Characterization of Escherichia coli from acute cases of bovine mastitis: some biochemical, serological and virulence properties

Vanessa Sánchez-Carlo
Iowa State University

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Sanchez-Carlo, Vanessa

CHARACTERIZATION OF ESCHERICHIA COLI FROM ACUTE CASES OF BOVINE MASTITIS: SOME BIOCHEMICAL, SEROLOGICAL AND VIRULENCE PROPERTIES

Iowa State University

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Characterization of \textit{Escherichia coli} \\
from acute cases of bovine mastitis: Some biochemical, serological and virulence properties  \\
by \\
Vanessa Sánchez-Carlo \\
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\textit{In Charge of Major Work} \\
Signature was redacted for privacy. \\
\textit{For the Major Department} \\
Signature was redacted for privacy. \\
\textit{For the Graduate College} \\
Iowa State University  \\
Ames, Iowa  \\
1983
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INTRODUCTION

During recent years, *Escherichia coli* has assumed a role of increasing importance in bovine mastitis. The preventive and control programs implemented during lactation and the antibiotic therapy administered in the dry period have brought no change in the incidence of coliform mastitis. As a result, gram-negative rods have become one of the major causes of the clinical form of the disease.

Mastitis caused by *Escherichia coli* is a severe and sometimes fatal infection which afflicts cows at any stage of lactation but most often during the peripartum period. The disease is characterized by rapid onset, may be systemic or only local, and of short duration without extensive damage of the mammary parenchyma. It is thought that infections are generally acquired directly from the environment since the low level of incidence and sporadic occurrence in a herd suggest little contagion. Healthy udders seem to be more prone to coliform infections than those exhibiting a mild leucocytosis or subclinical mastitis associated with other microorganisms.

Data provided by routine biochemical tests which are used for the laboratory identification of *E coli* and reports on the antigenic composition of the cell wall are available, but little is
known regarding the pathogenic mechanisms and virulence factors of the mastitis strains. Furthermore, the means to distinguish contaminating from pathogenic strains in milk samples have not been determined. However, thorough characterization of E coli associated with the disease conditions other than mastitis have provided information which has established certain markers of pathogenicity and have contributed to the elucidation of virulence mechanisms.

The objectives of this investigation are to characterize biochemically and serologically 184 E coli isolates from bovine mastitis. In addition, a search for pathogenic mechanisms and virulence factors which are present in E coli associated with diseases other than mastitis will be made. It is expected that some biological markers will be found which can be used to differentiate between nonpathogenic and E coli pathogenic for the bovine udder.

This dissertation is presented in the alternate format with two manuscripts to be submitted for publication in the American Journal of Veterinary Research. The manuscripts are preceded by a general introduction and a literature review. A general discussion and conclusions from the investigations follow at the end. The style and format of the manuscripts are in compliance with the guidelines for authors specified by the Journal except for the footnote notations which comply with the Iowa State University Graduate College Thesis Manual. A brief introduction indicating the objectives of the
investigation is followed by materials and methods, results and discussion. References are cited by order of appearance in the text and tables, and footnotes to tables are designated by symbols.

Additional literature cited includes the references to the general introduction and general discussion and are listed in a format consistent with the one used in the manuscripts.

The Ph.D. candidate, Vanessa Sánchez-Carlo, was the principal investigator for each of the studies and is the senior author for each manuscript. Co-authors are indicated in by-lines of each manuscript.
Biochemical Properties

The laboratory identification of *Escherichia coli* isolated from mastitis cases is based on colony morphology in selective and differential media and several routine biochemical tests. However, when *E. coli* isolates from disease conditions other than bovine mastitis are subjected to additional biochemical tests, it has been found that some unique metabolic reactions can be used in differentiating strains within a species. For example, invasive *E. coli* are characterized by their lack of lysine decarboxylase, nonmotility and slow lactose fermentation, and about 50% of the enterotoxigenic *E. coli* (ETEC) ferment adonitol compared to 7% non-ETEC. Several biotyping schemes have been developed to identify urinary pathogenic strains in humans and drug-resistant strains among those affecting domestic animals. Limited studies which have employed only a few metabolic and fermentative tests have been reported using mastitis strains.

Surface Antigens and Serotyping of *Escherichia coli*

An increased interest in the surface antigens of *E. coli* has been noted in recent years; mainly because of their possible involvement in pathophysiological processes, their use as epizootiological markers and their potential as immunogens. The identification
of these antigens is accomplished by a serologic analysis of the different structures present on the bacterial surface. Serotyping has become a useful tool in providing the means of subdividing the species *E. coli* into thousands of serogroups and serovars.\(^4\)

Three main antigens are recognized on the surface of *E. coli*: the 0 antigen or somatic antigen, which is polysaccharide in nature and heat-stable; the K antigen, capsule or envelope, which is an acidic polysaccharide and made up of three different varieties: L, A, and B; and the H or flagellar antigen, which is protein in nature.\(^3,11\) There are 164 0 antigen groups reported but only a limited number of these are associated with specific diseases. Almost all the septicemic,\(^12\) the human and animal enterotoxigenic,\(^13,14\) and urinary pathogenic strains\(^15\) belong to specific 0 groups and good correlations have been established between their antigenic composition and several pathogenic and virulence properties. In addition, 0 antigens have also been associated with protection under certain circumstances.\(^16\)

About 103 K antigens have been described of which 70 conform to the definition of an acidic polysaccharide.\(^3,11\) The remaining K antigens are other types of polysaccharides or protein.\(^11,17,18\) Certain capsular types as well as the amount of capsular material\(^19\) have been associated with virulence.\(^10\) In general, it has been postulated that the capsule is antiphagocytic,\(^10\) and anticomplementary and that
it protects the bacterial strain from the bactericidal activity of serum.\textsuperscript{20} Fifty-one H or flagellar antigens have been recognized.\textsuperscript{3} It has been proposed that the flagellum contributes to virulence by permitting chemotactic migration of the bacteria to the mucosal surfaces.\textsuperscript{16} Recently, H typing has gained in importance since the pathogenicity of some specific O groups of enteropathogenic \textit{E coli} seems to be related to particular H types.\textsuperscript{21}

Several reports regarding the serotyping of \textit{E coli} recovered from mastitis cases have been published. Eberhart and Buckalew\textsuperscript{22} identified 10 serotypes from among 17 \textit{E coli} mastitis isolates; McDonald et al,\textsuperscript{9} found 23 different O groups and 27 different serotypes from 32 \textit{E coli} cultures. Saran-Rosenzaig and Cohen\textsuperscript{23} found 48 serotypes represented among 60 strains isolated from mastitis. Linton et al\textsuperscript{24} reported 67 O groups among 217 O-typable strains recovered from mastitis. Similarly, Saran's\textsuperscript{25} serologic analysis of 109 strains from clinical mastitis cases shows a wide diversity of antigenic patterns. When these results were compared to serotypes of environmental strains, identical patterns were often observed. Likewise, Linton et al\textsuperscript{24} found the comparison between the serotypes of fecal strains from calves with those found in mastitis highly significant. These observations led the authors to postulate that \textit{E coli} occurring in mastitis was environmental and endogenous in origin.
Invasiveness

It is generally accepted that pathogenic *E. coli* may cause disease by: 1) elaboration of toxins (endotoxin and exotoxins) and 2) by the invasion and disruption of mucosal surfaces.\textsuperscript{12}

Invasive *E. coli* are enteropathogenic for humans.\textsuperscript{6,12} They produce a dysentery-like infection by penetrating and multiplying in the epithelial cells of the colon.\textsuperscript{12} Some biochemical characteristics of these *E. coli* are similar to *Shigella* and only a limited number of O antigenic groups are commonly associated with the disease.\textsuperscript{6}

Several methods are currently used to detect the ability of bacteria to invade epithelial surfaces: fluorescent antibody technique to visualize the organisms in the mucosa,\textsuperscript{12} the HEp-2 tissue culture test,\textsuperscript{26} and the Sereny (guinea pig eye) test.\textsuperscript{27} The latter is based on the production of keratoconjunctivitis after the instillation of a heavy suspension of *E. coli* into the eye of the guinea pig. The development of the lesions in the eye correlates well with the organism's ability to penetrate the surface of intestinal cells.\textsuperscript{12,27,28} This test has the advantage of being easy to carry out, and to give well-definable results.\textsuperscript{28} In addition to its use in the identification of *Shigella* spp., the test has the ability to distinguish between freshly isolated virulent and avirulent strains and to detect the ability of any enteric isolate to cause dysenteric disease.\textsuperscript{28} However, the assay has the disadvantage of being costly and painful to the animal.\textsuperscript{26}
The pathogenic mechanism of invasive strains has been related to the possession of 2 plasmid-mediated characteristics: increased virulence and resistance to serum due to presence of the ColV plasmid and the synthesis of a toxin and a surface antigen coded by the Vir plasmid.

