antigens were demonstrable. Antiserum derived from a second rabbit immunized with the 20p30 fraction of E. coli 014 (ECA-positive) revealed a single antigen peak. Figure 12 represents an interpolation of Figures 3 and 11. The two antigens which appear to be shared by the ECA-positive strain of E. coli 014 and its ECA-negative mutant are marked with an arrow.

C. ANTIGENIC ANALYSIS OF SALMONELLA TYPHIMURIUM

Figure 13 illustrates results of XIE obtained with a crude sonicate of S. typhimurium (ECA-positive) and antiserum (27-F) prepared against the 20p30 fraction of the same organism. Seven antigen peaks were demonstrable. Antiserum prepared in a second rabbit demonstrated only two peaks.

Figure 14 represents an interpolation of XIE results using E. coli 014 (ECA-positive) against its homologous antiserum

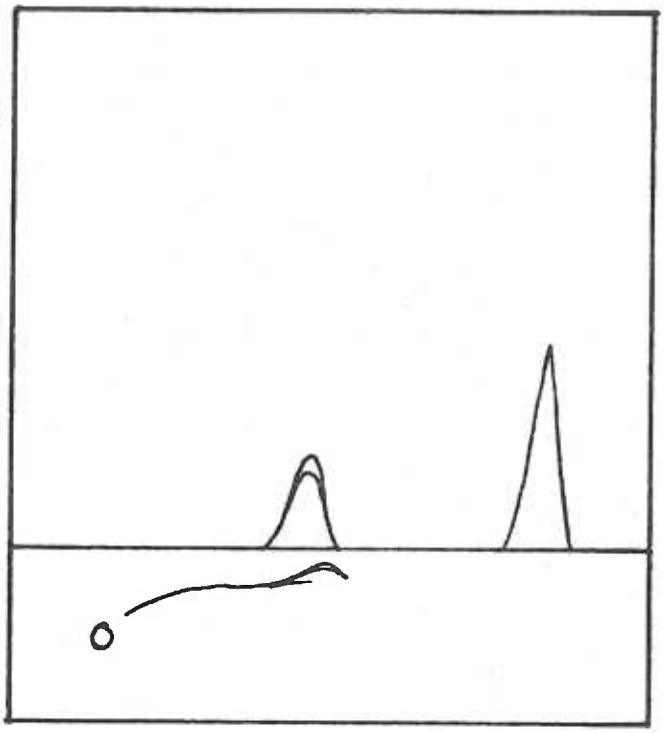


Figure 10

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-negative).

Source of antibodies: Antiserum (derived from rabbit 29-F) against the 20p30 fraction of E. coli 014 (ECA-negative).

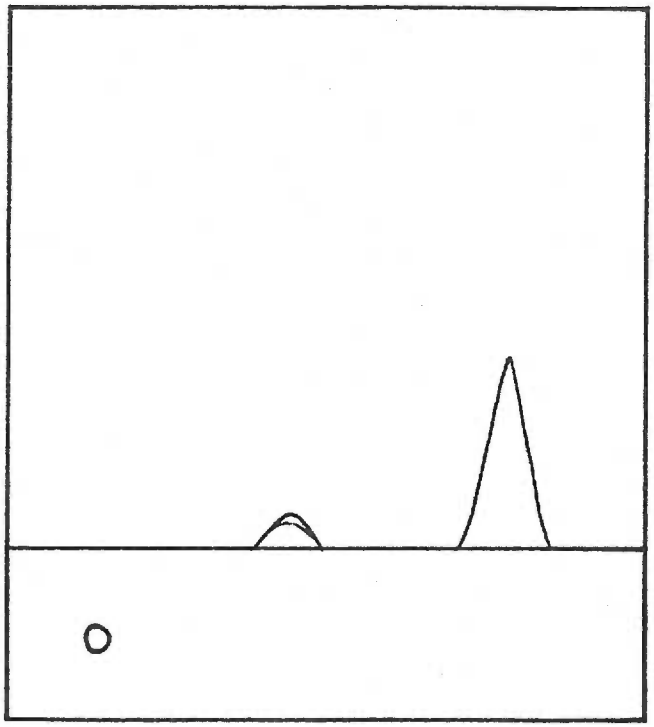


Figure 11

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-negative).

Source of antibodies: Antiserum (derived from rabbit 8-D) against the 20p30 fraction of E. coli 014 (ECA-positive).

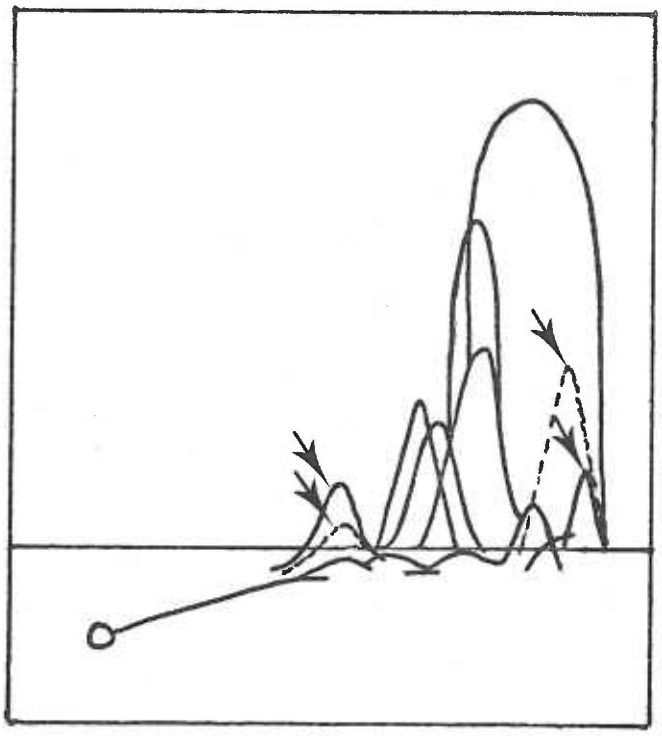


Figure 12

Interpolation of Figures 3 and 11.

(———): E. coli 014 (ECA-positive) versus antiserum prepared against the same organism (Figure 3).

(- - -): E. coli 014 (ECA-negative) versus antiserum prepared against E. coli 014 (ECA-positive) (Figure 11).

