CHARACTERIZATION OF FECAL COLIFORM ISOLATES BY ELECTROPHORETIC ANALYSIS OF PILI

FINAL REPORT

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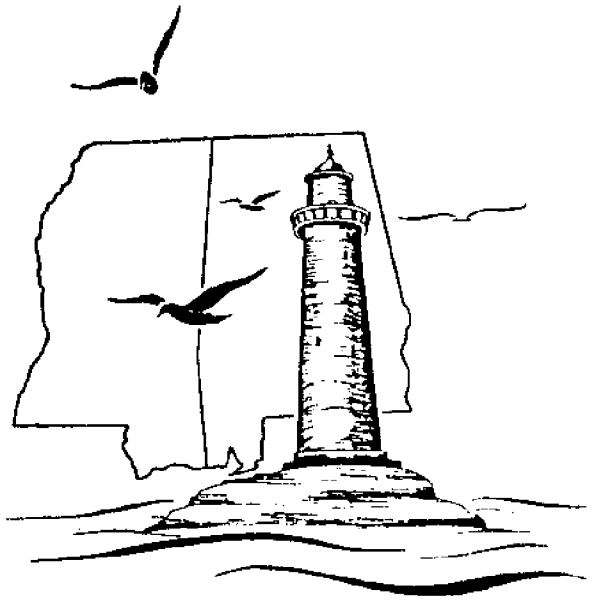

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Abstract

Escherichia coli bacteria isolated from the stool of normal, healthy individuals, a variety of other warm bloodied animals, and from the environment, were characterized according to a hemagglutination (HA) sequence and by electrophoresis of surface components. The hemagglutination method was rapid, inexpensive, and amendable to the analysis of many isolates. A total of 1280 isolates were analyzed by HA. The data revealed that *E. coli* strains exhibited a finite number (60 of 243) of HA combinations, but that isolates from different animals and environmental samples commonly produced the same HA pattern. The conclusion was that a more specific HA procedure, consisting of additional blood types, and analysis of non-finbriae associated HA, needed verification. Techniques used to isolate and purify pili were conducted on 112 isolates. The time required for bacterial growth and pili extraction were lengthy; only 4 isolates could be examined per week. Pili digests, separated by SDS-PAGE electrophoresis, demonstrated that isolates differed according to the number and the molecular weight of gel bands. Many protein bands outside the MW of pili were observed, indicating that the extraction procedure accumulated other contaminating surface proteins. As predicted by the HA method,

pili analysis will require a more detailed procedure to be able to differentiate between individual isolates.

Introduction

The term "fecal coliform" (FC) describes a subpopulation of Gram negative rods capable of growth in certain selective media, and at a temperature slightly higher (44.5) than body temperature. As a standard microbiological procedure, the fecal coliform test has been employed in a variety of ways and is often used to gauge the degree to which a sample is contaminated with human sewage or the excreta of other warm bloodied animals.

In this report, we do no take issue with the value of the test. Its stringent requirements have a proven history of public protection, and for this reason alone the test should be continued. However, as environmental sampling has increased in complexity and scope, the value of the test has been questioned. The test is often used to make delicate environmental distinctions, a task it was not designed to do. This ambiguity is perhaps best exemplified in numerous analyses of wastewater for enteric viruses; the fecal coliform index has never been shown to be able to indicate the presence of infectious virus in contaminated natural samples.

It is the specificity of the test which causes the greatest problems. The fact that the FC method represents more than one bacterial organism and is not able to distin-

guish the ultimate source of wastewater pollution is a significant drawback. A need for increased test specificity is obvious in many recent and current investigations. As a first step, several investigators are examining specific ways of detecting Escherichia coli in contaminated samples. In Texas, for example, Randell Nichelson is testing four procedures to quantitate E. coli in oyster tissue. Cameron Hackney and Marilyn Kilgen in Louisiana have found that when fecal coliform numbers rise in oysters during shipment, this does not imply a concomitant rise in the numbers of E. coli in the samples. Fred Howell and R.D. Ellender (Mississippi) have shown that fecal coliforms in storm drain waters flowing into the Mississippi Sound are not associated with human sewage pollution, but rather with surface runoff during periods of heavy rainfall. The persistence of these bacteria in coastal waters appears to result from a combination of factors including the lack of a flushing tidal effect, shallow depth, water temperature, low salinity, and organic content of the water. They have proposed that the levels of E. coli in storm drain waters may be a more appropriate indicator of the nature of the problem.

It was the latter study which prompted the testing described in this report. The two and a half year storm drain study used the FC standard procedure to attempt to specify the ultimate bacterial source in a closed watershed.

Additional test specificity was considered necessary to clarify the sources of FC contamination which contributed to the pollution levels observed.

Objectives

The original proposal described the use of pili profiles of fecal coliform isolates as a potential mechanism to designate a source of wastewater pollution. During the early investigations, a second method, hemagglutination (HA), was discussed with the Sea Grant authority, and included in the research phase. Hemagglutination was found to be a rapid and inexpensive means to characterize individual isolates; electrophoresis of pili proteins was also specific but was more expensive and time consuming. The pros and cons of each method are discussed in later sections.

Specific Objectives

To isolate fecal coliforms directly from the stool of humans and other animals and find a mechanism to rapidly designate an isolate as an E. coli.

To characterize E. coli isolates according to patterns of HA and purified pili analysis.

To determine if the characteristics of E. coli isolated from the same human or animal (or environmental sample) would vary temporaly and spatially.

To determine whether isolates collected according to the three standard methods of FC analysis share common characteristics.

Approach

1. Isolation of Fecal Coliforms

a. Fecal Samples: Samples were collected using sterile cotton swabs and in a manner which minimized environmental contamination. Samples were rapidly transferred to LTB broth and incubated at 35 C. Gas filled Durham tubes at 24 hr were considered positive and a portion of the culture was transferred via a sterile wooden applicator stick to a tube of EC broth. After 24 hr at 44.5 C , growth from positive tubes was transferred to 4-6 MacConkey agar plates. Individual colonies, typical of lactose (+) bacteria, were inoculated into 1% tryptone broth, and following 24 hr incubation at 35 C, were tested for the indole production.

This procedure is a variation of the method described in the original proposal. We found the modified method to be efficient, inexpensive, and capable of designating an isolate as an E. coli 95% of the time.

We could not isolate E. coli from certain humans; one child and one male adult were found to consistently yield Klebsiella sp. Other animals from which no E. coli could be isolated included dogs, rabbits and fish. This pattern persisted over several isolation attempts.

The isolation of E. coli from animal feces was often hampered by environmental contamination. In these

instances, large numbers of MacConkey colonies were tested to insure a minimum of 20 E. coli cultures for testing.

Certain animals, namely fish and oysters, were sacrificed to collect the fecal material and preculded replicate sample collection.

b. Environmental Samples: Grab samples were collected and processed as described above. Enterobacter and Klebsiella sp. (indole negative) were commonly encountered requiring the testing of up to 100 MacConkey isolates to find the required 20 specimens.

2. Storage of isolates:

Sample isolates were grown on fresh agar and colonies picked into a drop of fetal bovine serum containing 10% dimethylsulfoxide. The turbid mixture (approximately 0.1 ml) was drawn into sterile capillary tubes which were sealed by heat. Capillary tubes were placed in a 4 inch section of freezer tape and covered with a similar piece of tape. The tape was marked to designate the sample and stored at -70 C in an envelope.

3. Analysis of isolates:

Isolates to be subjected to HA or pili analysis were removed from the freezer and thawed rapidly. Cultures were transferred to CTA medium and grown at 35 C for 24 hr.

a. Hemagglutination: Bloods (red blood cells, rbc) to be used in this portion of the study were purchased (Flow

Laboratories, Rockville, Md.) diluted in Alsever's solution. Lots of African Green Monkey, Human type O, Bovine, Adult Chicken and Guinea Pig rbc's were requested when a sufficient number of bacteria were ready for analysis, usually 150-200. Cells for HA analysis were mixed as follows prior to isolate analysis: 1.8 ml of blood was drawn from each vial and mixed with 0.2 ml of a 3.8% citric acid solution prepared in distilled water; 1.0 ml each of the bloods was removed and placed into 5 tubes containing 4 ml of sterile PBS and a second group of 5 tubes containing 4 ml of PBS and 1% D-mannose. Red blood cells were used within the two week expiration period. It is assumed that these cells were not from the same animal (probably pools of rbc from several animals) and represent species specific rather than individual reactions. The presence of hemagglutination was indicated by rbc clumping in a two minute period at ambient temperature. Glass slides, used to perform HA testing, were washed in a lysol solution, 70% ethanol and distilled water, and dried prior to reuse. The collected data was compressed as shown in Figure 1.

b. Pili purification

Isolates were thawed and grown on CFA agar (1% casamino acids, 0.15% yeast extract, 0.05% $MgSO_4$, 2% Agar, pH 7.4) plates overnight at 35 C. The growth from these plates was