**Enterotoxins**

Two determinants of enteropathogenicity have been described from human and animal *E. coli* strains: 1) the ability to colonize and proliferate in the small intestine, and 2) the ability to elaborate enterotoxins. The latter consists of 2 types: a heat-stable toxin (ST) of small molecular weight and apparently nonimmunogenic and a heat-labile toxin (LT) of large molecular weight and immunogenic. The ability to synthesize enterotoxins is plasmid-mediated and occurs as ST or ST/LT, although some strains that produce LT only have been found.

Various assay methods have been developed to detect the LT. However, many of these tests are not suitable for routine clinical screening. The tissue culture system described by Donta, et al using the Y-1 adrenal tumor cells correlates well with the standard intestinal-loop system for LT assay. The specific determination of the ST is by means of the infant mouse test described by Dean et al. Because the original rabbit loop test was unreliable and the mouse intestinal-loop lacked uniformity in the results, infant mice were chosen for the test. The initial absence of bacteria in their
intestines, as well as their size and low cost prompted their use as the animal model. Bactericidal Activity/Serum Resistance

Bactericidal capacity of serum is a significant host defense mechanism against infection. Numerous nonspecific (complement, lysozyme, phagocytes, and iron-binding proteins) and specific (antibodies and lymphocytes) agents, alone or in combination, kill, lyse and prevent growth of many bacteria that come in contact with blood or serum. Although nonpathogenic and noninvasive strains are serum susceptible (SS), invasive and pathogenic strains are serum resistant (SR). The bactericidal activity of serum is mainly directed towards gram-negative rods. The biochemical basis by which serum kills is unknown, but it has been suggested that antibody and complement mediate events in the cell membrane depleting the cytopool of low molecular weight constituents, resulting in death of the bacterium.

Experimental evidence and clinical observations suggest that serum resistance is an important virulence factor of some gram-negative rods and that it contributes to the pathogenicity. Little is known about the capability of bacteria to resist, evade or inactivate the host defenses, but Taylor and Hughes suggested it to be a multifactorial property. Several bacterial mechanisms which resist antibacterial effects of serum have been
postulated. All of them are related to the accumulation of a
number of associated components at or near the cell surface. 1) the possession of fewer short-chain fatty acids and more long-chain fatty acids in both the outer and inner membrane may impart rigidity to the structure and repel the insertion of complement components; 2) the presence of a capsular antigen which provides bacteria with substantial SR increases their virulence, reduce their sensitivity to phagocytosis and shields cell surface structures which ordinarily activate serum; 3) the presence of lipopolysaccharide (LPS) O-side chains in the cell wall has an important role in SR but does not determine SR alone. Some O antigens have been associated with SR but Vosti and Randall found considerable variation in susceptibility and resistance to serum within the same O-groups; 4) plasmids have been found to increase the SR of gram-negative rods by modifying the bacterial surface structure. The SR factor has been found to reside in the traS and traT plasmid gene products which mediates surface exclusion. Several plasmids, R100 and R6-5 code for this protein. In addition, the ColV,I-K94 plasmid also confers SR but the genetic determinant is located at the iss gene; 5) the carriage of many extrachromosomal determinants has been related to SR. However, Hughes et al found that the average number of plasmids decreases as the SR increases among urinary isolates; 6) the amount of LPS seems
to be relevant to SR.\textsuperscript{48}

It has been reported that the ability of \textit{E. coli} to cause mastitis in normal mammary quarters was related to the SS or SR of the strain.\textsuperscript{49} Almost all the cultures recovered from mastitis are SR.\textsuperscript{37} The bactericidal substances reaching the milk from the blood are related to complement-dependent antibodies and are called bactericidins.\textsuperscript{50} Therefore, the susceptibility or resistance to the natural antibodies is a measure of the relative virulence of individual \textit{E. coli} in the mammary gland.\textsuperscript{49} Normal milk and mastitis exudate contain varying degrees of bactericidal activity, but the system's efficiency lies in the vast perfusion of serum components during an inflammatory episode.\textsuperscript{51} In spite of all the protection afforded by the bactericidins, a high somatic cell count in the udder seems to limit significantly the growth of either SS or SR strains.\textsuperscript{37}

Several methods for determining SS have been described.\textsuperscript{45,46,49} Some of the techniques are based in the actual count of colonies after incubation of serum and bacterial cultures; other methods measure the turbidity of growth in broth photometrically. DeMatteo et al\textsuperscript{52} reported a comparison between three methods for detecting serum bactericidal activity among \textit{Pseudomonas}. He concluded that the medium as well as the phase of growth of the culture affected the sensitivity of the test. Also, very few of the isolates were uniform in their reactions in the three different tests.
Hemagglutination

Adhesion of microorganisms to the host's epithelial surfaces is a prerequisite for many infections. The interaction between bacteria and host tissue is mainly a surface phenomenon where microbial surface structures, physical forces and host complementary receptors are involved. The bacterial ligands responsible for the binding to different cells have been defined as adhesins. The intercellular recognition is thought to be mediated by sugar residues on the host receptors to which bacteria attach by sugar-binding proteins akin to plant lectins. Among the gram-negative bacteria, the most common adhesins are in the form of pili or fimbriae. These structures are filamentous non-flagellar, proteinaceous appendages, rod-shaped, variable in length and with several functions. Pili are usually found among both commensal and pathogenic bacteria. The majority of bacteria that possess adhesins in the form of pili vary in a reversible, spontaneous and random way, from a piliated to a nonpiliated phase. The pilus phase variation is largely controlled by laboratory methods of cultivation, temperature of incubation and phase of growth.

The significance of pili for bacterial survival in vivo is uncertain; not all piliated bacteria are adherent or pathogenic and not all adherent pathogenic bacteria are piliated. Nevertheless, pili are considered important virulence
factors among bacteria colonizing mucosal surfaces. Attachment prevents the wash out of the microorganism by host defenses and also ensures an effective toxin delivery to host epithelium.

Pili may be classified antigenically, morphologically and by their ability to agglutinate red blood cells (RBC). Because of the correlation between pili and hemagglutination (HA) and agglutination of RBC is the basis of a test for the presence of pili on bacteria. The HA is simple, rapid, economical and the conventional way for screening large numbers of isolates for adhesiveness. However, not all piliated strains cause HA and not all HA is related to the possession of pili.

The biological activity of some pili can be inhibited by D-mannose or a derivative of β-methyl-D-mannoside. Based on this reaction, pili have been called mannose-sensitive (MS) and mannose-resistant (MR). Both types of pili function as possible adherence factors but the role of MS pili in human and animal diseases is still debatable. Mannose-sensitive hemagglutinins are found in 50-70% of all E coli without obvious relationship to virulence, although a recent publication reports their presence among virulent urinary strains.