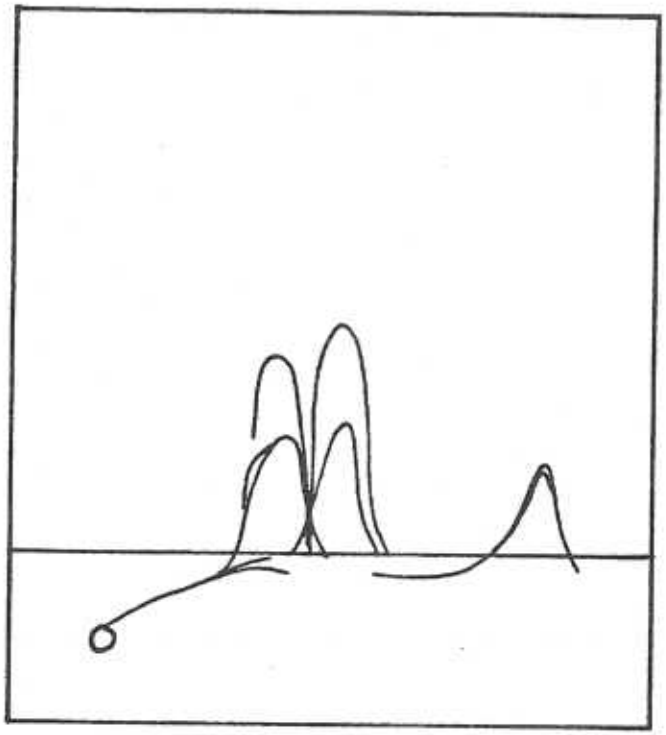


Figure 13

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of Salmonella typhimurium (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 27-F) against the 20p30 fraction of Salmonella typhimurium (ECA-positive).

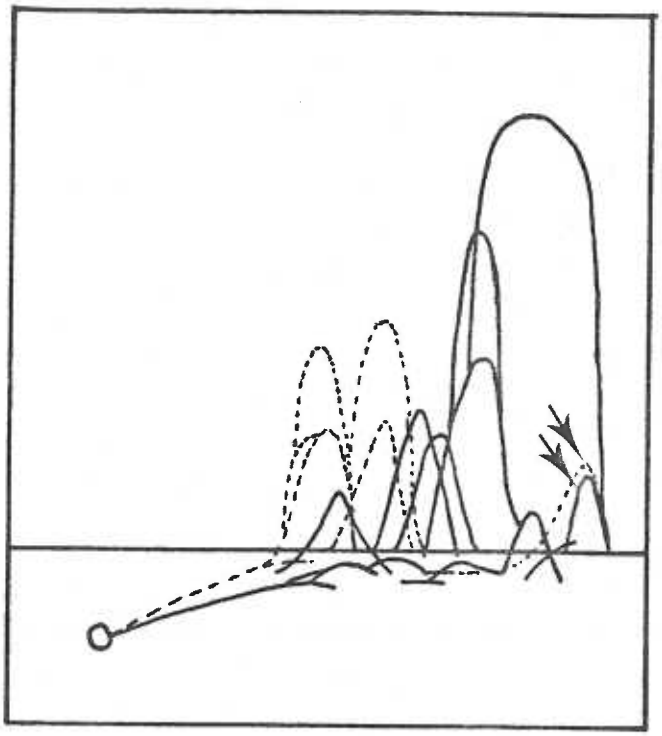


Figure 14

Interpolation of Figures 3 and 13.

(———): E. coli 014 (ECA-positive) versus antiserum prepared against the same organism (Figure 3).

(- - -): Salmonella typhimurium (ECA-positive) versus antiserum prepared against the same organism (Figure 13).

(Figure 3) and S. typhimurium (ECA-positive) against its homologous antiserum (Figure 13). The single antigen which appears to be shared by the two organisms is marked with an arrow.

D. ANTIGENIC INTER-RELATIONSHIP BETWEEN E. COLI 014 AND OTHER MEMBERS OF THE FAMILY ENTEROBACTERIACEAE

The crude sonicated antigen of S. typhimurium (ECA-positive) was tested by XIE using antiserum prepared against the 20p30 fraction of E. coli 014 (ECA-positive). Two antigen peaks were obtained (Figure 15). These results illustrate that at least two antigens of E. coli 014 (ECA-positive) and S. typhimurium (ECA-positive) are shared. The antigen peaks were labeled "a" and "b."

The crude antigen sonicate of E. coli 014 (ECA-positive) was tested by XIE using antiserum against the 20p30 fraction of S. typhimurium (ECA-positive). As seen in Figure 16, again two shared antigens (labeled "a" and "b") were observed.

When the ECA-negative mutant of E. coli 014 was tested by XIE using antiserum prepared against the 20p30 fraction of S. typhimurium (ECA-positive), only one antigen ("b") was detected (Figure 17).

E. coli 0111, a strain isolated in the U.O.H.S.C. laboratory from a stool culture, was also examined by IHA and XIE. IHA results (Table 2) demonstrated that E. coli 0111 produces ECA and that its ECA is immunogenic (IHA titer greater than 8192). XIE using the crude antigen sonicate of E. coli 0111

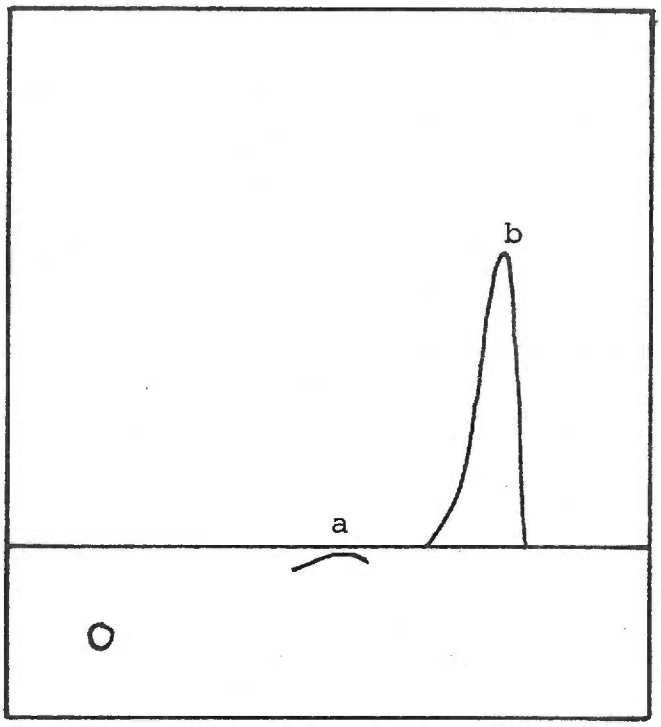


Figure 15

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of Salmonella typhimurium (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 8-D) against the 20p30 fraction of E. coli 014 (ECA-positive).

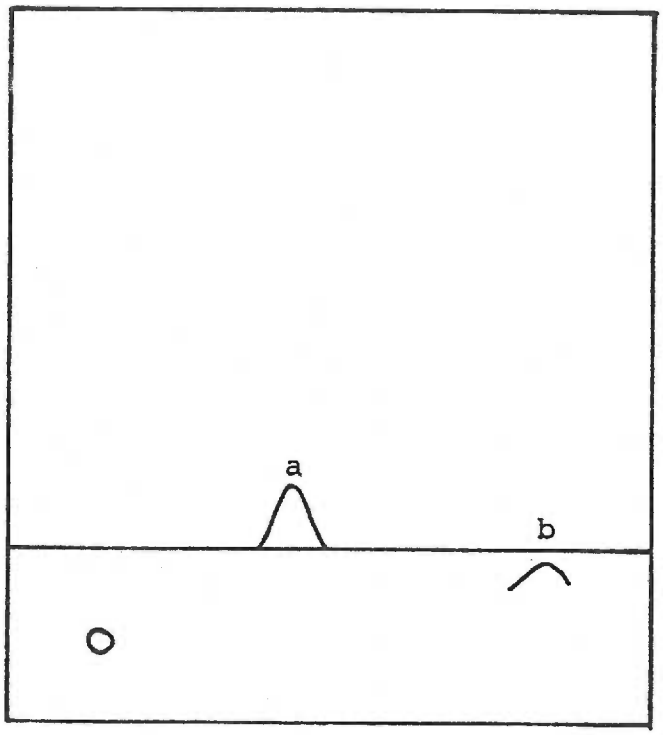


Figure 16

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 27-F) against the 20p30 fraction of Salmonella typhimurium (ECA-positive).