Figure 1

SAMPLE OF RESULTS : DATA PREPARATION : DOMINANT COMBINATIONS

ISOLATE #	NO MANNOSE AG	HO	BO	AC	GP	MANNOSE AG	HO	BO	AC	GP		AG	HO	BO	AC	GP		
1	+	+	+	+	+	-	-	+	+	+		S	S	R	R	R		
2	-	+	+	+	+	-	+	+	+	+		M	R	R	R	R		
3	-	-	-	+	-	-	-	-	+	-		M	M	M	R	M		
4	+	+	+	+	+	-	+	+	+	+		S	R	R	R	R		
5	+	+	+	+	+	+	+	+	+	+		R	R	R	R	R		
6	+	-	-	+	-	-	-	-	+	-		S	M	M	R	M		
7	-	-	-	+	-	-	-	-	+	-		M	M	M	R	M		
8	+	+	+	+	+	-	+	+	+	+		S	R	R	R	R	MMRRM	30%
9	+	+	+	+	+	+	+	+	+	+	+,-=S	R	R	R	R	R	SRRRR	25%
10	+	+	+	+	+	+	+	+	+	+	→+,+=R	R	R	R	R	R	RRRRR	25%
11	-	-	-	+	-	-	-	-	+	-	-,-=M	M	M	M	R	M	SSRRR	10%
12	-	-	-	+	-	-	-	-	+	-		M	M	M	R	M	MRRRR	5%
13	+	+	+	+	+	-	-	+	+	+		S	S	R	R	R	SMMRR	5%
14	+	+	+	+	+	-	+	+	+	+		S	R	R	R	R		
15	+	+	+	+	+	-	+	+	+	+		S	R	R	R	R		
16	-	-	-	+	-	-	-	-	+	-		M	M	M	R	M		
17	-	-	-	+	-	-	-	-	+	-		M	M	M	R	M		
18	+	+	+	+	+	+	+	+	+	+		R	R	R	R	R		
19	+	+	+	+	+	+	+	+	+	+		R	R	R	R	R		
20	+	-	-	+	+	-	-	-	+	+		S	M	M	R	R		

suspended in sterile PBS and the suspension used as inoculum for 10, 1000 cm^2 stainless steel pans containing CFA agar. The inoculum was spread over the entire surface of the agar using a sterile L-shaped glass rod. Incubation was at 35 C for 16-18 hr. Bacterial growth was scraped from the surface of the agar using the edge of a glass slide, and suspended in a minimum volume of ice cold Tris (hydroxymethyl aminomethane hydrochloride, pH 7.8) buffer. The slurry was homogenized in a cooled Waring blender for 5 minutes, then centrifuged at 10,000 x g for 30 min. The supernatant was removed and dialyzed against several changes of sodium acetate buffer (pH 4.2) using a minimum of 10:1, buffer to sample volume. Dialysis was performed at 4 C, and the buffer was magnetically stirred during this period. When dialysis was complete (usually at least 24 hr), the sample was removed from the tubing and centrifuged at 8,000 x g for 10 min. The supernatant was decanted and the pellet resuspended in Tris buffer (pH 7.8). Saturated ammonium sulfate was added to the precipitate, and the pili proteins collected by centrifuagation at 6,000 x g for 15 min. The supernatant was removed and the pellet resuspended in a small amount of Tris buffer, stored in small plastic vials, and frozen at -70 C for electrophoresis.

c. Control extractions:

To insure that no proteins in the CFA agar would con-

taminant pili extracts, the agar used for bacterial growth was subjected to the steps described in part b.

d. Electron microscopy:

Approximately seven pili extracts were examined by electron microscopy. Preparations contained hairlike filaments (Figure 3) characteristic of pili, although there appeared to be differences between preparations in the amount of pili protein found in a sample. One concentrate did not contain visible pili.

e. Electrophoresis:

A 1:2 dilution of a pili protein concentrate was prepared with buffer and the mixture heated at 100 C for 15 min. The solution was chilled, and 10 ul of bromphenol blue and 10 ul of 2-mercaptoethanol were added to 250 ul of the diluted sample. The mixture was either used immediately or stored at -70 C for later analysis. From the frozen condition, the mixture was briefly heated at 100 C to insure that all proteins were dissolved.

Protein separations were conducted using LKB instrumentation and reagents (see Appendix). Type 1 water (reverse osmosis purified) was used to prepare all reagents.

Slab gels of 5% polyacrylamide with 20 and 10 ul wells were used. A minimum of 2 wells were used for each sample. Molecular weight markers were also separated with each gel (range 12,300-78,000). Slabs for SDS Page analysis were

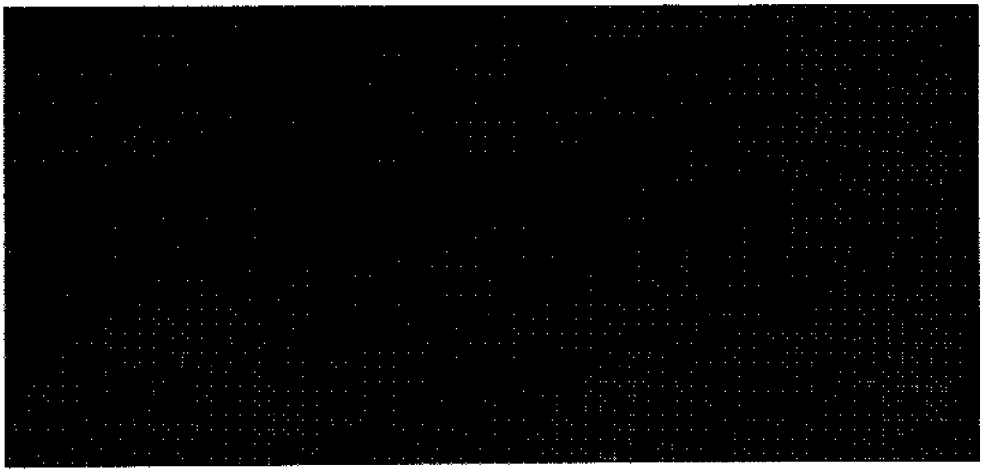
made 12 hrs before use. Preparation of the gels involved the mixing of proper quantities of phosphate buffer, acrylamide solution (containing acrylamide and Bis), ammonium persulfate and Temed (catalysts). The solution, with the exception of catalyst, was mixed in a vacuum flask and evacuated to remove air which retards polymerization. The catalysts were added, and the mixture used immediately to fill the moulding set. Polymerization occurred within 40 min. In an actual run, the Multiphor unit was filled with buffer (1.2L), the gel laid onto the cooling plate and wicks applied on each side of the gel, overlapping by 10-12 mm. For best results, wicks had to be parellel. Ice water was flowed through the cooling plate to avoid overheating of the gel during a run. The anticondensation lid was put in place and a pre-electrophoresis run was conducted at 150 mA for 30 min. This step removed any protein or impurities on the slab. The samples, containing bromphenol blue as tracking dye, were applied quickly to avoid diffusion. The power was set at 20 mA for 10 min to concentrate all proteins at the anode side of the well. The current was increased to 200 mA (constant current) for the remainder of the run (about 4 hr). The gels were immediately placed in fixing solution (1 hr) then stained for 2 hr. Destaining was performed until the gel background was clear. This required several changes of destaining solution over a period of a day or two. Gels

were preserved for 1 hr, wrapped in cellophane sheets and pressed until dry. Figure 2 shows the type of results obtained from electrophoresis of samples.

The molecular weight of each ban was calculated from a standard curve prepared by plotting the relative movement of the MW markers against the $\log_{10}$ MW of each marker.

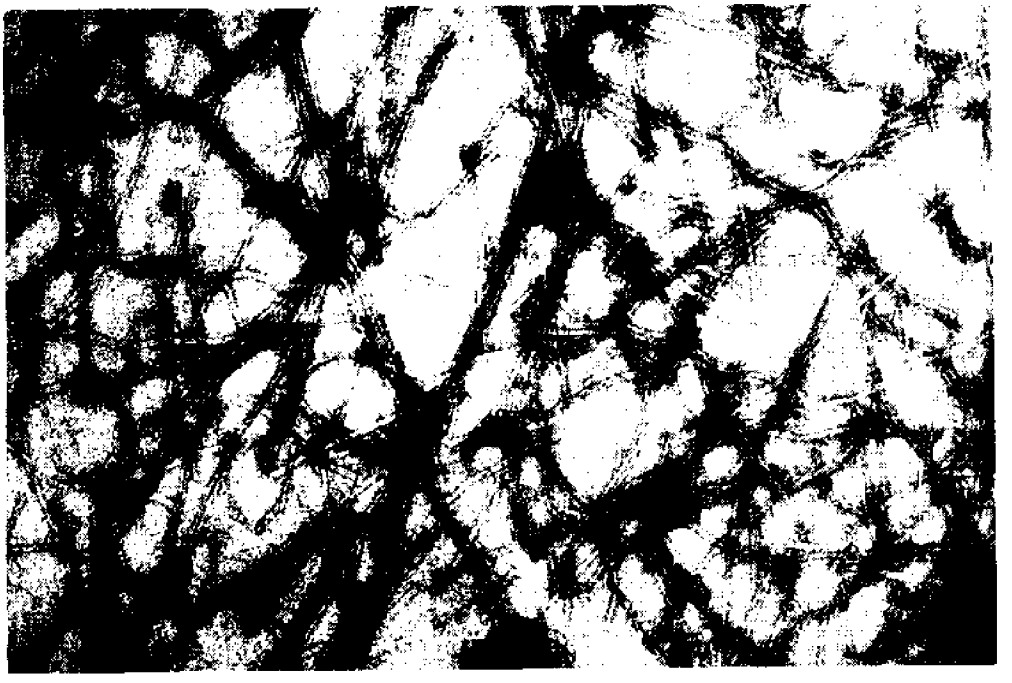
Figure 2. Representative electrophoretic patterns

Gel 35: lanes a&b marker; lanes c-l, horse #1 isolates; lanes m-t, fish isolates



Gel 28: lanes a&b marker; lanes c-l, cow #2 isolates; lanes m-t, horse # 3 isolates

Figure 3. Purified pili preparation, x 47000: Individual hair-like, hollow structures represent pili filaments prior to SDS digestion.