Antigenically, all MS pili are related; colonization of the large intestine by commensal flora may be due to this pilus.
The hemagglutinin that agglutinates guinea pig RBC in a MS way has been defined as common pilus, type 1 pilus,^66,69 or Pl.^^ Its chromosomal expression is largely suppressed by culturing on solid media.73 Mannose-resistant hemagglutinins are less widely distributed among E coli. These hemagglutinins are likely to be coded by different plasmids, are antigenically diverse,73 and may be serologically identified.77 Different patterns of HA are observed with RBC from different animal species.71,74,75,76 Some hemagglutinins are enhanced in the cold and some elute when warmed to room or higher temperature.76

Five different types of MR pili have been distinguished serologically: 2 types are from human ETEC and 3 are related to animal ETEC. In addition, the animal strains show a distinct pattern of MR-HA which aids in their identification. K-99 pilus isolated from ETEC from calves, lambs and pigs agglutinates sheep RBC,77 K88 recovered from ETEC from pigs agglutinates guinea pig RBC,77,78 while 987P does not hemagglutinate in vitro but adheres in vivo to epithelia of the small intestine.79,80

In addition to the adhesive properties of pili, other forms of adhesins are present in the outer membrane of the cell wall.72 The capsule of some porcine and bovine pathogens acts in concert with the pili to anchor the bacteria to the intestinal epithelium.77 Usually, the loss of the plasmid determined pili (K88, K99) is
associated with the loss of a virulence factor and hemagglutination capacity, therefore, these antigens are considered reliable markers for recognition of pathogenic strains. Although a good correlation exists between the presence of pili and the ability of a strain to attach to RBC, this type of adhesin does not necessarily imply adhesion to epithelial surfaces.
SECTION II. SOME BIOCHEMICAL AND SEROLOGICAL PROPERTIES OF ESCHERICHIA COLI ISOLATED FROM ACUTE CASES OF BOVINE MASTITIS
Some biochemical and serological properties of *Escherichia coli* isolated from acute cases of bovine mastitis

Vanessa Sánchez-Carlo¹,², M.S.
R. A. Wilson³, Ph.D.
J. S. McDonald², D.V.M., Ph.D.
R. A. Packer¹, D.V.M., Ph.D.

A manuscript to be submitted to the American Journal of American Research. From the Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011¹, the National Animal Disease Center, Ames, IA 50010², and the Department of Veterinary Science, Pennsylvania State University, University Park, PA 16802³.

Supported in part by funds provided under Broad Form Cooperative Agreement #12-14-3001-533 between the United States Department of Agriculture, Science and Education Agricultural Research Service, National Animal Disease Center and Iowa State University.
SUMMARY

Biochemical and serological characteristics were determined on 184 strains of Escherichia coli isolated from acute cases of bovine mastitis. The majority of the test reactions were in agreement with the results reported by others for E coli. In those characteristics reported as variable, no trends or patterns unique to the bovine strains were observed, except in the adonitol fermentation. Approximately half of the cultures were positive. Growth on T-7 agar plates exhibited three types of colony morphology. Rough and intermediate textures predominated over those with smooth colony surfaces. A large variety of O and K serogroups was detected and identified, but many K antigens showed multiple low titer agglutinations and therefore were untypable with available antisera. Only 2 isolates were found to contain the K99 antigen. The results did not provide markers to distinguish mastitis strains of E coli as a specific group of pathogens.
Escherichia coli is a frequent cause of bovine coliform mastitis.\textsuperscript{1} The isolation of this gram-negative rod from udder secretions may be difficult to interpret because of the possibility of contamination of the sample at the time of collection and the low numbers of organisms occasionally recovered from milk samples. No effective ways have been developed to differentiate between nonpathogenic, contaminating and pathogenic strains.

Strains of \textit{E coli} isolated from other diseases, namely neonatal diarrhea in animals and humans\textsuperscript{2-5} and septicemia and urinary tract infections in humans\textsuperscript{6-7} have been well-characterized. Biochemical and serological test reactions of these strains have been correlated with pathogenicity.

The purposes of this study were to identify \textit{E coli} from among coliform mastitis isolates, and to search for any physiological or serological marker which could aid in the recognition of an \textit{E coli} isolate as a specific udder pathogen.
Cultures—One hundred ninety-three cultures identified only as coliforms and isolated from naturally occurring cases of clinical mastitis in dairy cows were used in this study. Thirty cultures were obtained from the mastitis research herd at the National Animal Disease Center (NADC), Ames, Iowa. One hundred sixty-seven cultures were sent to us from 8 California dairy herds by Dr. DE Jasper. All the isolates were initially recovered on 5% bovine blood agar (BA) plates. The California isolates were transported on nutrient agar slants and transferred again to BA plates when received in our laboratory. All the cultures were frozen and stored at -51°C as previously described. The number of transfers was kept at a minimum.

Controls—The organisms listed in Table 1 were used as controls throughout this experiment. C-cultures and the ATCC strain were used as media and reagent controls for the various biochemical tests. Strains B41 (O141:K99:NM) and 123 (O43 K-:H28) were used as positive and negative controls respectively in the K99 slide agglutination test.

Biochemical tests—Prior to inoculation into test media, each frozen culture was transferred to a modified Tergitol-7 (T-7) agar

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a Department of Clinical Pathology, School of Veterinary Medicine, Davis, CA.

b Difco Laboratories, Detroit, MI.
TABLE 1—List of organisms used as control strains in the biochemical and serological tests

<table>
<thead>
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<th>Designation</th>
<th>Organism</th>
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<td>C-463*</td>
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<td>C-531</td>
<td><em>C</em>-cultures kindly provided by Dr. J. S. McDonald, NADC, Ames, IA.</td>
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<tr>
<td>C-533</td>
<td><em>C</em>-cultures kindly provided by Dr. J. S. McDonald, NADC, Ames, IA.</td>
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<td>ATCC 25922†</td>
<td>American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD.</td>
</tr>
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<td>B41†</td>
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<td>123†</td>
<td>American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD.</td>
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</table>

*American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD.**

†Courtesy of Dr. H. W. Moon, National Animal Disease Center, Ames, IA.
plate and to a tryptose agar (TA) slant. Growth on T-7 agar plates was used to verify typical \textit{E. coli} colony characteristics and texture. Growth on TA was used to inoculate all the differential media. The biochemical reactions were conducted according to the methods of Edwards and Ewing with the following modification: an additional 0.5 gram of glucose was added to Möller decarboxylase basal medium. The β-D-galactosidase test (ONPG) and the Kovac's indirect filter paper method for the cytochrome oxidase test were performed as described by MacFadden.

**Serological procedures**—All the coliforms identified as \textit{E. coli} were serotyped as described by Glantz. The slide agglutination test as described by Moon et al. was employed to detect the K99 antigen except that only Minca Isovitalex agar plates were used.

In addition, several K99 negative \textit{E. coli} isolates which appeared to be capsulated on Minca Isovitalex agar were randomly selected and individually grown on 5% bovine blood agar plates. The cultures were incubated at 37°C for 48 hours and at 21°C for one week to allow development of acapsular mutants. Slide agglutination

\textsuperscript{a}Difco Laboratories, Detroit, MI.

\textsuperscript{b}ONPG, o-nitrophenyl, β-D-galactopyranoside.

\textsuperscript{c}Baltimore Biological Laboratory, Inc, Cockeysville, MD.
procedures for K99 antigen on nine different mutant, translucent strains were made as described by Moon et al.\textsuperscript{13}
RESULTS

One hundred eighty-four \textit{E coli} cultures were identified from among the one hundred ninety-three gram-negative rods submitted for biochemical characterization. Nine isolates did not conform to the description of \textit{E coli} because they were positive to urease and citrate tests. Biochemical test results of the 184 isolates are shown in Table 2. In general, test results are in agreement with the typical reactions reported for \textit{E coli} except for the adonitol fermentation.

Colony appearance of \textit{E coli} isolates on T-7 agar plates was typically yellow with yellow halos and no reduction of the tetrazolium salts. Texture of the colonies was described as smooth, rough, or intermediate; the latter possessing some characteristics of each of the other two types. Table 3 indicates that approximately 50% of the cultures exhibited intermediate colony morphology, whereas the other 50% was about equally distributed between smooth and rough colony types.