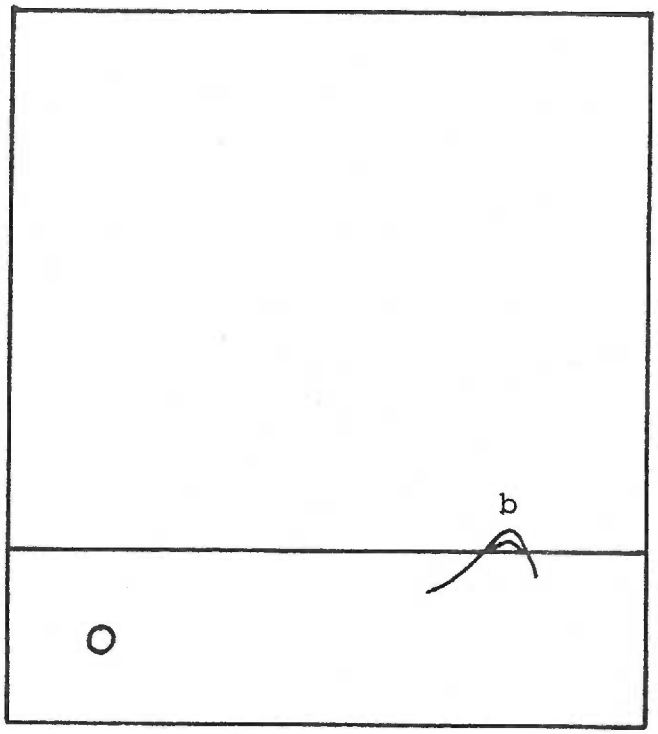


Figure 17

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-negative).

Source of antibodies: Antiserum (derived from rabbit 27-F) against the 20p30 fraction of Salmonella typhimurium (ECA-positive).

and antiserum prepared against the 20p30 fraction of E. coli 014 (ECA-positive) demonstrated the presence of three shared antigens (Figure 18). These antigens were labeled "a," "b" and "c."

In summary, there appears to be at least three cross-reacting antigens among E. coli and Salmonella. Antigen "a" was detected in the ECA-positive strains of E. coli 014, E. coli 0111, and S. typhimurium, but not in an ECA-negative mutant of E. coli 014. Antigen "b" was observed with all four bacteria. Antigen "c" was seen only with the ECA-positive strains of E. coli 014 and E. coli 0111.

E. IDENTIFICATION OF ENTEROBACTERIAL COMMON ANTIGEN THROUGH ADSORPTION OF ANTISERA WITH THE ECA-NEGATIVE MUTANT OF E. COLI 014

Antiserum prepared against the 20p30 fraction of E. coli 014 (ECA-positive) was adsorbed with the ECA-negative mutant of E. coli 014. Adsorbed antiserum prepared from a single rabbit (31-G) was tested by IHA and was found to have ECA-Ab titer greater than 8192. This antiserum was tested by XIE using the crude sonicate of E. coli 014 (ECA-positive). Results illustrated in Figure 19 demonstrate the presence of antigen "a." When the adsorbed antiserum was tested by XIE using the crude sonicate of E. coli 014 (ECA-negative), no antigen peaks were detected. This antiserum (which had been adsorbed with the ECA-negative mutant of E. coli 014) was subsequently readsorbed with E. coli 014 (ECA-positive)

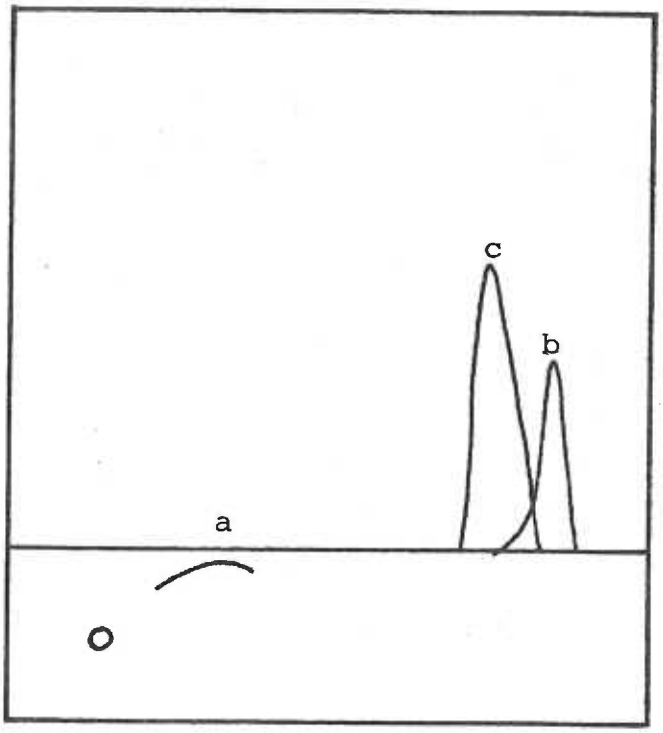


Figure 18

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 0111 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 8-D) against the 20p30 fraction of E. coli 014 (ECA-positive).

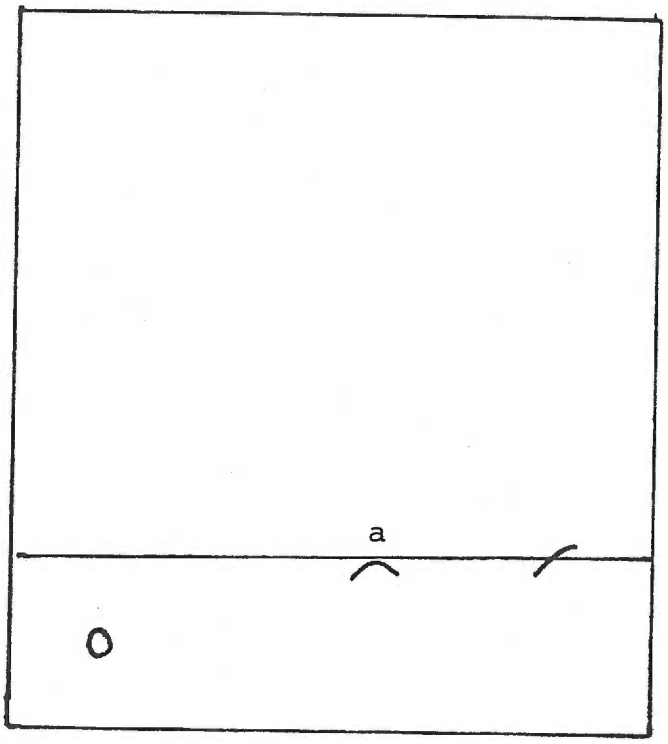


Figure 19

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 31-G) against the 20p30 fraction of E. coli 014 (ECA-positive) adsorbed with E. coli 014 (ECA-negative) mutant.

and then was tested by IHA. No ECA-Ab was detected (titer less than 8).