Results

1. Hemagglutination:

Table 1 summarizes the results of seven humans sampled on three separate dates. Other humans are included in later summary tables, but it was difficult to obtain replicate samples from them. The HA combinations shown for each date indicate the patterns which exhibited the greatest dominance followed by the percentage of that combination of the 20 isolates examined. Each combination (for ex. RMMSS) represents the summary of one or more bacteria producting a specific set of HA reactions. For example, the 20 E. coli taken from Human #1 (10-11-82) produced the following group of combinations: RMMRM (50), RMMRS (30), SMMRS (15), and RMSRS (5). In other words, 10 of the isolates produced a combination of RMMRM, 6 produced a combination of RMMRS, and so on.

There is a great amount of disparity in the results, even between samples taken from the same human on two consecutive days. However, there are also data from certain humans (i.e. #8) which show that a particular combination is found consistently, even in samples taken several months apart. These results suggest that factors such as diet, physiological conditions such as gastric acidity or degree of peristalsis can stimulate changes in the HA reactions

Table 1, page 1: HUMAN HA COMBINATIONS
-BY DATE-

HUMAN 1:	9-20-82	RMMSS (30)	RMRSS (30)	RSMSS (25)	RSRSS (5)
		RMMMM (5)	SSMSS (5)		
	9-21-82	RMRRS (45)	RMMRM (10)	RMRRM (10)	RMMRS (10)
		RMMSS (5)	SMMSS (5)	SMMRM (5)	SMRSS (5)
		SMMMM (5)			
	10-11-82	RMMRM (50)	RMMRS (30)	SMMRS (15)	RMSRS (5)
HUMAN 3:	10-19-82	RSMSS (55)	RMMSS (35)	RMMMS (5)	MMMMS (5)
	5-31-83	RRMRR (85)	RMMRR (15)		
	6-26-82	RMMSS (70)	RSMSS (15)	RSMSM (5)	RRMSM (5)
		RRMSS (5)			
HUMAN 4:	6-28-83	SSMSS (100)			
	7-29-83	SSMSS (80)	RSMSS (15)	SMMSS (5)	
	8-09-83	SMMSS (65)	SSMSS (25)	MMMSS (10)	
HUMAN 6:	5-31-83	RMMRR (95)	SMMRR (5)		
	7-11-83	MMMMM (100)			
	7-18-83	MMMMM (85)	SMMMS (10)	SSMSS (5)	
HUMAN 7:	7-13-83	MMMSS (45)	MMMMS (20)	MMMSM (20)	SMMSS (10)
		MSMSS (5)			
	9-14-83	SMMSS (65)	RMMSS (20)	MMMSS (10)	SSMSS (5)
	11-29-83	SMMSS (35)	SSMSS (25)	MMMSS (20)	MMMMM (5)
		SSMSM (5)	SMMMS (5)	MMMMS (5)	

Table 1, page 2: HUMAN HA COMBINATIONS
-BY DATE-

HUMAN 8:	7-05-83	RRMMM (85)	MRMMM (10)	SRMMM (5)	
	10-04-83	RRMMM (85)	SSMSS (10)	MRMMM (5)	
	11-21-83	RRMMM (90)	SRMMM (10)		
HUMAN 10:	7-13-83	MMMM (30) MMMSS (10) MSMSS (5)	RSMSS (15) SMMSS (10)	MMMS (10) MMMSM (5)	MSMRM (10) SSMRS (5)
	9-14-83	RRMMM (25) RRMMS (5)	RRMMR (25) MMMM (5)	RRMSR (20) RRMSM (5)	RRMSS (15)
	11-29-83	MRMMM (55) MSMMM (5)	MMMM (15)	SRMMM (15)	RRMMM (10)

types found in individual samples.

Table 2 is a summary of all data collected from humans. Examination of the results shows that certain combinations are more frequently encountered, but not to such an extent that any one combination could be designated as truly dominant. The five combinations which represent 50% or more of all isolates demonstrate the diverse nature of HA responses. This variation and the degree to which the data must be examined is exemplified in the status of combination RRMMM. This combination was found in 54 of 420 isolates (13%), and was the combination most often found in human isolates. A close inspection of the data, however, demonstrates that this combination was associated with 52 isolates taken from human #8 and 2 isolates from human #10. By contrast, the two combinations following RRMMM in prevalence (MMMM and SSMSS) were found in several humans (3 humans ((#'s 6,7,10)) and 5 humans ((#'s 1,4,6,7,8)), respectively).

HA combinations representing 3 temporal and spacial trials were also collected for two cats, one horse, one dog and two cows (Table 3). The combinations observed on a certain date were not usually consistent with those collected on a separate date. For example, cat #1 retained only one of 5 combinations observed on two sampling dates 7 days apart (7-05-83, 7-12-83). However, six months later, a

Table 2. DATA SUMMARY FOR ALL HUMANS

	#	%
RRMMM	54	13
RSMSS	25	6
RMMRR	22	5
RMMSS	21	5
RRMRR	17	4
RMMRM	12	3
RMMSS	11	3
RMRRS	9	2
RMMRS	8	2
RMRSS	6	1
RRMMR	5	1
RRMSS	4	<1
RRMSR	4	<1
RRMSM	2	<1
RMRRM	2	<1
RRMMS	1	<1
RMMMS	1	<1
RSMSM	1	<1
RSRSS	1	<1
RMMMM	1	<1
RMSRS	1	<1

	#	%
MMMMM	48	11
MMMSS	19	5
MRMMM	14	3
MMMMS	8	2
MMMSM	5	1
MSMRM	2	<1
MSMSS	2	<1
MSMMM	1	<1

	#	%
SSMSS	46	11
SMMSS	44	10
SRMMM	6	1
SMMMS	3	<1
SMMRS	3	<1
SSMSM	1	<1
SSMRS	1	<1
SMMRR	1	<1
SMMRM	1	<1
SMRSS	1	<1
SMMMM	1	<1

*TOTAL OF 40 COMBINATIONS FOUND IN ALL HUMANS
*420 ISOLATES EXAMINED

COMBINATIONS REPRESENTING =>50% OF TOTAL
RRMMM 13%
MMMMM 11%
SSMSS 11%
SMMSS 10%
RMMRR 5%

Table 3, page 1:

HA COMBINATIONS -3 TRIALS / ANIMAL-

-BY DATE-

CAT 1	7-05-83	SSMSS (45)	RSMSS (35)	RMMSS (10)	SMMSS (10)
	7-12-83	RRMSS (75)	RRMSR (10)	RSMSS (10)	SRMSS (5)
	11-21-83	RRMSS (70) SRMSS (5)	RSMSS (15)	RMMSS (5)	SMMSS (5)
CAT 2	8-05-83	SSMSR (70) MMMM (5)	SMMSS (10)	SSMSS (10)	SSMSM (5)
	8-15-83	SSMSS (60)	SMMSS (30)	MMMSS (5)	MMMM (5)
	9-01-83	MMMRM (50)	MMMSM (30)	MMMM (20)	
HORSE 1	9-20-82	MMMSM (50)	MMMM (45)	MMMSS (5)	
	9-21-82	MMMM (70)	SSMSS (20)	SMMM (10)	
	11-14-82	MMMSM (30) SMMSS (10)	MMMSS (25)	MMMM (20)	MMMMS (15)

Table 3, page 2:
HA COMBINATIONS -3 TRIALS / ANIMAL

-BY DATE-

DOG 1	9-20-82	MMMM (100)				
	7-24-83	SSMSS (100)				
	8-28-83	MMMM (70)	SSMSS (30)			
COW 1	7-24-83	SSMSS (70)	MMMM (10)	SMMSM (10)	SSMRS (5)	SMMMS (5)
	8-27-83	MMRSS (70)	MMRMS (20)	MMRSM (10)		
	8-28-83	MMRSS (65)	MMRMS (20)	MMRSM (15)		
COW 2	11-14-82	MMMM (95)	MMMSM (2)			
	7-24-83	SSMSS (90)	SSSSR (10)			
	8-14-83	SSMSS (60)	SMMSS (40)			

sample from the same animal contained four combinations that had been observed earlier. Cat #1 contained a total of 7 separate combinations (all dates); cat #2 contained a total of 8 separate combinations (all dates). Of the 15 combinations, 13 did not match (86%), representing only a 14% agreement between the two animals.

A total of seven different combinations were found for E. coli isolated from horse #1 on three trial dates. Samples taken one day apart contained only one identical combination (MMMM). Whether these results represent distinct differences in E. coli isolates or differences in the HA procedure are not clear at this time.