The serotypes of all the isolates and their frequency of occurrence are shown in Table 4. Seventy-six percent of the cultures could be O-typed with the antisera presently available. Five percent of the cultures exhibited low titer agglutinations with
TABLE 2--Biochemical characterization of 184 *Escherichia coli* isolates from clinical cases of bovine mastitis

<table>
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<th>Typical reactions % positive</th>
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<td>Jordan tartrate</td>
<td>184</td>
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<td>97.6</td>
</tr>
<tr>
<td>β-D galactosidase (ONPG)^†</td>
<td>184</td>
<td>100</td>
<td>NA^†</td>
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<tr>
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<td>Mucate</td>
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<tr>
<td>Oxidase</td>
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<td>0</td>
</tr>
</tbody>
</table>

^*P. R. Edwards and W. H. Ewing.*

^†TSI = Triple sugar iron; ONPG = o-nitrophenyl β-D-galactopyranoside; NA = Not available.
TABLE 3—Growth characteristics on T-7 agar of 184 *Escherichia coli* strains isolated from clinical cases of bovine mastitis

<table>
<thead>
<tr>
<th>Texture of colony</th>
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<tr>
<td></td>
<td># of isolates</td>
</tr>
<tr>
<td>Smooth</td>
<td>43</td>
</tr>
<tr>
<td>Intermediate</td>
<td>90</td>
</tr>
<tr>
<td>Rough</td>
<td>51</td>
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TABLE 4—Serotypes of 184 Escherichia coli isolates from bovine cases of clinical mastitis

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<thead>
<tr>
<th>Serotype</th>
<th># isolates</th>
<th>Serotype</th>
<th># isolates</th>
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<td>K</td>
<td>H</td>
<td>O</td>
</tr>
<tr>
<td>1a</td>
<td>-</td>
<td>nm</td>
<td>1</td>
</tr>
<tr>
<td>1a</td>
<td>32</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td>1a</td>
<td>-</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td>1ab</td>
<td>-</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td>1ab</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>2a</td>
<td>.</td>
<td>-</td>
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<tr>
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<td>.</td>
<td>nm</td>
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<tr>
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<td>-</td>
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<td>-</td>
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<tr>
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<td>7</td>
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</tr>
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<td>.</td>
<td>nm</td>
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</tr>
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</tr>
<tr>
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<td>3</td>
</tr>
<tr>
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<td>48</td>
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</tr>
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<td>40</td>
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<td>2</td>
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<tr>
<td>40</td>
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<td>1</td>
</tr>
<tr>
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<td>32</td>
<td>1</td>
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</tr>
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<td>32</td>
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<tr>
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<td>24/76</td>
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<tr>
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<tr>
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<td>-</td>
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<tr>
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<td>-</td>
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Table 4 (Continued)

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<th># isolates</th>
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<td>-  39  2</td>
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<td>157 -  1  1</td>
<td>-  48  2</td>
<td></td>
<td></td>
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<td>160 -  1  1</td>
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<td>X-3 - 26  1</td>
<td>-  76  1</td>
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1 = Also high titer cross reactions with 065, 05ac and 070 antisera.
2 = Also high titer cross reactions with 0138 and 0139 antisera.
3 = Also high titer cross reactions with H6, H7 and H12 antisera.
4 = Also high titer cross reactions with H7 and H8 antisera.
5 = Also high titer cross reactions with H3, H6 and H8 antisera.
6 = Also high titer cross reactions with K41, K49 and K50 antisera.
7 = Also high titer cross reactions with K10 and K54 antisera.
8 = Also high titer cross reactions with H6, H7 and H8 antisera.
= Low titer agglutination with many antisera.
- (O and K antigens) = Lack of O or K antigen, or detection of specific antigen not possible with available antisera.
- (H antigen) = Presence of undetectable H antigen with available antisera.

nm = Nonmotile; no H antigen present.
several O antisera. Eighteen percent of the cultures were negative for the O antigens tested. Fifty-seven different O groups were represented among the 141 O-typable isolates. Thirty O groups appeared with a frequency of 1. Only 12 O groups had at least 4 isolates per group.

It is interesting to note that several O groups were limited in the range of H antigens with which they were associated. Groups Ola, O16, and O65 when found in motile isolates, appeared with H55, H48, and H30, respectively.

Only 20% of the isolates were successfully K-typed. Twenty-nine different K antigens, appearing singly or in several combinations, were identified. The majority of the cultures possessed K types undetectable with available K antisera. Most of these K typing reactions were multiple, low titer agglutinations. The highest frequency of a given K type was three and the antigen was identified as Kl.

One hundred sixty-eight isolates were judged motile following inoculation into motility test medium in our laboratory. When the same cultures were grown in a different motility test medium for the identification of the H antigen, 145 cultures were classified

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aDifco Laboratories, Detroit, MI.

bE coli Reference Center, Department of Veterinary Science, The Pennsylvania State University, University Park, PA.
as motile. Fifty-four percent of these motile strains were typed with available H antisera. Forty-one percent of the isolates could not be placed into a recognized H group and only 3% of all the motile cultures showed low titer agglutination with available antisera.

Only 2 of the 184 cultures of *E. coli* were positive on the slide agglutination test for K99 antigen: (O*:K*:H11 and O":K*:H39).

None of the nine translucent acapsular mutants that developed on BA agglutinated with K99 antiserum.
DISCUSSION

The overall characterization of the *E coli* isolated from acute cases of bovine mastitis indicates that these strains exhibit a uniform and typical pattern of biochemical behavior in all tests except one: adonitol fermentation. The differences observed between our results and those reported by others\textsuperscript{10,11,15,16} are within the normal range of variability expected in metabolic reactions.

In Table 5, selected biochemical characteristics of *E coli* from mastitis are compared to *E coli* recovered from other sources. It should be noted that mastitis strains and enterotoxigenic *E coli* (ETEC), both from bovine origin, exhibit a tendency to ferment adonitol. Forty-five and six-tenths percent and fifty percent, respectively, were positive in contrast to the low rates of fermentation encountered among *E coli* from other sources. Braaten and Myers\textsuperscript{2} suggested the use of adonitol fermentation as an indicator of enterotoxigenicity. Our findings in this regard do not support this statement since only three of the mastitis strains produced any kind of enterotoxin (Section II). It is possible that adonitol fermentation is a characteristic of isolates from cattle and not necessarily a marker for enterotoxigenicity or pathogenicity in bovine strains. Based on the results of the biochemical profile
TABLE 5—Comparison of some biochemical reactions* of *Escherichia coli* isolated from different sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Reference</th>
<th>No. of strains</th>
<th>Adon-</th>
<th>Ino-</th>
<th>Ornithine decarboxylase</th>
<th>Lysine decarboxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastitis</td>
<td>This study</td>
<td>184</td>
<td>45.6</td>
<td>0.5</td>
<td>74.4</td>
<td>98.9</td>
</tr>
<tr>
<td>Heterogenous</td>
<td>Edwards &amp; Ewing (10)</td>
<td>...†</td>
<td>6</td>
<td>0.6</td>
<td>68</td>
<td>89.5</td>
</tr>
<tr>
<td>Heterogenous</td>
<td>Cowan (15)</td>
<td>...</td>
<td>-†</td>
<td>-</td>
<td>d†</td>
<td>+†</td>
</tr>
<tr>
<td>Heterogenous</td>
<td>Brenner et al (16)</td>
<td>84</td>
<td>0-10</td>
<td>NA†</td>
<td>26-74</td>
<td>75-89</td>
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<tr>
<td>Urinary tract</td>
<td>Buckwold et al (6)</td>
<td>959</td>
<td>8</td>
<td>0</td>
<td>NA</td>
<td>92</td>
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<td>Septicemia</td>
<td>Silva et al (7)</td>
<td>97</td>
<td>0</td>
<td>0</td>
<td>48.3</td>
<td>0</td>
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<td>Enterotoxigenic</td>
<td>Braaten &amp; Myers (2)</td>
<td>18</td>
<td>50</td>
<td>12</td>
<td>50</td>
<td>83</td>
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<tr>
<td>Nonenterotoxigenic</td>
<td>Braaten &amp; Myers (2)</td>
<td>15</td>
<td>7</td>
<td>27</td>
<td>87</td>
<td>93</td>
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<tr>
<td>Domestic animals</td>
<td>Tshiguro et al (28)</td>
<td>80</td>
<td>4</td>
<td>NA</td>
<td>74</td>
<td>100</td>
</tr>
<tr>
<td>Milk</td>
<td>Yang &amp; Jones (17)</td>
<td>3</td>
<td>33</td>
<td>0</td>
<td>d</td>
<td>33</td>
</tr>
</tbody>
</table>

*Data expressed as % positive.

†... = Unspecified large number of isolates; - = > 90% negative; d = different reactions by different strains; + = > 90% positive; NA = not available.
of the mastitis strains, there are no features that could be used in
the identification of \textit{E. coli} as a specific udder pathogen. No
relationship could be established between adonitol fermentation and
serotype or colony morphology. However, all the isolates of groups
040 (n = 8), 01a (n = 5), and 065 (n = 6) fermented adonitol.