XIE was repeated using the crude sonicate of E. coli 014 (ECA-positive) and adsorbed antiserum prepared in a second rabbit (60-F). Adsorption was performed using the ECA-negative mutant of E. coli 014. Three antigens were detected (Figure 20); they were labeled "a," "c" and "d." These antigens were not removed by further adsorption with the ECA-negative mutant.

Figure 21 illustrates XIE results when a crude antigen sonicate of S. typhimurium (ECA-positive) was tested against antiserum (prepared in rabbit 29-G) to the 20p30 fraction of E. coli 014 (ECA-positive), after adsorption with the ECA-negative E. coli 014 mutant. A single cross-reacting antigen ("a") was demonstrable. These results were duplicated when XIE was performed using antisera against the 20p30 fraction of E. coli 014 (ECA-positive), derived from two other animals following adsorption with an ECA-negative mutant of E. coli 014.

E. coli 0111, a laboratory strain found to be ECA-positive by IHA was also tested by XIE using antiserum to E. coli 014 (ECA-positive) that had been adsorbed with the ECA-negative mutant of E. coli 014. A single antigen peak, labeled "a," was observed (Figure 22).

Crude sonicates of E. coli 014 (ECA-positive) and S. typhimurium (ECA-positive) were analyzed by TXIE using antiserum against the 20p30 fraction of E. coli 014 (ECA-positive)

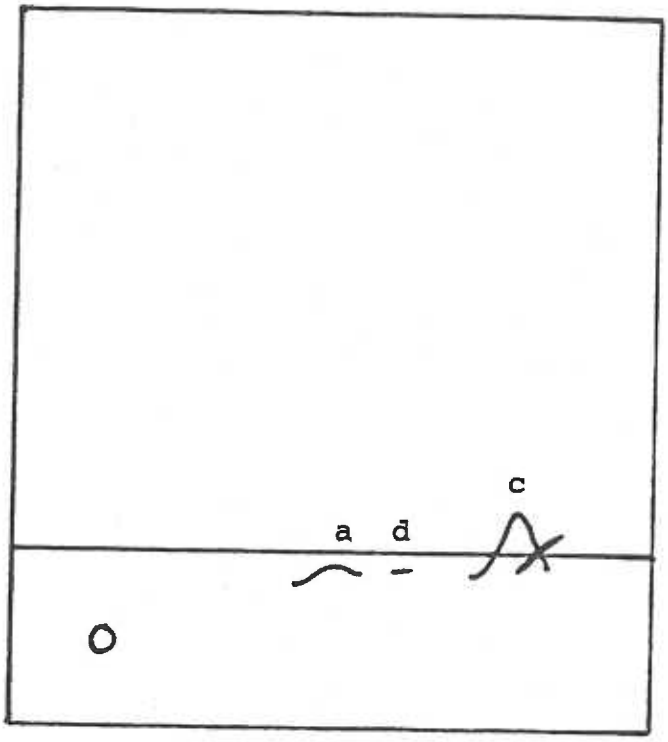


Figure 20

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 60-F) against the 20p30 fraction of E. coli 014 (ECA-positive) adsorbed with E. coli 014 (ECA-negative) mutant.

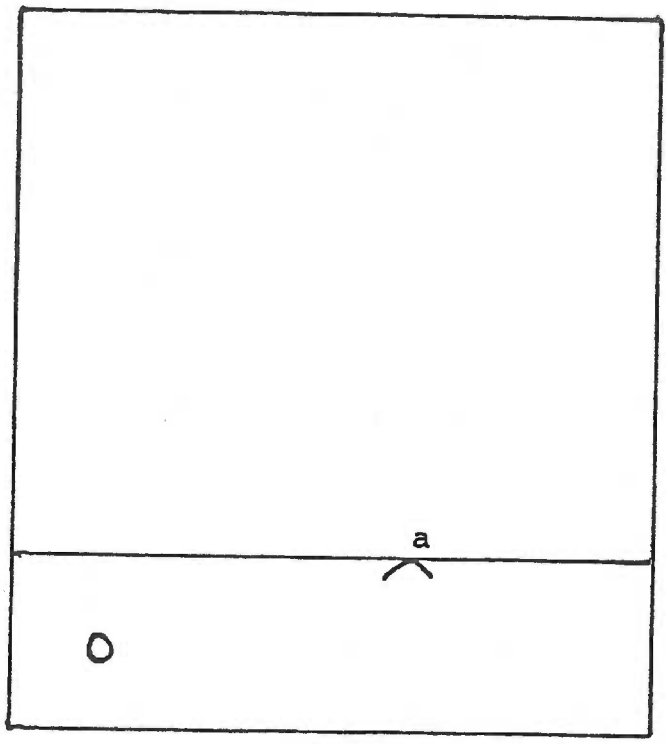


Figure 21

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of Salmonella typhimurium (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 29-G) against the 20p30 fraction of E. coli 014 (ECA-positive) adsorbed with E. coli 014 (ECA-negative) mutant.

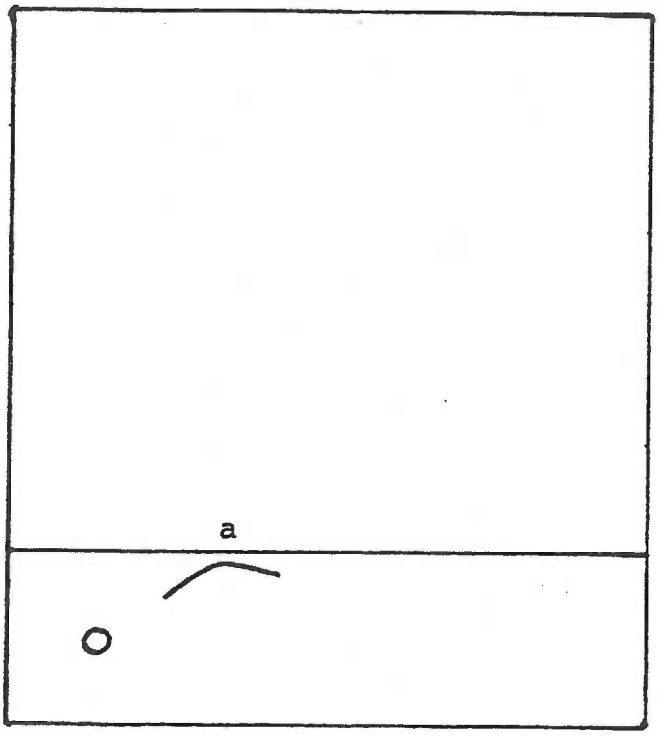


Figure 22

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of E. coli 0111 (ECA-positive).