On occasion, certain samples yielded only one HA pattern (dog #1; Table 3). All bacteria tested gave a MMMM pattern on the first date of sampling, but a modified profile (SSMSS) was observed nine months later. One month after the second sample, a third sample yielded a population with a mixture of the previous 2 patterns.

The above results demonstrate that certain animals display similar HA variation expressed earlier for humans, and produce many of the same HA patterns. There are significant HA differences between temporal and spacial responses that occur in the same animal, and together with the results obtained from humans, they appear to preclude the use of this HA methodology as a means of species different-

iation within environmental samples.

Table 4 summarizes all HA combinations from all animals. The first two pages of the table contain the results discussed previously and include the data obtained from animals from which only one, or perhaps 2 samples could be obtained (human 9, horse 1, horse 3, horse 4, dogs 2&3, and cat 1). These data also appear to contain inter- and intraspecies variation and on occasion, for example from horse #1, yield HA patterns not found in other animals.

The third page of Table 4 summarizes the results obtained from other animals and environmental samples. These animals were difficult to sample (with the exception of the goat), and certain of the animals had to be sacrificed to obtain a suitable fecal sample. A significant amount of background contamination was found in these samples, resulting in the elimination of many samples containing few or no E. coli. The water samples collected were likewise difficult to analyze due to high levels of non-E. coli growth in the media employed. Several combinations, (i.e. MMMM, RMMRR, SSMSS, MMMSM and others) found in the higher animals, were also noted in the animals included in Table 3, page 3. Certain combinations (SSSSS and RRRRR) found in this grouping of animals, were not found in any of the other samples tested, or were associated with only one additional species.

Table 4, page 1:

SUMMARY

ALL ANIMALS - ALL COMBINATIONS

ANIMAL:	TEMPORAL REPLICATE	# COMBINATIONS	DOMINANT COMBINATIONS(S)
HUMAN 1	1	6	RMMSS (30) RMRSS (30) RSMSS (25)
	2	9	RMRRS (45)
	3	4	RMMRM (50)
HUMAN 3	1	4	RSMSS (55) RMMSS (35)
	2	2	RRMRR (85) RMMRR (15)
	3	5	RMMSS (70)
HUMAN 4	1	1	SSMSS (100)
	2	3	SSMSS (80)
	3	3	SMMSS (65) SSMSS (25)
HUMAN 6	1	2	RMMRR (95)
	2	1	MMMM (100)
	3	3	MMMM (85)
HUMAN 7	1	5	MMMSS (45) MMMMS (20) MMMSM (20)
	2	4	SMMSS (65) RMMSS (20)
	3	7	SMMSS (35) SSMSS (25) MMMSS (20)
HUMAN 8	1	3	RRMMM (85)
	2	3	RRMMM (85)
	3	2	RRMMM (90)
HUMAN 9	1	1	SRMMM (100)
	2	2	SSMSS (75) SMMSS (25)
HUMAN 10	1	9	MMMM (30) RSMSS (15)
	2	7	RRMMM (25) RRMMR (25)
	3	5	MRMMM (55) MMMM (15)

Table 4, page 2:

SUMMARY

ALL ANIMALS - ALL COMBINATIONS CONTINUED

ANIMAL:	TEMPORAL REPLICATE	# COMBINATIONS	DOMINANT COMBINATIONS(S)
HORSE 1	1	4	SMMRS (80)
HORSE 2	1	3	MMMSM (50) MMMM (45)
	2	3	MMMM (70)
	3	5	MMMSM (30) MMMSS (25) MMMM (20)
HORSE 3	1	2	MMMM (80) MMMS (20)
	2	2	SSMSS (75) SMMSS (25)
HORSE 4	1	5	SMMSS (40) MMMS (35)
	2	4	SMMSS (80)
COW 1	1	5	SSMSS (70)
	2	3	MMRSS (70) MMRMS (20)
	3	3	MMRSS (65) MMRMS (20)
COW 2	1	2	MMMM (95) MMMSM (5)
	2	2	SSMSS (90) SSSSR (10)
	3	2	SSMSS (60) SMMSS (40)
DOG 1	1	1	MMMM (100)
	2	1	SSMSS (100)
	3	2	MMMM (70) SSMSS (30)
DOG 2	1	1	RSMSR (100)
DOG 3	1	4	SMMSS (85) SSMSS (5) MMMSS (5) SMMSM (5)
CAT 1	1	4	SSMSS (45) RSMSS (35)
	2	4	RRMSS (75)
	3	5	RRMSS (70)
CAT 2	1	5	SSMSR (70)
	2	4	SSMSS (60) SMMSS (30)
	3	3	MMRM (50) MMMSM (30) MMMM (20)
CAT 3	1	2	MMMM (60) MMMSM (40)

Environmental water samples collected from the Courthouse Rd storm drain system exit into the Mississippi Sound; isolates from the 16th street headwall (which leads to the Courthouse Rd exit), and the Ocean Springs small craft harbor were also analyzed by the HA procedure (Table 4, page 3). A degree of HA homology could have been expected between the 16th street and Courthouse Rd samples of 6-28-83. These sampling points are related; water flowing past the 16th street station is known to exit the Courthouse Rd. drain. However, only two of the nine combinations detected were identical (SMMSS and SSSSS). Two hundred yards separate these two sampling points, and the intrusion of other water is a definite possibility, along with bacterial contaminants from animal or plant life within the wooded part of the system. However, in view of the HA variation observed among the animals tested, it is as likely that the number of isolates tested was not compatable with the degree of significance desired.

Two additional analyses were conducted using the HA method of comparison. First, mice, housed in a closed environment were tested on 3 separate occasions (Table 5). Little similarily is seen in isolates collected 5 months apart (M1 and M2), but there exists a definite similarily between mice (M3 and M4) sampled on the same day. This could be a result of these mice being subjected to the same

Table 4, page 3: SUMMARY

ADDITIONAL ANIMALS AND ENVIRONMENTAL SAMPLES

ANIMAL:	DATE	# COMBINATIONS	COMBINATION(S)
DOVE	3-07-83	4	SSMRS (55) SMMRS (35) MMMRS (5) MSMRS (5)
DOVE	3-07-83	4	SSSRS (55) SSMRS (25) SMMRS (15) SMSRS (5)
CHICKEN	9-21-82	6	MMMM (60) SMMSS (15) SMMM (5) SMMMS (5) SSMSS (10) SSSSS (5)
CROAKER	6-28-83	3	MMMM (80) MMMS (15) MMMR (5)
CROAKER	6-28-83	2	MMMM (60) MMMSM (40)
CATFISH	6-28-83	4	SSMRR (70) SMMRR (20) SMMRM (5) SSMRS (5)
CATFISH	6-28-83	2	SSMRR (90) SMMRR (10)
MULLET	7-15-83	2	RRRRR (65) SSMSS (35)
GOAT	5-24-83	3	RMMRS (60) SMMRS (30) RMMRR (10)
OYSTER	6-28-83	6	SRMRR (65) SSMRR (10) SRMRS (10) MSMRR (5) MRMRR (5) SMMRR (5)

ENVIRONMENTAL WATER SAMPLES

COURTHOUSE	6-28-83	5	SMMSS (40) MMMSS (30) SSSSS (15) MMMM (10) MMMS (5)
COURTHOUSE	7-15-83	8	SSMSS (35) SMMSS (20) SMMSR (15) SSMSR (10) RSMSR (5) SSMRS (5) RMMRR (5) RSMSS (5)
OCEAN SPRINGS	6-28-83	6	MMMM (55) SMMSS (15) RRMMS (10) MMMMS (10) RRMMM (5) MMMSS (5)
16TH STREET	6-28-83	4	SSMSS (60) SMMSS (25) SSSSS (10) SMMSM (5)

Table 5

HA COMBINATIONS OF E. COLI ISOLATED FROM MICE HOUSED IN A CLOSED ENVIRONMENT

ANIMAL:	DATE	# COMBINATIONS	COMBINATION(S)
M1	3-22-83	5	MMRS (40) SMMRR (20) SMMRS (20) SSMRS (15) SMMRM (5)
M2	8-30-83	2	SSMSS (50) SMMSS (50)
M3	9-06-83	3	SSMSS (45) SMMSS (40) MMMSS (15)
M4	9-06-83	2	SSMSS (55) SMMSS (45)

food, water and bedding. It may be that other closely quartered animals share common E. coli, but this determination remains to be performed.

Table 6 examines the results of a single fecal sample from which fecal coliforms (ultimately E. coli) were isolated by three FC standard methods. These primary combinations (RSMSS, RMMSS and SSMSS) were found by all of the methods. The MPN procedure gave the least number of combinations, the A1 method the greatest. On first observation, it appears that the A1 method should not be used since it produced a greater degree of variation than the other methods. However, the opposite may also be true representing a selective pressure by the MPN and MF procedures, and a loss of certain HA biotypes. Additional research is needed to clarify this point.

All of the HA data accumulated during this study is summarized in Table 7. Our bias in this table is the use of combinations beginning with R, M or S, and, therefore, the assumption, that the reaction of an isolate with African Green Monkey rbcs (the first reaction in the sequence) is of primary significance. We do not have data to support this method, but there is no indication in the literature that other paths of analysis would be more productive.