One-half of the \textit{E. coli} isolates grown on T-7 agar plates
exhibited either smooth (23%) or rough (27%) colony texture. The
other half showed an intermediate type of colony (Table 3). These
results agree with those of McDonald et al\textsuperscript{8} who also reported rough
and intermediate colony types as the predominant colony type of \textit{E. coli}
isolated from mastitis. Yang and Jones\textsuperscript{17} in their study with 3
pathogenic and 3 nonpathogenic \textit{E. coli} isolated from pasteurized
milk established a correlation between rough and intermediate type
and pathogenic strains, and between smooth colonies and nonpathogenic
strains. Based on these observations, it would be difficult to
interpret the role of smooth colonies in mastitis. Either colony
morphology is not related to pathogenicity of \textit{E. coli} from mastitis
or Yang and Jones' classification is irrelevant to mastitis isolates.
Since all three types of texture were represented among our isolates,
colony morphology doesn't seem to be a useful diagnostic tool.

Fifty-seven different 0 serogroups were identified from 141
0-typable mastitis cultures. This large number of different 0-
antigen groups is not consistent with the trend of \textit{E. coli} from
other disease processes, where a limited number of 0 groups are
involved. Eberhardt and Buckalew and McDonald et al. typied a small number of E. coli from mastitis cases and obtained similar results regarding the occurrence of a wide variety of O antigen groups. Twenty-four O groups were identified from 49 E. coli intra-mammary infections. The highest frequency of a given O group, in the above mentioned studies was three. Linton et al. serogrouped 279 E. coli strains from clinical cases of bovine mastitis in England and found 67 different O antigens. The frequency of identical O antigens was usually low, with the majority of the O-groups occurring in single infections in a herd. These results lend support to the contention that E. coli mastitis is not highly contagious and that it is likely acquired from an environmental pool of serologically different strains.

In an unrelated study, Howe et al. found a significant correlation between their mastitis O groups and those obtained from fecal samples from calves. This finding led them to postulate that coliform mastitis was endogenous in origin and that infection resulted from environmental exposure. In Israel, Saran serotyped 75 E. coli environmental strains isolated from milker's hands and milking equipment, and 109 E. coli from acute mastitis cases. In some cases, identical O antigenic patterns were observed between the two sources. When our groups were compared to those of Howe et al. and Saran many common specific O-groups were found in the three studies. The similarities between the serogroups from calves' fecal samples
and mastitis isolates and the fact that a higher than expected percentage of calves' ETEC and mastitic strains fermented adonitol suggests common serological and biochemical characteristics among bovine *E. coli*. It is interesting to note that the same 0 antigens are often encountered in widely different geographical areas.

While antigenic structure and biochemical behavior are the criteria often used to identify pathogenic strains of *E. coli*, neither our biochemical or serologic data were able to provide a definition which could be used to identify mastitis pathogens.

The antigenic diversity exhibited by the mastitis isolates in the serologic analysis and the absence of predominant serogroups from a herd suggests that there are no specific 0 antigens associated with the disease, and that intramammary infections probably are caused by nonspecific environmental, opportunistic *E. coli*. Except for epizootiological data in problem areas, serotyping is not useful in differentiating pathogenic from contaminating strains of *E. coli* isolated from milk samples.

Considerable difficulty was encountered in attempting to type the K antigens among the mastitis isolates. Eberhart and Buckalew also reported many untypable K antigens and reported them as K dot (K*). Since the *E. coli* antigenic scheme has been largely developed for human isolates, further research is suggested in the serotyping of animal strains, especially those from
mastitis. Whether the K untypable mastitis strains possess single or multiple, as yet undescribed, antigens is not known. It would be interesting to know if the envelope antigen(s) plays a role in the pathogenicity of E coli in the mammary gland. It has been found that toxicity and virulence of several enteropathogenic, enterotoxigenic and invasive strains associated with human and animal diseases are related to the K antigen.24,25,26

The fact that many motile isolates could not be serotyped with the available antisera might be attributed to the limitations of the antigenic scheme used. There are possibly undescribed H antigens from animal sources whose role, if any, in disease is not established. From our data, no specific group(s) of H antigens could be associated with mastitis.

Several authors3,13,18 have reported that K99 antigen is an important virulence attribute of enterotoxigenic E coli in calves and that its expression is correlated with specific O groups.18 Only two mastitis isolates were positive in the K99 agglutination test and both fermented adonitol. Neither of them could be O serotyped.

It has been reported that capsular antigens could mask the K99 antigenic determinants.27 In order to overcome the difficulty presented by capsulated, mucoid isolates, we developed acapsular translucent mutants from nine different isolates and repeated the
agglutination test. None of the mutant colonies agglutinated with K99 antiserum. Since there was not enough evidence to indicate that K99 was present in bovine mastitis isolates, no further agglutination tests were made.

Based on the data from this study it is not possible to characterize biochemically or serologically distinct E. coli from mastitis episodes. No marked biochemical differences were found that could distinguish the strains associated with the disease nor did the serologic analysis offer the discriminating information we were looking for.
REFERENCES


SECTION II. A SEARCH FOR VIRULENCE FACTORS AMONG ISOLATES OF ESCHERICHIA COLI RECOVERED FROM ACUTE CASES OF BOVINE MASTITIS
A search for virulence factors among isolates of *Escherichia coli* recovered from acute cases of bovine mastitis

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SUMMARY

Several pathogenic and virulence mechanisms commonly associated with *Escherichia coli* isolated from various disease processes were studied. Only one mastitis strain was found to produce heat-stable toxin in the infant mouse test and two strains were positive for the heat-labile toxin production in the Y-1 adrenal tumor cell bioassay. None of the cultures were found to be invasive in the Sereny test and all the isolates were serum resistant. The majority of the *E. coli* agglutinated guinea pig red blood cells in a mannose-sensitive (MS) manner suggesting the presence of type 1 pilus, and rabbit red blood cells in a mannose-resistant (MR) way, indicating another type of adhesin. Based on our results, it appears that serum resistance is the only characteristic common to all isolates that could possibly be related to virulence.
INTRODUCTION

Coliform mastitis caused by *Escherichia coli* is responsible for considerable economic loss in dairy herds as indicated by lower production and quality of milk, cost of treatment, and occasional death. The opportunistic nature of the microorganism in causing mastitis is dependent on certain host physiological and immunological factors as well as on microbial pathogenic factors. Adhesins in the form of pili, endotoxin, enterotoxin, invasiveness of epithelial surfaces, and serum resistance have been related to the pathogenicity of *E. coli* in certain animal and human disease processes.

Except for the endotoxin and serum resistance, the virulence factors of *E. coli* associated with mastitis have not been reported. It has been suspected that if the pathogenic mechanisms or virulence factors of *E. coli* in the mammary gland were understood, more effective ways could be developed for its treatment and control.

The purpose of this study was to examine some representative isolates of *E. coli* from acute cases of bovine mastitis for the presence of recognized virulence factors such as ability to produce enterotoxins, possession of adhesins in the form of pili, invasiveness and serum resistance.
MATERIALS AND METHODS

Bacterial strains--One hundred eighty-four E. coli strains recovered from acute cases of bovine mastitis and previously identified were used in this study. Details of the sources, propagation and handling have already been described (Section I). Data and purposes pertaining to the E. coli strains used as controls are given in Table 1.

Enterotoxin production--Preparation of the toxin filtrates was done according to Isaacson and Moon with some modifications. Briefly, each E. coli strain was inoculated into 8 ml of trypticase soy broth (TSB)\textsuperscript{a} and incubated overnight. One-tenth ml of the broth culture was transferred to 25 ml of TSB and incubated at 37°C with vigorous shaking. After 20 hours of incubation, the broth was centrifuged at 15,000 x g, the supernatant filtered through a Millex\textsuperscript{b} filter unit (0.45 μm) and stored at 4°C for further use.

Heat stable toxin (ST)--The infant mouse bioassay, as described by Moon et al\textsuperscript{14} was followed to detect ST. Three two-day-old mice were individually given 0.1 ml of a culture filtrate (0.3 ml) mixed with 1 drop of Evans blue dye (0.01%), intragastrically. Each set

\textsuperscript{a}Baltimore Biological Laboratory, Inc, Cockeysville, MD.