Source of antibodies: Rabbit antiserum (derived from rabbit 29-G), against the 20p30 fraction of E. coli 014 (ECA-positive), adsorbed with E. coli 014 (ECA-negative) mutant.

following adsorption with the ECA-negative mutant of E. coli 014. As seen in Figure 23, antigen "a" of E. coli demonstrated complete immunochemical identity with antigen "a" of S. typhimurium (marked with an arrow). Antiserum against E. coli 014 (ECA-positive) derived from a different rabbit and adsorbed with the ECA-negative mutant demonstrated the same results; i.e., complete immunochemical identity between E. coli antigen "a" and "a" of S. typhimurium.

E. coli 014 (ECA-positive) and E. coli 0111 (ECA-positive) were tested by TXIE using antiserum to E. coli 014 (ECA-positive) that had been adsorbed with the ECA-negative mutant of E. coli 014. A line of complete identity was demonstrable between antigen "a" of E. coli 014 and antigen "a" of E. coli 0111, as marked by the arrow (Figure 24). Adsorbed antiserum derived from a second rabbit demonstrated the same results.

F. IMMUNOCHEMICAL IDENTIFICATION OF OTHER COMMON ANTIGENS BY TANDEM TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS (TXIE)

Figure 25 illustrates the results of TXIE obtained when crude sonicates of E. coli 014 (ECA-positive) and a mutant strain of E. coli 014 which lacks ECA were tested using antiserum to the 20p30 fraction of E. coli 014 (ECA-positive). One cross-reacting antigen (marked with an arrow and labeled "b" in Figure 25) demonstrated complete immunochemical identity.

The crude sonicates of E. coli 014 (ECA-positive) and S.

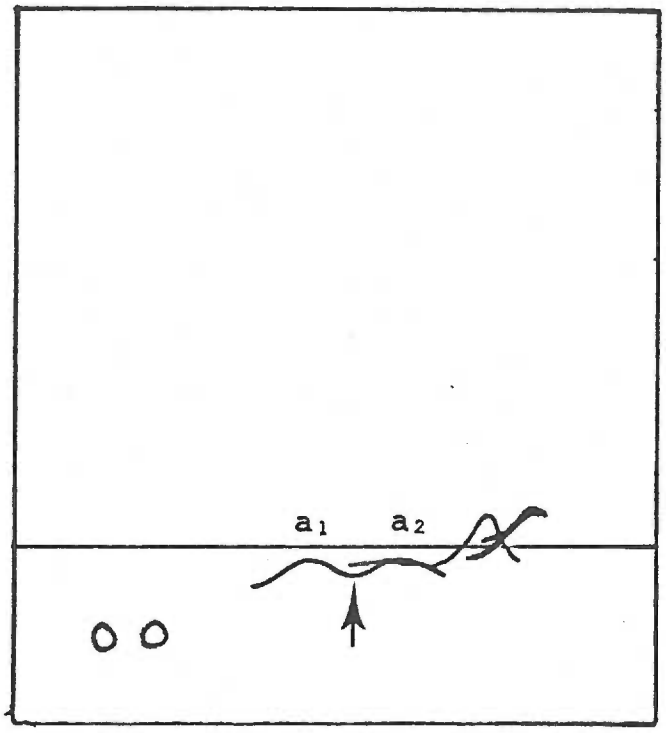


Figure 23

Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).

Antigen 2: Sonicated preparation of Salmonella typhimurium (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 60-F) against the 20p30 fraction of E. coli 014 (ECA-positive) adsorbed with E. coli 014 (ECA-negative) mutant.

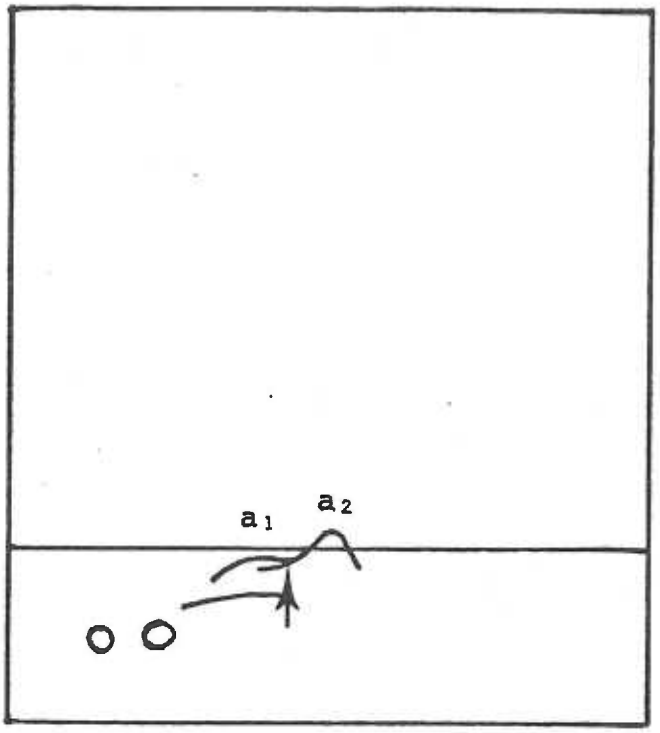


Figure 24

Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).

Antigen 2: Sonicated preparation of E. coli 0111 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 29-G) against the 20p30 fraction of E. coli 014 (ECA-positive) adsorbed with E. coli 014 (ECA-negative) mutant.

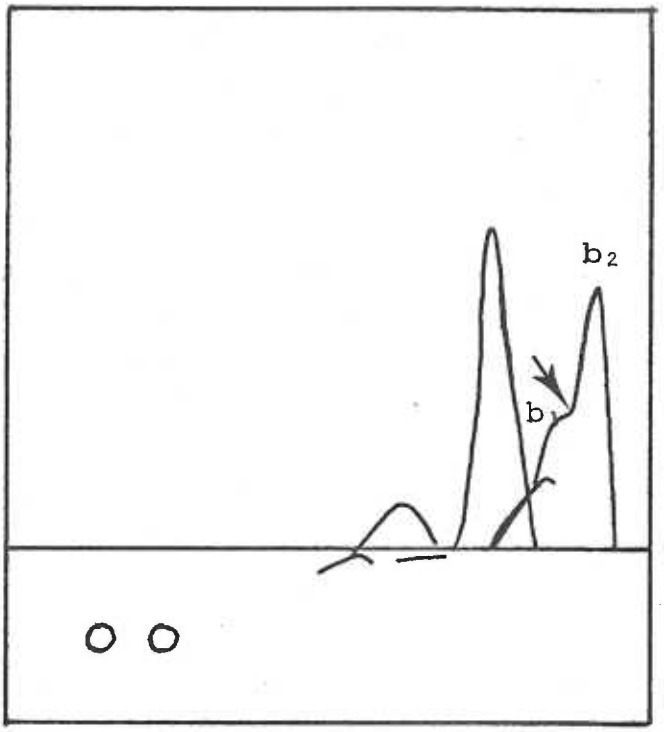


Figure 25

Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).

Antigen 2: Sonicated preparation of E. coli 014 (ECA-negative).