With this in mind we have summarized all HA combinations into those beginning with R, M or S, determined the

Table 6

METHOD OF FC ISOLATION

FECAL SAMPLE TAKEN FROM HUMAN 1 (11-29-93)
AND FECAL COLIFORMS ISOLATED BY EACH OF THE THREE METHODS

METHOD	# COMBINATIONS	COMBINATIONS
A1	7	RSMSS (30) RMMSS (30) SSMSS (15) SMMSS (10) RMMMS (5) RMMRS (5) RSMRS (5)
MF	4	RMMSS (40) RSMSS (30) SMMSS (20) SSMSS (10)
MPN	3	RSMSS (55) RMMSS (30) SSMSS (15)

Table 7, page 1

FINAL SUMMARY (R)

-ALL ANIMALS; ALL COMBINATIONS-
DECREASING FREQUENCY

COMBINATION(S)		TOTAL
RRRMMM	54 (H), 1 (E)	55
RSMSS	25 (H), 12 (C), 5 (D), 1 (E)	43
RMMSS	32 (H), 3 (C), 1 (D)	36
RRRMSS	29 (C), 4 (H)	33
RSMSR	30 (D), 1 (E)	31
RMMRRR	22 (H), 2 (G), 1 (E)	25
RMMRS	12 (G), 8 (H), 1 (D)	21
RRRRRR	13 (F), 5 (I)	18
RRMRRR	17 (H)	17
RMMRM	12 (H)	12
RMRRS	9 (H)	9
RMRSS	6 (H)	6
RRRMSR	4 (H), 2 (C)	6
RRRMMR	5 (H)	5
RRRMMS	2 (E), 1 (H)	3
RRRMSM	2 (H)	2
RMRRRM	2 (H)	2
RMMMS	1 (H)	1
RSMSM	1 (H)	1
RSRSS	1 (H)	1
RMMMM	1 (H)	1
RMSRS	1 (H)	1
	TOTAL =	329

(H) HUMAN; (D) DOG; (C) CAT; (I) HORSE; (F) FISH; (G) GOAT;
(A) BIRDS; (C) COW; (E) ENVIRONMENTAL SAMPLE

(H) FOUND IN	20 OF 22 COMBINATIONS
(D)	4
(C)	4
(I)	1
(F)	1
(G)	2
(A)	0
(B)	0
(E)	5

Table 7, page 2

FINAL SUMMARY (M)

-ALL ANIMALS; ALL COMBINATIONS-
DECREASING FREQUENCY OF OCCURRENCE

COMBINATION(S)		TOTAL
MMMM	51 (I), 48 (H), 34 (D), 28 (F), 21 (B), 18 (C), 13 (E), 12 (A)	225
MMMSM	16 (I), 14 (C), 8 (F), 5 (H), 1 (B)	44
MMMMS	22 (I), 8 (H), 3 (F), 3 (E)	36
MMMSS	19 (H), 7 (E), 6 (I), 1 (D), 1 (C)	34
MMRSS	27 (B)	27
MMMRM	10 (C), 4 (D)	14
MRMMM	14 (H)	14
MMMRS	7 (D), 1 (I), 1 (A)	9
MMRMS	8 (B)	8
MMRSM	5 (B)	5
MSMRM	2 (H)	2
MSMSS	2 (H)	2
MSMMM	1 (H)	1
MRRRR	1 (I)	1
MSMRS	1 (A)	1
MMMMR	1 (F)	1

TOTAL=424

(H) FOUND IN	8 OF 16 COMBINATIONS
(D)	4
(C)	4
(I)	6
(F)	4
(G)	0
(A)	3
(B)	5
(E)	3

FIANL SUMMARY (S)

-ALL ANIMALS; ALL COMBINATIONS-
DECREASING FREQUENCY OF OCCURRENCE

COMBINATION(S)		TOTAL
SSMSS	46 (H), 44 (B), 30 (D), 23 (C), 21 (I), 19 (E), 7 (F), 2 (A)	191
SMMSS	44 (H), 24 (D), 31 (I), 20 (E), 11 (C), 8 (B), 3 (A)	141
SMMRS	16 (I), 10 (A), 6 (D), 6 (G), 3 (H)	41
SSMRR	32 (F)	32
SSMSR	14 (C), 4 (D), 2 (E), 1 (F)	21
SSMRS	16 (A), 1 (H), 1 (B), 1 (E)	19
SMMMS	8 (I), 3 (H), 1 (B), 1 (A)	13
SSSRS	11 (A)	11
SMMRR	6 (F), 2 (I), 1 (H)	9
SMMSR	3 (D), 3 (E), 1 (I)	7
SRMMM	6 (H)	6
SSSSS	5 (E), 1 (A)	6
SMMMM	3 (I), 1 (H), 1 (A)	5
SMMRM	2 (I), 1 (H), 1 (F)	4
SMMSM	2 (I), 1 (D), 1 (B)	4
SRRRR	4 (I)	4
SSMSM	1 (H), 1 (B), 1 (C), 1 (E)	4
SSSSR	2 (B)	2
SRMSS	2 (C)	2
SSRRR	2 (I)	2
SMRSS	1 (H)	1
SMSRS	1 (A)	1
	TOTAL =	527

(H) FOUND IN	11 OF 22 COMBINATIONS
(D)	6
(C)	5
(I)	11
(F)	4
(G)	1
(A)	9
(B)	7
(E)	7

number of times that an HA combination appeared for a particular species, and ranked them according to order of occurrence. Table 7, page 1, shows that 22 R combinations (representing 329 isolates or 25% of all isolates) were encountered during this study. RRMMM was observed on 55 occasions, representing 16.7% of those combinations beginning with R, but only 4% of all combinations found in this study. Twenty (91%) of the twenty-two combinations beginning with R could be found in association with human isolates, but at this time, there is no way to determine if this result is an artificial number created by the larger number of human isolates tested. Humans were not found to contain isolates giving 2 HA combinations (RRRRR and RSMSR). None of the isolates taken from birds or cows began with an R reaction.

Combinations which began with M (Table 7, page 2) represented 33% of all isolates (424 or 1280). The most common pattern, MMMM, was observed on 225 of 1280 occasions (17.5%), and isolates from all species and environmental samples (except the goat) contained this combination. Human isolates were represented by only 8 of 16 M combinations (50%), a drop of 41% when compared to those which began with R. More isolates from horses were represented by M as compared to R combinations.

Those combinations beginning with S (Table 7, page 3)

were found more often (527 of 1280; 41.7%) than those beginning with either R or M. The most frequent pattern in this group was SSMSS, representing 36% of those patterns beginning with S, and 14.9% of all isolates collected.

The combination SMMSS was second in this group, representing 141 isolates. In all, 22 combinations were observed to begin with S. Human and equine isolates were found among 11 or the 22 combinations. By comparison, more species were represented by S combinations than by either R or M, although there was equal opportunity for other patterns to develop.

These results (Table 7) suggest that isolates of E. coli collected from animal feces and environmental samples alike exhibit a wide variety of HA combinations. Particular combinations do not appear to be characteristic of a particular animal or sample, although a trend toward human isolates to begin with R is noticeable. However, human isolates also began with M and S patterns as do isolates of other species.

While it may appear through a cursory examination of the data that HA may afford little value toward the accomplishment of the objectives of this research, there are other facts which dispute this point. It must be pointed out that E. coli appeared to exhibit a limited number of combinations (only 60 out of a possible 243, or 25%), and

that the HA method employed could have several inherent flaws which contribute to the degree of variation observed. For example, Adult chicken rbc easily hemolyzed, making it difficult to observe this HA reaction. Also, we looked for immediate HA to occur and could have missed delayed reactions. Likewise, it can be expected that certain isolates could have produced a limited HA reaction which was difficult to see with the unaided eye. We feel that this first trial of HA characterization of non-pathogenic FC isolates could lead to more advanced systems with greater accuracy and reproducibility.

It must also be noted that only 5 of the 60 combinations (8.3%) represent 51.3% (656 or 1280) of all isolates: MMMM 225 (17.5%); SSMSS 191 (14.9%); SMMSS 141 (11.0%); RRMMM 55 (4.2%); MMMSM 44 (3.4%). It required an additional 9 combinations (14/60; 23%) to reach >75% of all isolates. Thus, 46 of 60 combinations (or 77%) represented only 25% of the combinations observed, implying either: 1) that 25% of E. coli isolates produce abberant HA combinations, or 2) that the data collected was in error due to the reading of the HA response.

If we examine the 14 combinations representing >75% of all bacterial isolates, we observe the following result:

Combination	No. isolates	Animal(s) represented
MMMM	225	I H D F B C E A
SSMSS	191	I H D F B C E A

SMMSS	141	I H D B C E A
RRMMM	55	H E
MMMSM	44	I H F B C
RSMSS	43	H D C E
SMMRS	41	I H D A G
RMMSS	36	H D C
MMMMS	36	I H F E
MMMSS	34	I H D C E
RRMSS	33	H C
SSMRR	32	F
RSMSR	31	D E
MMRSS	27	B

From this result, certain observations can be made. Combinations representing >10% of all isolates (the first three) represent the majority of the animals tested. only two combinations represent a single species and include SSMRR and MMRSS. Humans are represented by 11 or the 14 combinations. Three combinations (RRMMM, RRMSS and RSMSR) represent only 2 sample types. All other combinations fall somewhere in between. These results imply that although there is a finite degree of variation among E. coli, enough is available to prevent a separation of species on this basis.