\textsuperscript{b}Millipore Corporation, #CLHA 02505, Bedford, MA.
### TABLE 1—Background data and use of *Escherichia coli* control strains *",†

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Source</th>
<th>Serotype</th>
<th>Toxin type</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1261 (2176EB)</td>
<td>Pig</td>
<td>0138:K98:NM</td>
<td>ST</td>
<td>Positive control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IMT</td>
</tr>
<tr>
<td>263</td>
<td>Pig</td>
<td>08:K87,88ab:H19</td>
<td>LT-ST</td>
<td>Positive control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Y-1 ATCA MS-HA (guinea pig RBC)</td>
</tr>
<tr>
<td>123</td>
<td>Pig</td>
<td>043:K7:H28</td>
<td>None</td>
<td>No HA of sheep RBC</td>
</tr>
<tr>
<td>1300 (1624 * 56)</td>
<td>Human</td>
<td>0144:K-:NM</td>
<td>NK</td>
<td>Positive control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sereny test</td>
</tr>
<tr>
<td>Eng</td>
<td>Human</td>
<td>NK</td>
<td>NK</td>
<td>SR to guinea pig serum</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SS to human serum</td>
</tr>
<tr>
<td>B41 (1471)</td>
<td>Calf</td>
<td>0101:K99:NM</td>
<td>ST</td>
<td>MR-HA (sheep RBC)</td>
</tr>
<tr>
<td>987P</td>
<td>Pig</td>
<td>09:K103:NM</td>
<td>ST</td>
<td>Negative control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HA</td>
</tr>
</tbody>
</table>

*Courtesy of Dr. H. W. Moon, National Animal Disease Center, PO Box 70, Ames, IA 50010.*

†ST = heat-stable toxin; IMT = infant mouse test; LT = heat-labile toxin; Y-1 ATCA = Y-1 adrenal tumor cell assay; MS-HA = mannose-sensitive hemagglutination; RBC = red blood cells; K' = K unknown; NK = not known; SR = serum resistant; SS = serum sensitive; MR-HA = mannose-resistant hemagglutination.
of mice was placed in the bottom of a 100 x 15 mm petri plate, covered with a piece of filter paper and incubated for 4 hours at 37 C. Positive results for ST were recorded when 4 or more diarrheal spots were detected on the filter paper.

**Heat labile toxin (LT)**—The production of LT was tested in mouse Y-1 adrenal tumor cells, derived originally from radiation-induced adrenocortical tumor. The test was conducted as described by Isaacson and Moon. Plastic trays with 24, 16 mm diameter wells were seeded with 1 drop of the Y-1 cell suspension and 1 ml of the nutrient medium. When confluent growth was observed, 0.1 ml of the *E. coli* culture filtrate was added to each of the 2 wells used per sample. Plates were incubated 24-48 hours in a humidified atmosphere of 95% air and 5% CO₂. Morphological changes expressed as rounding up of cells were considered a positive result for LT activity. The number of units of toxin activity was defined as the reciprocal of the highest dilution showing the rounding of cells.

**Invasiveness**—The Sereny test was used to detect invasiveness among the mastitis strains. Guinea pigs weighing about 350 g, obtained through the Animal Supply Unit of the National Animal Disease Center, were used in this study. Two drops of a heavy

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\[a\] Fisher Scientific Co, 1600 W Glenlake Ave, Itasca, IL.

\[b\] Multi-Dish-Disposo Trays, HFB-16-24-TC, Linbro Chemical Co, Inc, 681 Dixwell Ave, New Haven, CT.
suspension of a young culture, grown on 5% blood agar, were
instilled in the conjunctival sac of a guinea pig eye. The
development of keratoconjunctivitis by 24 hours postinoculation
was indicative of a positive test for invasiveness.

Serum resistance—A variation of the Eng procedure\(^{16}\) for the
screening for serum resistant strains was conducted using whey,
calf, pooled cow, human and guinea pig sera. The sera were used
undiluted and kept frozen at -70 C. The \textit{E coli} isolates were
inoculated onto trypticase soy agar\(^{a}\) (TSA) slants and incubated
overnight at 37 C. A bacterial suspension equivalent to McFarland
tube #3 was prepared from agar slant growth using phosphate
buffered saline (PBS) (pH 7.2). Twenty-five hundredths ml of the
suspension was mixed with 5 ml of melted and cooled TSA and 2 ml
were poured into 60 x 15 mm petri plates.\(^{b}\) Once solidified,
small holes measuring 2.7 mm in diameter were made in the agar and
4 \(\mu l\) of each serum were dispensed into individual wells. Plates
were kept at 4 C for 4 hours and then incubated at 37 C overnight.
Serum sensitivity or serum resistance was determined by the diameter
of the zone of growth inhibition (if any) around each well.

Mannose-resistant and mannose-sensitive hemagglutination (MR-HA,
MS-HA)—Rabbit, sheep and guinea pig red blood cells (RBC) were

\(^{a}\)Baltimore Biological Laboratory, Inc, Cockeysville, MD.

\(^{b}\)Falcon Becton Dickison and Co, Oxnard, CA.
separated from freshly collected blood as specified by Varga and Pesti. Three percent (v/v) RBC suspension in PBS with and without 1% D-mannose was prepared. Hemagglutination tests were performed over a bed of crushed ice and in duplicate for each species of red blood cell. Multi-well plastic plates (1 x 0.07 cm) as described by Varga and Pesti were used. All the mastitis isolates were cultured in TSB overnight and transferred to 0.5 ml E broth enriched with 1% minimal essential medium vitamins for four hours at 37 C. Enriched E medium growth was used to inoculate Minca Isovitalex agar and broth without glucose; heart infusion agar (HIA) and broth (HIB) with 0.2 yeast extract, and TSA plates supplemented with 5% bovine blood. Twenty-four hour cultures from solid medium and 48 hour cultures grown in broth were used for HA tests. If a pellicle was not developed on the broth culture, transfers were made every 48 hours, up to six times. Dense bacterial suspensions from all the solid media were prepared in PBS. One drop of each suspension (broth or agar) was delivered into each of four wells. One drop of RBC with 1% mannose was added to each of two wells. The same procedure was followed with RBC in PBS (without mannose) in the other two wells.

^Difco Laboratories, Inc, Detroit, MI.

^Linbro/Titerek Flow Laboratories, Inc, McLean, VA.

^Baltimore Biological Laboratories, Inc, Cockeysville, MD.
Plates were incubated at 4 C overnight. Test results were read with the aid of a microtiter reader, as mannose-sensitive (hemagglutination only with RBC without mannose) or mannose-resistant (hemagglutination of RBC with and without mannose). Plates were again incubated at 37 C for 24 additional hours and elution of hemagglutinins were recorded.

\(^{a}\)Cooke Laboratory Products, Alexandria, VA.
RESULTS

The results of the search for some pathogenic mechanisms and virulence factors among 184 *E coli* recovered from mastitis cases are shown in Table 2. Of all the isolates examined, only one strain exhibited ST activity in the infant mouse test. The control mice, inoculated with a strain positive for ST, showed the expected number of diarrheal spots as specified in the assay procedures. In order to preclude any false positive or false negative results, a known negative control strain was included in the test. Three mice were inoculated with a nonenterotoxigenic *E coli*. None of these mice exhibited any type of reaction to the culture filtrate. Two isolates were positive for LT activity as determined by the Y-1 adrenal tumor cell bioassay. The toxin preparation from cultures C-639 and C-636 were titrated and tested for heat lability and antibody neutralizability. The latter two criteria are used to identify heat-labile toxin. Toxin titers expressed as the reciprocal of the highest dilution showing LT activity were 16 and 4, respectively. Both toxins were inactivated after heating at 100 C for 15 minutes, but none were neutralized by the antisera prepared in pigs with strain 263 or with normal pig serum.

None of the cultures submitted to the Sereny test caused any type of lesion in the eyes of the guinea pigs, whereas the control
Table 2—Results of the search for selected virulence factors among 184 Escherichia coli strains isolated from acute cases of bovine mastitis

<table>
<thead>
<tr>
<th></th>
<th># positive results</th>
<th># negative results</th>
<th>% positive results</th>
<th>serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><em>ST</em> in vitro production</em>*</td>
<td>1</td>
<td>183</td>
<td>0.5</td>
<td>0151:K17:H-</td>
</tr>
<tr>
<td><em><em>LT</em> in vitro production</em>*</td>
<td>2</td>
<td>182</td>
<td>1.0</td>
<td>0138:K:H8</td>
</tr>
<tr>
<td>Invasiveness</td>
<td>0</td>
<td>184</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Serum resistance</td>
<td>183</td>
<td>1†</td>
<td>95.5</td>
<td>0154:K*:NM</td>
</tr>
</tbody>
</table>

*ST = heat-stable toxin; LT = heat-labile toxin.