Source of antibodies: Antiserum (derived from rabbit 8-D) against the 20p30 fraction of E. coli 014 (ECA-positive).

typhimurium (ECA-positive) were tested by TXIE using antiserum to the 20p30 fraction of E. coli 014 (ECA-positive). The results are illustrated in Figure 26. Cross-reacting antigen "b" (marked with an arrow) was clearly visualized.

E. coli 014 (ECA-positive) and S. typhimurium (ECA-positive) were also tested by TXIE using antiserum to the 20p30 fraction of S. typhimurium (ECA-positive). A line of complete identity between two antigens (marked with an arrow and labeled "b") was seen (Figure 27).

Figure 28 illustrates TXIE results of the crude antigen sonicates of E. coli 014 (ECA-positive) and E. coli 0111 (ECA-positive) versus antiserum to the 20p30 fraction of E. coli 014 (ECA-positive). A line of complete immunochemical identity was observed between two antigens (labeled "b" and marked with an arrow).

It should be noted that the cross-reacting antigen "a" peak was also demonstrated in the TXIE experiments illustrated in Figure's 26-28; however, this antigen peak was often difficult to visualize due to the presence of overlapping antigens.

Photographs of representative results obtained using XIE and TXIE are presented on pages 84 and 85, respectively.

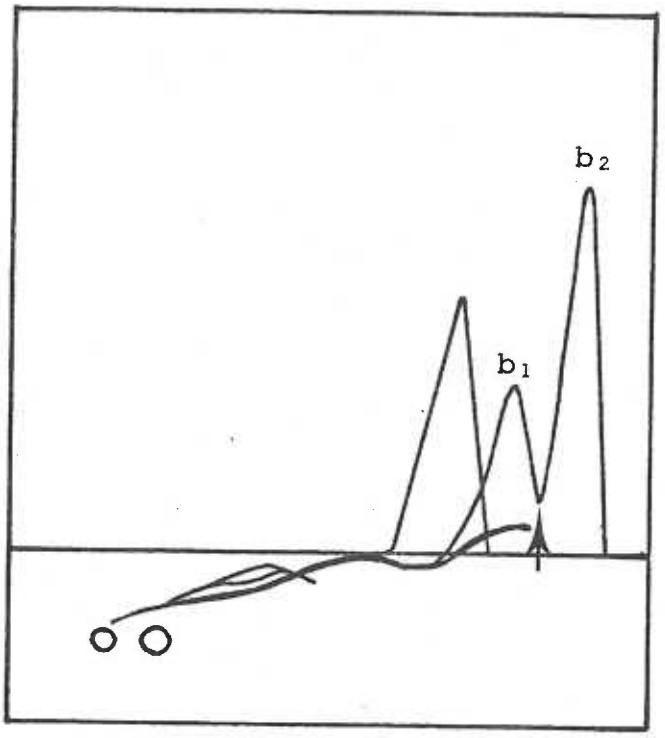


Figure 26

Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).

Antigen 2: Sonicated preparation of Salmonella typhimurium (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 8-D) against the 20p30 fraction of E. coli 014 (ECA-positive).

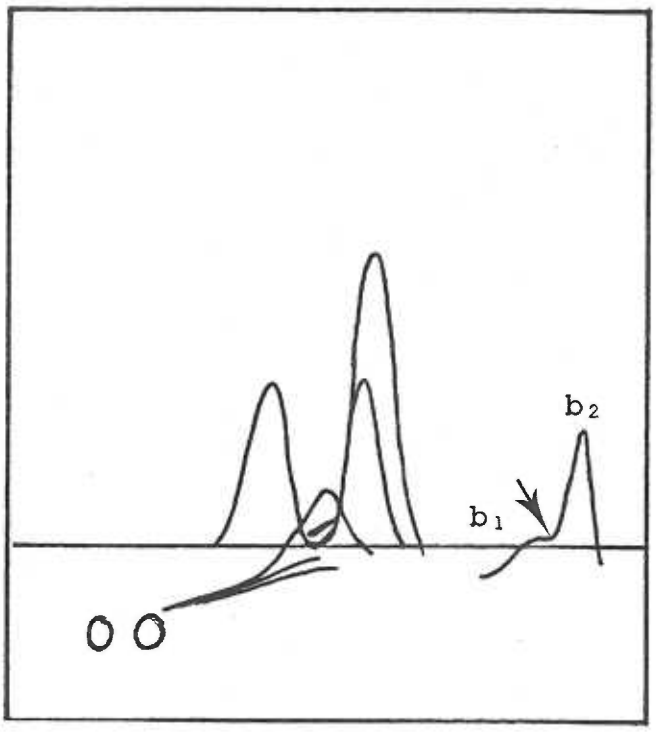


Figure 27

Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).

Antigen 2: Sonicated preparation of Salmonella typhimurium (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 27-F) against the 20p30 fraction of Salmonella typhimurium (ECA-positive).

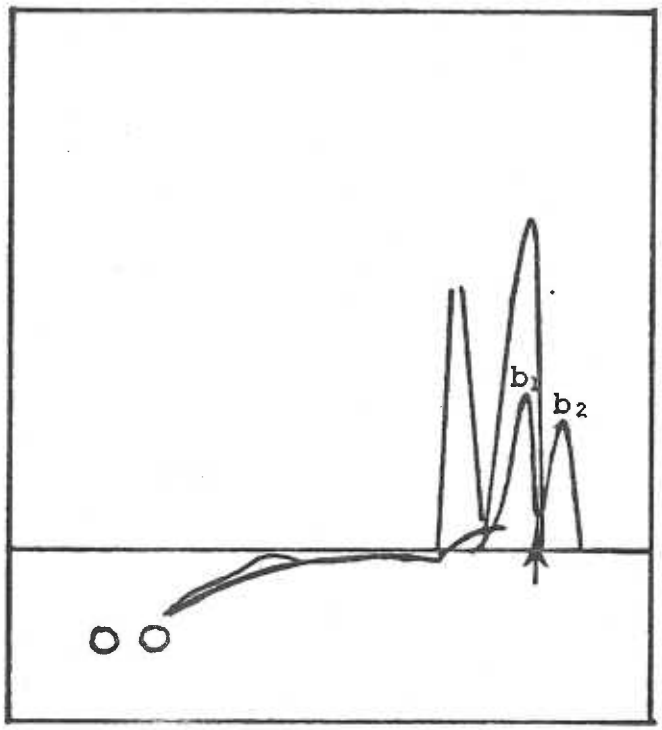


Figure 28

Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).

Antigen 2: Sonicated preparation of E. coli 0111 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 8-D) against the 20p30 fraction of E. coli 014 (ECA-positive).

PHOTOGRAPH 1

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of *E. coli* 014 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 60-F) against the 20p30 fraction of *E. coli* 014 (ECA-positive).

PHOTOGRAPH 2

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of *Salmonella typhimurium* (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 27-F) against the 20p30 fraction of *Salmonella typhimurium* (ECA-positive).

PHOTOGRAPH 3

Two dimensional crossed immunoelectrophoresis analysis of a sonicated preparation of *E. coli* 0111 (ECA-positive).