The degree of variation can be expressed as the number of combinations per animal of each type:

	R	M	S	Total	%
H	20	8	11	39	64.0
I	1	6	11	18	29.5
E	5	3	7	15	24.6
D	4	4	6	14	22.9
C	4	4	5	13	21.3
A	0	3	9	12	19.6
B	0	5	7	12	19.6
F	1	4	4	9	14.7
G	2	0	1	3	4.9

Humans demonstrate the greatest amount of variation (64%), followed by the horse (29.5%). The fact that humans represent a wide variation underscores the need to analyze additional isolates associated with other species so that equal numbers of species isolates are compared.

No doubt the order and type of the red blood cells used for the HA determinations influences the status of a combination. Assuming, however, that all isolates are compared using the same order, the results should be equivocal. The number of types of blood used is also of significance. Five is a practical number in terms of ease of procedure, but additional types of rbcs would be more functional in terms of separation of isolates. It follows that smaller numbers of types of rbcs used would create fewer biotypes.

As shown in Table 7, which used the rbc sequence AG-HO-B-AC-GP, a total of 60 combinations result (23R, 16M, 22S). If we drop the AG reaction, we find the following result:

RMMM	68(H), 1(E)	69
RMSS	31(c), 4(A	35
RRRR	13(F), 10(I)	23
RMRR	17(H)	17
RMSR	4(H), 2(C)	6
RMMR	5(H)	5
RMMS	2(E), 1(H)	3
RMSM	2(H)	2
	Total	160
SMSS	73(H), 44(B), 35(D), 35(C), 21(I), 20(E), 2(A)	237
SMSR	34(D), 14(C), 3(E), 1(F)	52

SMRR	32(F)	32
SMRS	17(A), 1(H), 1(B), 1(E)	20
SSRS	11(A)	11
SSSS	5(E), 1(A)	6
SMSM	2(H), 1(B), 1(C), 1(E)	5
SSSR	2(B)	2
SSSR	2(I)	2
SMRM	2(H)	2
SRSS	1(H)	1
SMMM	1(H)	1
	Total	371

MMMM	79(H), 55(I), 34(D), 28(F) 21(B), 18(C), 13(E), 13(A)	261
MMSS	84(C), 37(I), 27(E), 25(D) 15(C), 8(B), 3(A)	199
MMRS	18(G), 17(I), 14(D), 11(A) 11(H)	71
MMMS	31(I), 11(H), 6(F), 1(B), 1(A)	50
MMSM	18(I), 14(C), 8(F), 2(B), 1(D)	48
MMRR	23(H), 6(F), 3(E), 2(G)	34
MRSS	24(B), 6(H)	30
MMRM	13(H), 10(C), 4(D), 2(I), 1(F)	30
MRRS	9(H)	9
MRMS	8(B)	8
MRSM	5(B)	5
MRRM	2(H)	2
MSRS	1(H), 1(A)	2
	Total	749

A drop occurs in the number or combinations which began with each letter (22 R to 8 R; 22 S to 12 S; 16 M to 13 M); R & S combinations also lose representation in numbers of isolates (329 R drops to 160 R; 527 S drops to 371 S), but there is a sizeable rise in the number of combinations which begin with M (424 rises to 749). This means that fewer isolates reacted with human type O blood than had reacted with African Green Monkey rbcs.

If we continue this process and drop the reactions of both AG and Ho rbc's, we find the following: the number of R combinations drops to 6, the number of representative isolates to 78; the number of S combinations drops to 3, the number of representative isolates to 21; and there are now 9 M combinations representing 1181 isolates.

Thus, as rbc types are excluded there is a drop in the possible number of combinations. This trend is apparant in the scheme below as each successive level of rbc is dropped:

	R	M	S	Total
AG-Ho-B-AC-GP	22	16	22	60
Ho-B-AC-GP	8	13	12	33
B-AC-GP	6	9	3	18
AC-GP	3	3	3	9
GP	1	1	1	3

Approximately, one-half of the number of combinations (in one case 2/3) are lost with each deletion of an rbc type (starting with AG and proceeding to GP) implying that additional rbc types, such as ox, might double the number of combinations observed, and possibly lead to a greater degree of species specific reactions.

If we examine the number of isolates represented by each succesive deletion of an rbc type and the % of each value, the following is observed:

	Isolates Represented		
	R	M	S
AG-Ho-B-AC-GP (AG)	329(25.7)	424(33.1)	527(41.1)
Ho-B-AC-GP (Ho)	160(12.5)	749(58.5)	371(28.9)
B-AC-GP (B)	78 (6.0)	1181(92.2)	21 (1.6)
AC-GP (AC)	225(17.5)	398(31.0)	627(48.9)
GP (GP)	175(13.6)	425(33.2)	680(53.1)

As this table demonstrates, there is more likelihood that an isolate will not react with bovine rbcs (92.2%), a 58.5% chance that an isolate will not react with Ho rbcs, but only a 33.2, a 31.0 and a 33.1% chance that an isolate will not react with GP, AC and AG rbcs, respectively. Further research should take these values into consideration and create a species key based on rbcs producing the highest levels of reactivity.

2. Electrophoresis:

Other studies have analyzed pili from individual enteropathogenic E. coli, but have described only a limited number of isolates. In their studies, the nature of the analyses could be lengthy and more cumbersome due to the small number of strains examined. To be appropriate for the analysis of many isolates, and thus, toward the development of a method to distinguish E. coli isolates from specific animals, a procedure had to be rapid, basically inexpensive, and reproducible. Pili extraction was found to have several negative procedural characteristics. First, the volume of

E. coli needed to produce a suitable quantity of pili protein was approximately 60 ml of bacterial paste; this required a growth surface of about 10,000 cm^2 Next, the time required to process a single isolate was 2.5 days, allowing for the extraction of up to 4 isolates per week. The steps of electrophoresis were rapid, amendable to the simultaneous analysis of several extracts, and, on a per sample basis, inexpensive. Therefore, the limiting factor in the analysis was the accumulation of pili protein concentrates.

During the course of the analyses, 112, extractions were conducted, representing 3 cats (14 isolates), 8 humans (40 isolates), 4 horses (20 isolates), 3 dogs (15 isolates), 3 fish (13 isolates) and 2 cows (10 isolates).

Table 8, pages 1 to 4 summarize these results. All strains tested were isolated in the manner described previously, and were characterized by the HA method so as to facilitate comparison.

The pattern of HA variation expressed earlier for groups of 20 E. coli collected from a single animal on a specified date appears to hold for the isolates tested here. There is little agreement on the number of pili bands or the HA pattern observed. Commonly, isolates which produce the same number of bands, produce radically different HA patterns (horse 1, isolates 4 & 5). Conversely, isolates pro-

Table 8, page 1

Animal	Isolate #	# pili bands	Date	HA pattern
Horse 1 (E1OG)	1	4	9/20/82	SSRRRR
	2	8	"	MRRRRR
	3	7	"	SRRRRR
	4	3	"	RRRRRR
	5	3	"	SMMRM
Horse 2 (E2YM)	1	3	11/14/82	MMMSS
	2	1	"	MMMSS
	3	1	"	MMMMS
	4	1	"	MMMSM
	5	6	"	MMMSM
Horse 3 (E3C)	1	6	10/18/82	MMMMS
	2	6	"	MMMMM
	3	6	7/19/83	SMMSS
	4	1	"	SSMSS
	5	1	"	SSMSS
Horse 4 (E4M)	1	1	10/18/82	MMMMS
	2	1	"	MMMMS
	3	1	7/19/83	SMMSS
	4	1	"	SMMSM
	5	1	"	SMMSS
Cow 1 (B2C)	1	5	11/14/82	MMMMM
	2	3	"	MMMMM
	3	1	"	MMMMM
	4	1	"	MMMMM
	5	1	"	MMMMM
Cow 2 (B2C)	1	5	7/24/83	SSMSS
	2	1	"	SSMSS
	3	1	8/27/83	MMRSM
	4	1	"	MMRSS
	5	1	8/28/83	MMRSS

Table 8, page 2

Animal	Isolate #	# pili bands	Date	HA pattern
Cat 1	1	3	9/27/83	MMMSM
(F)	2	7	"	MMMSM
	3	2	"	MMMSM
	4	0	"	MMMSM
Cat 2	1	3	7/05/83	RMMSS
(F2FC)	2	2	"	RMMSS
	3	0	"	SMMSS
	4	1	11/21/83	RSMSS
	5	1	"	RMMSS
Cat 3	1	2	8/05/83	SSMSR
(F3CC)	2	1	"	SSMSR
	3	1	8/15/83	MMMSS
	4	2	"	SSMSS
	5	4	9/01/83	MMMM
Dog 1	1	0	9/20/82	MMMM
(C1M)	2	0	"	MMMM
	3	0	"	MMMM
	4	1	7/24/83	SSMSS
	5	1	"	SSMSS
Dog 2	1	2	9/21/82	MMRM
(C2W)	2	2	"	SMMRS
	3	1	"	SMMRS
	4	2	"	MMMRS
	5	6	"	MMMRS
Dog 3	1	6	7/05/83	SMMSS
(C3TD)	2	3	"	SMMSS
	3	5	"	SMMSS
	4	5	"	SMMSM
	5	5	"	SMMSS