†Weak reaction.
strains induced acute keratoconjunctivitis. One *E. coli* isolate was weakly susceptible to calf, pooled cow, human and guinea pig sera. All the mastitis *E. coli* were resistant to milk whey. The control strain (Eng) was inhibited by guinea pig serum, but was resistant to human and the remaining animal sera.

One hundred thirty-eight strains grown on agar agglutinated one or more species of the RBC used in this study. Likewise, 114 isolates grown in broth hemagglutinated (data not shown). Twenty-six cultures failed to agglutinate any of the species of RBC even after six broth transfers. Table 3 shows the results of HA of rabbit, guinea pig, and sheep RBC by mastitis isolates grown in different media. Minca agar and HI agar do not show any significant differences in regard to the expression and detection of MS hemagglutinins by any species of RBC. However, HI agar appeared to be a better medium for the development of MR hemagglutinins. Although strains grown in HIB exhibited more MS-HA than strains grown in Minca broth, the differences observed are minimal. Of all the media used, blood agar seemed to be the least suitable for detection of hemagglutinins from mastitis isolates. Several mastitis isolates showed MS (34%) or MR (9%) hemagglutination only; 42% of the strains exhibited both types. Table 4 summarizes the distribution of the hemagglutinins in relation to media and species of RBCs used. Approximately equal numbers of MS and MR hemagglutinating cultures were recorded when
TABLE 3--Summary of hemagglutination reactions* of 184† *Escherichia coli* recovered from acute cases of bovine mastitis

<table>
<thead>
<tr>
<th>Medium</th>
<th>Species of RBC</th>
<th></th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbit</td>
<td>Guinea pig</td>
<td>Sheep</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>MS†</td>
<td>36</td>
<td>60</td>
<td>3</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>MR†</td>
<td>54</td>
<td>15</td>
<td>5</td>
<td>64</td>
</tr>
<tr>
<td>Minca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broth</td>
<td>MS</td>
<td>14</td>
<td>55</td>
<td>2</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>MR</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Heart Infusion</td>
<td>Agar</td>
<td>MS</td>
<td>33</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>MR</td>
<td>50</td>
<td>33</td>
<td>22</td>
<td>105</td>
</tr>
<tr>
<td>Heart Infusion</td>
<td>Broth</td>
<td>MS</td>
<td>2</td>
<td>76</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>MR</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Blood</td>
<td>Agar</td>
<td>MS</td>
<td>9</td>
<td>29</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>MR</td>
<td>22</td>
<td>1</td>
<td>2</td>
<td>25</td>
</tr>
</tbody>
</table>

*Data expressed in number of isolates exhibiting hemagglutination.

†Some strains were positive in more than one category.

‡MS = Mannose-sensitive hemagglutination; MR = Mannose-resistant hemagglutination.
TABLE 4—Distribution of hemagglutinating patterns* among 184 Escherichia coli recovered from acute cases of bovine mastitis

<table>
<thead>
<tr>
<th>Hemagglutination of</th>
<th>Medium</th>
<th>MS-HA†</th>
<th>MR-HA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit RBC†</td>
<td>Agar or broth</td>
<td>36</td>
<td>54</td>
</tr>
<tr>
<td>Guinea pig RBC</td>
<td>Agar or broth</td>
<td>76</td>
<td>33</td>
</tr>
<tr>
<td>Sheep RBC</td>
<td>Agar or broth</td>
<td>5</td>
<td>22</td>
</tr>
</tbody>
</table>

*Data expressed as the largest number of isolates exhibiting hemagglutination.

†MS-HA = Mannose-sensitive hemagglutination; MR-HA = Mannose-resistant hemagglutination; RBC = red blood cells.
grown on agar. However, broth appeared to be a superior medium for
detecting MS adhesins but an inhibitor of MR hemagglutinins.
Rabbit RBCs were better indicators of MR-HA. Likewise, guinea
pig RBCs were better for detection of MS adhesins. Sheep RBCs
were rarely agglutinated, but in those instances of HA, the
hemagglutinins were also detected by rabbit and/or guinea pig RBCs.

The most common pattern of MS-HA was observed in guinea pig
RBCs by strains grown in broth. Conversely, MR-HA was more
frequently detected with agar grown strains in rabbit RBCs.

Results of the elution tests indicate that in the majority of
the cases MS-HA was eluted. Eluting and noneluting hemagglutinins
were found among the MR hemagglutinating strains. The elution
patterns were found not to be consistent with the three different
species of RBCs used nor with the media employed (data not shown).
Resistance to calf, pooled cow, human, and guinea pig sera, and milk whey was the only common characteristic present in all the *E. coli* isolates from mastitis which could be related to virulence. This finding is in agreement with the results reported by Carroll et al.\(^{12}\) in which the majority of the *E. coli* isolated from mastitis were serum resistant (SR). They postulated that the capability of *E. coli* to produce mastitis was related to serum resistance.

The ability to evade the bactericidal activity of serum has long been recognized as an important virulence attribute of *E. coli*, and one which contributes to their pathogenicity.\(^{10}\) It has been reported that serum resistance is multifactorial and that it results from the accumulation of several components at or near the cell surface.\(^{19,20,21}\) The acidic polysaccharide capsule,\(^{10,22}\) the lipopolysaccharide O side chains,\(^{20}\) and plasmids\(^{10,21,23}\) among others, have been related to serum resistance. Usually, nonpathogenic, noninvasive strains of *E. coli* are serum susceptible (SS), while invasive or pathogenic strains which come in contact with serum are SR.\(^{23}\) The fact that SS strains rarely are isolated from bacteremia\(^{24}\) and mastitis\(^{25}\) confirms the important role of the serum in limiting the number of microorganisms invading the blood vascular system and the mammary gland, respectively. Considerable research
has been conducted concerning the basis of SR among invasive and urinary tract isolates. However, very little has been published about this phenomenon in mastitis strains. Ward and Sebunya reported that resistance of \textit{E coli} in mastitis cases was greatest in strains possessing large amounts of the heat-labile capsule.

It must be noted that mastitis strains benefit from SR as a virulence factor in a limited way. While SS strains do not seem to survive within the mammary gland, SR strains grow rapidly at the beginning of an infection. As a result of histopathological changes in the udder, diapedesis of polymorphonuclear cells (PMNs) and perfusion of serum components are observed. However, clearance of the infection will likely follow by phagocytosis. The ability of \textit{E coli} to survive in serum is of little value in the presence of PMNs.

Invasion of the tissues and elaboration of toxins are the two main pathogenic mechanisms by which \textit{E coli} produces disease. The Sereny test for invasiveness has been correlated with the ability to invade the intestinal epithelium. The fact that \textit{E coli} isolates from mastitis did not penetrate the epithelial cells of the conjunctiva of guinea pigs suggested that mastitis cultures are not invasive. This observation agrees with Frost et al who reported histopathological findings following \textit{E coli} infection of the mammary gland in which there was no indication of attachment of bacteria to the surface epithelium. The lesions were confined to
the surface of the collecting ducts, without involvement of the secretory tissue. Blood et al.\(^2^8\) also described *E. coli* as a non-invasive microorganism. Both authors give support to their statement by citing the rapid clinical resolution of the infection and restoration of infected glands to normal levels of milk production. In addition, our previous serologic characterization (Section I) showed that none of the serotypes commonly associated with invasiveness were among our mastitis strains. This fact correlates with the observed difference between the biochemical reactions of the invasive and mastitis groups. Furthermore, the clinical signs of coliform mastitis do not suggest that bacteremia is part of the syndrome.

The guinea pig eye test has also been used to distinguish between virulent and avirulent gram-negative bacteria.\(^2^9\) Based on our negative results, the mastitis isolates could be regarded as lacking virulence also.

The contribution of endotoxin to the pathogenesis of mastitis was documented by Schalm and Ziv-Silberman;\(^1^1\) and recently, Brooker et al.\(^3^0\) postulated an additional cytotoxin, a heat-labile toxin, as the cause of lesions in the surface epithelium. However, these investigators did not determine the elaboration of ancillary toxins such as enterotoxins from mastitis strains. Our results from the infant mouse test and the Y-1 adrenal tumor cell bioassay did not
indicate any significant number of either heat-stable or heat-labile enterotoxin producers among the isolates. Apparently, enterotoxin production is not involved in the pathogenesis of mastitis. Other types of labile toxins such as Vero toxin and whole-cell-lysate toxin have not been reported from mastitis strains. Their mechanisms of pathogenicity are not clear but are probably different from the conventional LT which is related to increased levels of adenyl cyclase and alteration of mucosal electrolyte transport.