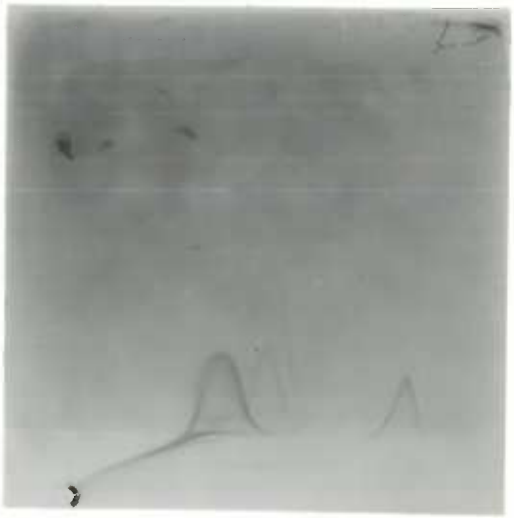
Source of antibodies: Antiserum (derived from rabbit 29-G) against the 20p30 fraction of *E. coli* 014 (ECA-positive), adsorbed with *E. coli* 014 (ECA-negative) mutant.

PHOTOGRAPHS OF

TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS

Photograph 1

Corresponds to Figure 3



Photograph 2

Corresponds to Figure 13

Photograph 3

Corresponds to Figure 22

PHOTOGRAPH 4

Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).
Antigen 2: Sonicated preparation of Salmonella typhimurium (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 60-F) against the 20p30 fraction of E. coli 014 (ECA-positive) adsorbed with the E. coli 014 (ECA-negative) mutant.

PHOTOGRAPH 5

Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).
Antigen 2: Sonicated preparation of E. coli 0111 (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 29-G) against the 20p30 fraction of E. coli 014 (ECA-positive) adsorbed with E. coli 014 (ECA-negative) mutant.

PHOTOGRAPH 6

Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).
Antigen 2: Sonicated preparation of E. coli 014 (ECA-negative).

Source of antibodies: Antiserum (derived from rabbit 8-D) against the 20p30 fraction of E. coli 014 (ECA-positive).

PHOTOGRAPH 7

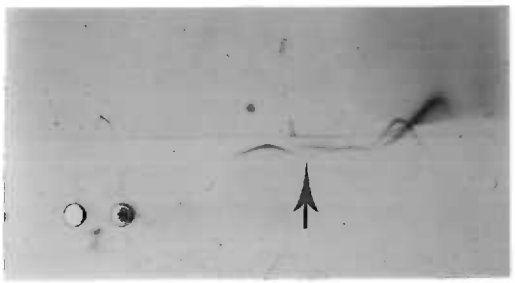
Tandem two dimensional crossed immunoelectrophoresis analysis

Antigen 1: Sonicated preparation of E. coli 014 (ECA-positive).
Antigen 2: Sonicated preparation of Salmonella typhimurium (ECA-positive).

Source of antibodies: Antiserum (derived from rabbit 27-F) against the 20p30 fraction of Salmonella typhimurium (ECA-positive).

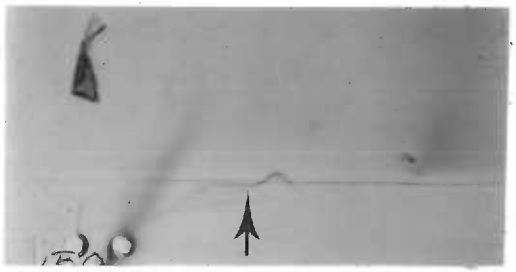
PHOTOGRAPHS OF TANDEM

TWO DIMENSIONAL CROSSED IMMUNOELECTROPHORESIS



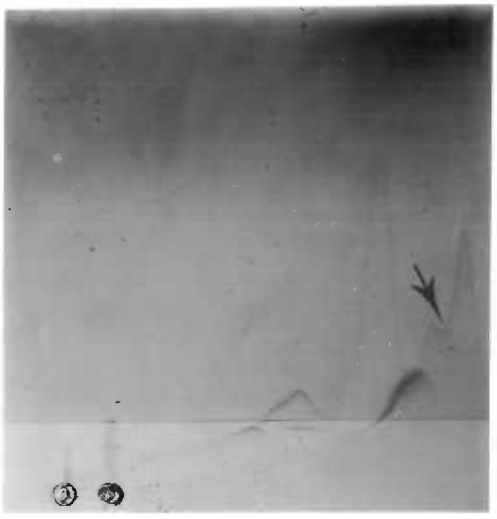
Photograph 4

Corresponds to Figure 23



Photograph 5

Corresponds to Figure 24



Photograph 6

Corresponds to Figure 25

Photograph 7

Corresponds to Figure 27

DISCUSSION

Although two dimensional crossed immunoelectrophoresis (XIE) has been used to analyze the antigenic composition of a variety of microorganisms, there are no known studies of its use to evaluate Enterobacteriaceae. Svendsen and Axelson utilized XIE to study Candida albicans and were able to demonstrate 67 different antigens (116). Likewise, Hoiby et al., detected 64 different antigens in Pseudomonas aeruginosa (117-121). Roberts et al., characterized 60 immunoprecipitates in Mycobacterium tuberculosis, M. bovis, M. scrofulaceum and M. phlei (122). Vestergaard et al., identified six antigens by XIE in Herpes simplex virus (123-126). Similarly, Caldwell et al., analyzed Chlamydia trachomatis, and were able to demonstrate 19 antigens (127). Doern used XIE for characterizing the antigenic heterogeneity of H. influenzae Type b and identified six separate antigens (128). Multiple antigens have also been demonstrated in other organisms, including Actinomyces israelii (129,130), and Mycoplasma species (131,132).

Studies aimed at characterizing ECA have been hampered by the inability to isolate this antigen in a pure state. Isolations of the so-called "pure" ECA have produced preparations which are no longer biologically active. Such ECA preparations lost their immunogenicity, erythrocyte coating ability, indirect hemagglutination activity (IHA), hemagglutination inhibition activity (HAI), or gel precipitation.

The most recent attempt at characterizing ECA was performed by Männel and co-workers utilizing S. montevideo (21,26). Their "purified" product retained its ability to coat erythrocytes, and was active in IHA, but it was no longer immunogenic.

Domingue and Johnson described a fractionation method for the isolation of ECA which preserved its native properties (61). This method resulted in three fractions: the 20p30 fraction (intact cell envelope), the 144p120 fraction (membrane vesicles and envelope fragments) and the 144s120 fraction (solubilized cytoplasmic constituents). In E. coli 014 the highest ECA activity was demonstrated in the 20p30 fraction. The present study used the 20p30 fraction derived from E. coli 014 and S. typhimurium for immunizing rabbits. The antisera produced was tested by XIE against different antigenic preparations of E. coli and Salmonella. XIE analysis of the 20p30 fraction demonstrated the presence of several antigens. As many as 13 antigens could be detected in E. coli 014. Likewise, 7 different antigens were demonstrable in S. typhimurium. These results illustrate the complex antigenic composition of Enterobacteriaceae. Although these results may not be a comprehensive representation of the total number of antigens present in Enterobacteriaceae, they nevertheless illustrate the complex antigenic composition of this group of organisms, and clearly indicate that the 20p30 fraction is not a pure preparation.