Table 8, page 3

Animal	Isolate #	# pili bands	Date	HA pattern
Human 1 (H1)	1	1	9/20/82	RMRSS
	2	0	"	RSRSS
	3	2	9/21/82	RMRRS
	4	3	10/19/82	SSSSS
	5	1	"	SSMSS
Human 3 (H3R)	1	0	10/19/82	RMMMS
	2	0	"	RSMSS
	3	2	"	RSMSS
	4	2	"	RMMSS
	5	2	"	RMMSS
Human 4 (H4EW)	1	0	6/28/83	SSMSS
	2	0	"	SSMSS
	3	3	7/29/83	SSMSS
	4	4	"	SSMSS
	5	0	8/09/83	SMMSS
Human 6 (H6W)	1	0	5/31/83	RMMRR
	2	0	"	RMMRR
	3	0	7/11/83	MMMMM
	4	0	"	MMMMM
	5	0	7/18/83	MMMMM
Human 7 (H7C)	1	6	7/13/83	MMMSS
	2	6	"	MMMSS
	3	6	"	MMMMS
	4	3	11/29/83	SMMSS
	5	3	"	MMMSS
Human 8 (H8T)	1	5	7/05/83	RRMMM
	2	5	"	RRMMM
	3	5	11/21/83	SRMMM
	4	0	"	SRMMM
	5	0	"	RRMMM
Human 9 (H9TW)	1	8	11/21/83	SSMMM
	2	5	"	SSMMM
	3	0	"	SRMMM
	4	0	"	SMMMM
	5	2	"	SMMSS
Human 10 (H10CW)	1	6	9/14/83	RRMSS
	2	4	"	RRMMM
	3	4	"	RRMMM
	4	4	11/29/83	MRMMM
	5	4	"	MRMMM

Table 8, page 4: Fish and Misc. Animals

Animal	Isolate #	# pili bands	Date	HA pattern
Catfish 1	1	9	6/28/83	SSMRR
	2	6	"	SSMRS
	3	4	"	SSMRR
Catfish 2	4	1	"	SSMRR
	5	2	"	SSMRR
Croaker 1	1	5	"	MMMM
	2	4	"	MMMR
	3	4	"	MMMS
Croaker 2	4	4	"	MMMM
	5	4	"	MMMM
Mullet 1	1	2	7/15/83	SSMSS
	2	2	"	SSMSS
	3	3	"	RRRRR
Chicken 1	1	1	9/20/82	MMMM
	2	1	"	MMMM
	3	0	9/21/82	SMMSS
	4	2	"	MMMM
	5	1	"	MMMM
Dove 1	1	0	3/07/83	SSSRS
	2	0	"	SSSRS
Dove 2	3	3	"	SSMRS
	4	2	"	SSMRS
	5	4	"	MSMRS
Mouse 1	1	2	3/22/83	SSMRS
Mouse 2	1	6	"	SSMRS
Mouse 3	1	2	8/30/83	SMMSS
Mouse 4	1	5	9/06/83	SSMSS
Mouse 5	1	5	"	SSMSS
Goat 1	1	4	5/24/83	RMMRS
	2	3	"	RMMRS
	3	4	"	RMMRS
	4	3	"	RMMRS
	5	1	"	RMMRS

ducing identical HA patterns (cow 1, isolates 1,2,3), often yield different numbers of pili bands on electrophoresis assay. As could be expected, some agreement was observed (dog 1, human 6, human 10, croaker 2, chicken 1, goat 1), but rarely did all isolates from a species contain the same number of bands and the identical HA pattern. We purposely analyzed species isolates collected from the same animal on different dates, and found: a). that both the HA pattern and the number of bands could change (horse 1, chicken 1), b). that the HA pattern might change and the number of bands remain the same (horse 4), or c). that the HA patterns may remain unchanged (human 4, isolates 1-4), but with different numbers of bands. There appeared to be less variation between isolates collected on the same day, as compared to isolates taken from an animal on 2 or more successive dates. No apparent relationship between the number of pili bands and the HA pattern observed could be implied, nor was the finding of no pili bands associated with an isolate consistent with a non-reactive HA pattern (MMMM). It would have been interesting to determine whether 2 subcultures of the same isolate, treated as described above, would yield identical numbers and molecular weights of bands.

A major question to be answered concerning the bands produced by several isolates from the same animal involves the molecular weight (MW) of individual proteins. The 112

isolates examined yielded a total of 344 bands of which 94 (27%) represented all isolates. Table 9 tabulates the individual MW of these 94 bands, and the number of times that a band was found in association with a particular species. 45 of 94 bands (48%) represented a single species, 24 of 94 bands (26%) represented 2 species, and 18 of 94 bands (19%) represented 3 species of animals. Seven bands (7%) were found in 4 or more species. No band was associated with all of the species tested (total of 12); the greatest number of species associated with a single band was 6 (band # 42658).

Although there is variation in the number and MW of bands associated with different species, and intraspecies variation as well, it should be noted that there is agreement between this part of the results and the overall objective of the project. Again, there appears to be a limit in the number of bands that might be associated with an isolate as a result of the observation that certain bands are multi-species associated. However, multi-species (3 or more) bands are low in number, representing only 29% of the total number of bands. These bands may represent E. coli which are not significantly modified by passage through the GI tract of various animals, or a common form of pili or other protein associated with many varients of E. coli and coded for on the bacterial chromosome rather than plasmid associated.

Table 9, page 1

Band	Species Represneted (No. times)	Total
	0 - 19,999	
0	H(15) D(3) C(2) A(2) Ch(1)	23
8511	B(1) C(1)	2
8710	H(3)	3
9120	I(1)	1
9333	I(1)	1
10471	I(1)	1
10715	I(1)	1
10965	C(2)	2
11220	B(1) Mo(2)	3
11749	I(1)	1
12023	H(4)	4
12303	I(2)	2
12589	Ch(1)	1
13183	H(1)	1
13489	C(1) H(2)	3
13804	I(1)	1
14454	H(3)	3
14791	D(5) H(1)	6
15136	Ca(1) Ch(2)	3
17378	B(1) H(2)	3
17783	H(1)	1
18197	Mo(1)	2
18621	A(1)	1
19498	Mo(1) I(3) D(1)	4
19953	H(3)	3
	20,000 - 39,999	
20417	M(2) D(1)	3
20893	C(2) I(1) D(1)	4
21380	I(1) H(1)	2
21878	I(2) H(2) G(1)	5
22387	H(1) Co(1) A(1) G(2)	5
22909	D(1) M(1) Mo(1)	3
23442	I(2) B(5) D(2)	7
23988	C(1) H(2) Ch(2)	5
24547	A(1)	1
25119	H(2)	2
25704	A(1) G(1)	2
26303	I(3) H(1) M(2)	6
26915	I(1) D(1) H(4)	6

H, human; I, horse; B, bovine; G, goat; D, dog; C, cat; Mo, mouse; M, mullet, Co, croaker; Ch, chicken; A, dove.

20,000 - 39,999 continued

27542	I(1) C(2) D(5) H(4)	12
28184	G(3)	3
28840	C(4)	4
29512	B(1) D(1) H(3) Co(3)	8
30199	I(5) B(2) H(3) Ca(2) Mo(2)	14
30903	D(1) H(2)	3
31623	I(2) Co(2)	4
32359	I(1)	1
33113	A(1)	1
33884	C(1) H(5) Co(2)	8
34674	C(1) Ca(1) G(1)	3
36308	H(5)	5
38019	I(2) B(2) A(2) G(1) M(1)	8
39811	D(1)	1

40,000 - 59,999

40738	M(1)	1
41687	C(3) H(1) M(2)	6
42658	B(3) D(4) H(1) Co(3) Mo(4) G(1)	16
43652	C(3) H(7)	10
44668	H(1)	1
46774	I(3) M(1) D(2)	6
47863	I(1) D(3) H(2) Ca(5) A(1)	12
48978	I(1) D(1) H(1)	3
51286	H(3)	3
52481	D(2)	2
53703	I(1) D(1) H(3)	5
54954	G(1)	1
56234	I(3)	3
57544	I(4)	4
58884	I(5) C(1)	6

60,000 - 79,999

60256	I(1)	1
61660	Co(3) G(1)	4
64565	D(1) Ca(3) G(2)	6
66069	I(1)	1
67608	I(1) H(1)	2
69183	B(1)	1
70795	A(1)	1
74131	D(1)	1
75858	I(1) C(1) H(3)	5
79443	I(2) H(6)	8

80,000 - 99,999

81283	H(1) Co(2)	3
83176	I(1) M(2)	3
85114	I(1) Co(3)	4
87096	H(2) M(1)	3
89125	I(2) D(3) H(3)	8
91201	Ca(1)	1
93325	B(3) C(1)	4
95499	B(1) C(1) D(1) H(2) Co(2)	7
97724	C(1) H(4) M(2)	7

>100,000

102229	Ca(1) M(1)	2
104713	Ca(3)	3
114815	I(2)	2
120226	B(1) C(1)	2
123027	Ca(1)	1
128825	Ca(2)	2
138038	C(1)	1
141254	Ca(1)	1
147911	C(1)	1

Intraspecies variation is a critical factor and its limits must be known to produce a viable method of species separation. In this study, human isolates were tested more often than those of other species (40/112; 35.7%). Table 10 indicates the level of variation observed. 38 bands were found in the pili extracts of the 40 human isolates; no pili bands were found in 15 of the isolates. Isolates collected on the same day were more likely to produce identical patterns than isolates from the same human collected on different dates (for ex. the 3 human 7 isolates of 7/13/83 all contained 6 bands; 4 months later, 2 isolates from the same human contained 3 bands each, none of which had been observed on the previous date). None of the 5 isolates taken from human 6 on 3 separate dates produced an extract banding pattern. This was not always the case and in several instances (humans 8,1 and 9), isolates collected on the same date yielded: 2 isolates with no bands, 1 isolate with 5 bands (h8); 1 isolate with no bands, 1 isolate with 1 band (h1); 2 isolates with no bands, 3 isolates with 8,5 and 2 bands, respectively (h9).