Enterotoxins and several of the colonization factors (pili) are encoded by plasmids. These are properties that usually occur together in a bacterial strain. Between 76 and 95% of the enterotoxigenic E. coli and between 0 and 14% of the nonenterotoxigenic strains from calves and lambs do possess the genetic determinant for the pilus K99. None of our isolates that were positive for K99 exhibited enterotoxic activity and conversely, none of the cultures showing enterotoxic activity were positive for K99 pilus. The absence of relationship between them suggests that this is not a pathogenic mechanism of mastitis isolates, as it is in enteric strains.

The majority of the mastitis strains were found to possess agglutinating activity against various species of RBC. A great diversity of HA patterns emerged as a result of the use of different RBCs, culture media and saline solutions with and without D-mannose.
Hemagglutinating activity has been associated with adherence to epithelial surfaces and attachment to host tissue has been determined to be an important virulence factor of many strains of *E. coli*. However, since there is no evidence to indicate that epithelial adherence is a feature of coliform mastitis, the significance of HA in mastitis isolates cannot be evaluated. Frost et al. could not demonstrate adherence of *E. coli* to mammary epithelium during experimental infection. Likewise, Anderson et al. were unable to show *E. coli* attachment to the mammary gland in their mouse model. Therefore, in spite of the possession of hemagglutinins which mediate adhesion to RBC, there is no evidence that mastitis strains attach to the mammary epithelium.

Adhesion to mannose-containing receptors has generally been regarded as the means of attachment of commensal flora, whereas attachment to cell receptors containing sugars other than mannose has been typical of pathogenic strains. However, the finding of a dual pattern of HA or mechanism of adherence (MS and MR) in mastitis cultures is not a unique property nor characteristic of mastitis strains. This observation has also been recorded from urinary strains by several authors and it has been speculated that both types of hemagglutinins mediate adhesion to different receptors and participate in pathogenesis.
A strong relationship exists between virulence of urinary and enterotoxigenic *E. coli* and the presence of MR hemagglutinins. The presence of these adhesins seems to be important for colonization of the mucosa and the establishment of an infection. Contrary to enteric strains from animals and humans, mastitis isolates possess several different hemagglutinins as shown by the heterogeneity in their HA patterns. Several authors have reported more than one kind of MR hemagglutinin in a given strain. Our elution data lend support to this finding since elution, a common characteristic of MR hemagglutins, was not consistent in the three species of RBC used. It is possible that *E. coli* strains from mastitis possess different kinds of MR hemagglutinins; or that these hemagglutinins are not in the form of pili; or that varying degrees of affinity exist between the cell receptors and the bacterial surface.

Mannose-sensitive adhesins are found in 50 to 70% of all *E. coli* strains. Seventy-six percent of our isolates showed this type of HA, either alone or in combination which is in agreement with Kuch et al. and Fein who found a similar incidence among urinary strains. The MS adhesins can be present on the bacterial surface as cell wall structures tightly attached to the outer membrane or as pili. When pili are detected by HA of guinea pig-RBC and their biological activity is inhibited by mannose, they are referred as type 1 pili or P1.
Our data indicate that many mastitis isolates exhibit a similar pattern of HA suggesting the presence of PI. The phenotypic expression of this pilus is markedly affected by the cultural conditions employed\textsuperscript{39,46} and production is largely suppressed by cultivation on solid media.\textsuperscript{36} However, the type of media used in this study did not affect the expression of MS adhesins to a great extent. Duguid et al,\textsuperscript{35} Clegg,\textsuperscript{45} and Fein\textsuperscript{46} were able to detect some MS-HA in different \textit{E coli} strains grown on agar. However, Clegg\textsuperscript{45} could not obtain MS-HA with urinary strains grown on solid media.

It is likely that some HA in our strains was not due to PI, but to cell wall adhesins. These adhesins have been shown to be present among urinary strains,\textsuperscript{40,46} and to have a biological activity similar to the pili. The evidence to suspect this kind of adhesive mechanism is provided by the elution data which show many instances of elution at 37 C. According to Old and Duguid,\textsuperscript{48} MS-HA does not elute at room or higher temperatures.

It is not clear whether PI can be related to virulence of \textit{E coli}. Conflicting reports exist regarding its role in urinary infections.\textsuperscript{36,38,49} As for the enteric strains, Awadh-Masalmeh et al\textsuperscript{50} could not demonstrate adhesion of diarrheic strains to the small intestine via PI. Although adherence by MS and MR hemagglutinins does not seem to be a requirement for colonization or for the
establishment of an udder infection, the role of PI should be carefully evaluated. Further research is needed to elucidate the part PI plays in the clearance of an \textit{E coli} infection after diapedesis of polymorphonuclear cells (PMN) into the mammary gland as suggested by PI capability to bind to PMNs and initiate phagocytosis.\textsuperscript{41}
REFERENCES


GENERAL SUMMARY

The examination of the biochemical test profile of the *Escherichia coli* isolates from bovine cases of mastitis did not reveal a usable marker which could differentiate mastitis pathogens from contaminating or nonpathogenic *E coli* strains. The mastitis cultures exhibited all the essential characteristics of typical *E coli* except for adonitol fermentation. This test seems to be of limited value in differentiating mastitis strains as a group since only 47% of the isolates were positive.

It was expected that the serotyping of the mastitis isolates would provide the necessary information to characterize the cultures. The results from serotyping confirms what others have reported: no definite O groups were associated with mastitis. Contrary to *E coli* found in some animal and human diseases, where a limited number of O groups are involved, mastitis strains showed a great diversity of serotypes. The present study supports the belief that mastitis organisms are acquired from the environment because of the similarities encountered between the serotypes of mastitis and environmental isolates. Furthermore, the diversity of serotypes is compatible with the idea that *E coli* mastitis strains are relatively not contagious between cows.

The problem encountered in typing accurately the majority of the K antigens offer some hope in the elucidation of pathogenicity. It
was not determined whether the untypable antigen(s) were one or several common or distinct antigens. Since there is a good correlation between some capsular types and virulence, it would be worth investigating. Capsules have been associated with antiphagocytic properties and serum resistance, yet a concentration of 500,000/ml PMN's of milk is capable of protecting the udder of an *E coli* infection regardless of the resistance or sensitivity of the *E coli* strain to serum. Any microorganism which is capable of inducing a severe and sometimes fatal infection must possess some pathogenic mechanism or virulence factors which mediate the disease process. Except for endotoxin, which has been reported to be responsible for many of the clinical signs of coliform mastitis, no other property has been associated with mastitis strains that could account for the severity of the disease. However, endotoxin effects are usually secondary in importance when other toxins such as enterotoxins are elaborated. The question whether *E coli* from mastitis possess ancillary toxins mediating inflammation arose. The results from our assay for ST and LT enterotoxins showed positive results with 1 isolate for ST; and 2 other cultures for LT. This fact indicates that enterotoxin production is not involved in the pathogenesis of *E coli* mastitis. The elaboration of enterotoxin is correlated with the presence of the K99 pilus. However, only two of our cultures were K99 positive and these strains were different
from those exhibiting ST and LT. Furthermore, very few of our cultures exhibited a pattern of HA characteristics of K99 pilus. The majority of the strains showed hemagglutination with at least one species of RBC, suggesting the presence of more than one type of hemagglutinin. In addition, HA patterns were diverse with little relationship among them. Since HA is associated with adherence and the present evidence of \textit{E. coli} in the mammary gland does not indicate attachment to the udder epithelium, the role of the hemagglutinins as a virulence factor does not seem to be important with the evidence available. The finding of MS hemagglutinins presumably in the form of pilus may offer some insight into the rapid phagocytosis of \textit{E. coli} in the mammary gland. Experimental evidence indicates that PI attaches to PMNs. If this event takes place in the udder, then the clearance of an infection after the inflammatory response may be facilitated.

The results of the characterization of the \textit{E. coli} from mastitis cases indicate that aside from the whey and serum resistance observed among all the isolates, no other virulence factors could be identified.
ADDITIONAL LITERATURE CITED


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