Data presented in this thesis demonstrate the variable response of rabbits to immunization with the 20p30 fraction.

For example, immunization of three rabbits of approximately the same age and weight with the same antigen (20p30 fraction of E. coli 014) resulted in the production of 13 precipitins in one animal, and only 8 precipitins in the other 2 rabbits. The variable antibody response in different animals of the same species has been reported previously (121,133,134, 135).

Although XIE has been employed for identifying cross-reacting antigens, and characterizing specific microbial antigens, there are no published reports for its use in identifying antigens of Enterobacteriaceae. Hoiby used XIE to identify 10 cross-reacting antigens between P. aeruginosa and 33 other gram positive and gram negative organisms (136). Vestergaard et al., demonstrated a type-specific antigen in Herpes simplex virus type 1 (137). Other type-specific antigens have likewise been identified for Chlamydia trachomatis and Lymphogranuloma venereum (138) and Haemophilus influenzae Type b (128).

The present study used XIE to detect cross-reacting antigens in E. coli and Salmonella. Two cross-reacting antigens, labeled "a" and "b" were demonstrated in these organisms. Antigen "a" has been identified as ECA. Supporting evidence for this thesis is the following:

1. Antigen "a" is found in ECA-positive strains of E. coli 014, E. coli 0111, and S. typhimurium.
2. Antigen "a" could not be detected in an ECA-negative mutant of E. coli 014.

3. Antiserum prepared against E. coli 014 (ECA-positive) retained its ECA antibodies (IHA titer 8192) following adsorption with an ECA-negative mutant of the same organism. When the adsorbed antiserum was tested by XIE using a crude antigen sonicate of E. coli 014 (ECA-positive) a single precipitin line, "a," was detected. Readsorption of the antiserum with E. coli 014 (ECA-positive) resulted in loss of ECA antibodies (IHA titer <8).
4. Tandem XIE demonstrated complete immunochemical identity between antigen "a" of E. coli 014 and antigens "a" of S. typhimurium and E. coli 0111.

Antigen "b" is not the enterobacterial common antigen of Kunin because it was detected in an ECA-negative mutant of E. coli 014. The exact nature of this antigen is not known. Several cross-reacting antigens have been described in gram negative bacteria (98-110). One common antigen, shared between Enterobacteriaceae, Pseudomonas aeruginosa and Neisseria meningitidis was shown by Kaisjer et al., to be an acidic glycoprotein. It was named the High Mobility Antigen (HMA). It could be demonstrated by immunoprecipitation and was capable of sensitizing erythrocytes for hemagglutination (109, 110,111). It is not known if the cross-reacting antigen "b" is the same as the HMA antigen. However, the existence of the HMA antigen makes it difficult to attribute the results of the IHA test totally to ECA, when polyvalent antiserum is used.

A third antigen, "c," was detected in two E. coli strains (E. coli 014 and E. coli 0111), but not S. typhimurium. This may represent a species-specific antigen of E. coli.

The identification in the present study of the antigenic peak which corresponds to ECA allows for the isolation of the antigen in a pure state for chemical characterization. Studies which dealt with chemical analyses of ECA have not presented convincing evidence of the purity of the antigenic preparation.

In addition, characterization of ECA also provides a means for the production of monospecific antisera to this antigen. Such antisera have been produced against the species-specific antigen of Chlamydia (138) and the type-specific antigen of Herpes simplex type 1 (139). These antisera were prepared by cutting the appropriate precipitin peak from the unstained gel, sonicating it in buffer and injecting the mixture intravenously into rabbits with Freund's adjuvant (138,139).

Rapid tests such as counterimmunoelectrophoresis (CIE) or radioimmunoassay (RIA) could be used for the detection of ECA in body fluids of patients suspected of having gram negative bacillary infections. Such techniques have become increasingly popular in the diagnostic laboratory for the rapid diagnosis of certain infections. The limiting factor for the use of CIE or other tests used to identify microbial antigens is the quality of the available antisera. The lack of monospecific antisera to organisms such as Streptococcus pneumoniae and Neisseria meningitidis have undoubtedly con-

tributed to impairment of the specificity of that test (140, 141).

The RIA test can be meaningfully applied for the detection of ECA only if either ECA antigen or its antibody is available in a pure state. Sanford et al., used this test recently to detect ECA antibodies in serum of patients with pyelonephritis. They used an antigen preparation derived from E. coli 014 which was not pure ECA. The investigators reported antibodies to ECA in 72% of the patients tested. The results were compared to the indirect fluorescent antibody procedure which measured titers of serum antibody to the patient's own infecting bacteria, and individual tests of RIA and IFA agreed in 75% of the cases (142). However, the use of an impure ECA preparation in the RIA technique raises the question as to whether ECA antibodies were measured. If produced, monospecific antiserum to ECA could be used in RIA or other similar tests for detecting ECA.

SUMMARY

This thesis characterizes for the first time the enterobacterial common antigen (ECA) by means of two dimensional crossed immunoelectrophoresis (XIE). The antigenic compositions of ECA-positive strains of E. coli 014 and S. typhimurium were evaluated by XIE. Immunochemical identity of antigens was determined by tandem two dimensional crossed immunoelectrophoresis (TXIE). Most antisera utilized in the XIE and TXIE techniques were prepared in rabbits against intact cell envelope fraction extracted according to the method of Domingue and Johnson.

XIE results demonstrated the complex antigenic composition of Enterobacteriaceae. As many as 13 distinct antigens could be detected in E. coli 014 (ECA-positive); at least 7 antigens could be demonstrated in the ECA-positive strain of Salmonella typhimurium.

Two common antigens (designated "a" and "b") were detected in E. coli 014, E. coli 0111, and S. typhimurium. Antigen "a" was thought to represent ECA. Evidence in support of this hypothesis is the following:

1. Antigen "a" was found in ECA-positive strains of E. coli 014, E. coli 0111, and S. typhimurium.
2. Antigen "a" was not detected in an ECA-negative mutant of E. coli 014.
3. Antiserum prepared against E. coli 014 (ECA-positive) retained ECA-reactive antibodies (IHA titer >8192)

following adsorption with an ECA-negative mutant of the same organism. When the adsorbed antiserum was tested by XIE using a crude antigen sonicate of E. coli 014 (ECA-positive), a single precipitin line, "a" was detected. Readsorption of the antiserum with E. coli 014 (ECA-positive) whole cells resulted in loss of ECA-reactive antibodies (IHA titer <8).

4. TXIE demonstrated immunochemical identity between antigen "a" of E. coli 014, and the "a" antigens of S. typhimurium and E. coli 0111 respectively.

The identity of the second common antigen, "b," is not known. The fact that antigen "b" could be demonstrated in the ECA-negative mutant of E. coli 014 indicates that it is not enterobacterial common antigen.

The ability to characterize ECA by XIE provides a means for isolating the antigen in a pure state, and for preparing monospecific antiserum. Neither pure ECA which is biologically active nor monospecific antiserum reactive with ECA are currently available.

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