These results imply that isolates of E. coli produce a variety of extractable surface proteins, not all of which may be derived from pili. Although the extraction procedure was designed to collect and purify pili, it is not known whether other bacterial surface contaminating proteins were

Table 10. Banding patterns of isolates collected from humans

Band #	H1					H3					H4					H6					H7					H8					H9					H10				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
0		x					x	x				x	x				x	x	x	x	x								x	x			x	x							
8710																					-	-	-																		
12023																							-	-								-	-								
13183																												-												
13489			-	-																																				
14454								-	-	-																														
14791																																								
17378																																-	-							
17783																												-												
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23988																										-	-													
25119	-		-																																					
26303																																				-				
26915								-	-	-																														-
27542																																						-	-	-	-
29512																					-	-	-																	
30199													-																			-	-							
30903																										-	-													
33844																																						-	-	-	-	-
36308																					-	-	-										-	-						
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81283																																						-			
87096												-	-																											
89125																										-	-	-												
95499																									-															
97724																																						-	-	-	-	-

x = no protein bands found in this isolate

present, and are identifiable as part of the complement of bands. Extracts of the agar used for isolate growth consistently produced gels with no protein bands, and it is unlikely that any of the bands listed in Table 9 represent proteins from the growth medium.

The procedures used to digest the purified pili have been employed in previous investigations, and should be capable of suitable pili digestion. However, it cannot be conclusively shown that the large MW bands represent independent subunits or whether they represent unhydrolyzed pili protein. Another problem concerns the amount of protein in an extract. Although all strains were grown in the same manner, variation in the amount of protein in the final concentrates may have contributed to the variation in banding observed. A standard procedure could be formulated to insure that the amount of protein being exposed to electrophoresis be equal for each isolate. A protein measuring refractometer could be used for rapid analysis, or the Lowry method if low values are consistently observed.

Discussion

Hemaggultination: The procedures used during this investigation to attempt to identify an E. coli according to animal of origin have been studied by other workers. However, the unique aspect of this study was the application of the methods. To date, the primary types of E. coli exhaustively examined by either HA or pili banding have been enteropathogenic or enterotoxigenic strains. The reasons for the substantial amount of literature on this subject involves an understanding of microbial surface factors which will lead to either the development of a vaccine, or a way to modify the epidemiology of these strains which produce gastro-intestinal or other forms of disease in man and animals.

By far the greater amount of research has concerned hemagglutination which has been described for isolates of E. coli since 1908. The grouping of this bacterium according to HA patterns began with the work of Duguid in 1955, who characterized three groups of E. coli strains producing different patterns of HA against rbcs of different animals, and a fourth group that was non-hemaggultinating. Group 1 strains were HA(+) in most rbc types and produced this reaction best following growth in liquid medium. These strains contained pili and were found to be mannose sen-

sitive. Group 2 varients gave an HA reaction in a variety of rbc types best seen at a low temperature of reactivity (3-5 C) following growth on agar. Pili were present on these bacteria, but the HA reaction was not sensitive to the presence of mannose. Elution of bacteria from rbcs was observed when the mixture was warmed to ambient temperature. Group 3 strains were identical in all respects to Group 2 with the exception of pili.

Our classification of intestinal and commensial isolates followed this scheme (R = mannose resistant, Groups 2 and 3; S = mannose sensitive, Group 1; M = no HA activity, Group 4). One difference was that many strains exhibited an R reaction at room temperature, a step more ammendable to rapid isolate analysis. If a cold HA reaction had been observed for each isolate, possibly additional R reactions would have occurred. Our method appears to favor an S reaction, but the results do not bear this out in all rbc types.

Group 1 (S) reactions were found more often when AG, AC or GP rbc were used. Only a small percentage (8.0%) of the 1280 strains examined hemagglutinated bovine rbcs, and 41% of the strains produced a HA(+) reactions with human type O cells. The group 1 isolates studied by Duguid reacted best with guinea pig cells, but he reported that most animal bloods were suitable to designate an S type reaction. In

our study, a clear distinction could be made between guinea pig, adult chicken cells, and monkey rbcs versus human type O and bovine red blood cells. The latter two rbc types appeared least likely to respond to pili containing E. coli.

RBC type	Group 1	Group 2/3	Group 4
Ag	527	329	424
Ho	371	160	749
B	21	78	1181
Ac	627	225	398
Gp	680	175	425

Although it is of interest to classify isolates according to group, it is apparent that the possession of S or R hemagglutinins does not correlate with the source of the bacteria. To underscore this point, another scheme for HA classification is presented below.

A second and more detailed scheme was developed to type human E. coli isolates (Evans et al. 1980, J. Clin. Micro. 12:235-242). Our goals, as expressed earlier, remain the same, but in this instance, further divisions of MS and MR hemagglutinins was possible and therefore conducted. Our steps of analysis used the general steps presented in this paper. Evans divided isolates into 7 HA types:

HA group	H	B	Ck	Mk	Gp
1A	R	R	R	M	M
1B	R	R	R	S	S
2A	M	R	R	M	M
2B	M	R	R	S	S
2C	S	R	R	S	S
3A	M	M	S	S	S
4A	S	M	S	S	S
4B	M	M	M	M	M
5A	R	M	M	M	M
5B	M	R	M	M	M
5C	M	M	R	M	M
5D	M	M	M	R	M
5E	M	M	M	M	R
5F	R	M	S	S	S
6A	R	M	R	R	M
6B	R	M	R	R	S
6C	R	M	M	R	M
6D	R	M	S	R	S
6E	R	M	R	R	R
6F	R	M	S	R	R
7A	R	R	M	M	M
7B	R	R	M	S	S
7C	R	R	S	S	S

A re-evaluation of the isolates examined in this study using the rbc sequence of Evans produced the following result:

Evans HA group	# strains in Evans group	# strains with single varients
1A	191	5
2A		7
2B		9
2C		2
3A	141	141
4A		70
4B	225	110
5A	14	
5B		8
5C	14	21
5D	1	1
5E	1	25
6C	55	14
6D	33	6

6E	17	
6F	6	1

The following strains contained equal to or greater than 2 rbc varients: RRRRR, 18; MRSRS 6; MSRRS, 1; MMRSR, 9; MMRSM, 4; MSRSS, 1; MRSMS, 27; MMRRS, 21; SMRSR, 32; SMRSS, 19; SSRSS, 11; SSSSS, 6; SSSSR, 2; SMRMM, 2; SMRMS, 1; SMSRM, 1; SRSRS, 1.

The number of R combinations droped from 22 to 13; M combinations increased from 16 to 30, and S combinations dropped from 22 to 17. The 5 major patterns which represented approximately 50% of all combinations in our analysis, fall directly into the HA groups of Evans, with one exception. 54% of all possible isolate combinations fall into the 7 groups, and if the isolates containing one rbc varient are included, this figure rises to 87%. Evans also observed that 36% of stool isolates were group 4B. In our case this figure is 29%.

It was not the intention of the Evans investigation to separate species-associated E. coli. However, it must be noted that the procedure would not be sufficient for species differentiation due to the finding that isolates from different animals and environmental samples remain in single groups and are not able to be separated further with the present data. Whether HA typing of E. coli can ultimately allow species separation must await a finer mechanism of

analysis.

Electrophoresis: The large number of bands found (Table 9) in isolates used in this study do not all represent pili proteins. Common pili proteins of E. coli can range from approximately 15,000 to 25,000 molecular weight. Purified pilin should have a molecular weight of around 17,000. Only a small number of the isolates examined in this study contained MWs at or near 17000. The remainder of the bands probably represent other types of surface proteins such as flagella, porins, or envelope proteins.

The problem appears to be one of purification. The SDS solubilization procedure seems to detect pili present, but also components of the cell envelope. It is also possible that pili may be refractory to solubilization by this technique. If solubilization does not occur, pili will not enter the gel, and this could account for the low number of samples with bands of approximately 17000. Conversely, isolates could exhibit more than one type of pili subunit giving rise to different bands in the gel, or exhibit different conformational or charge states for pili subunits in the SDS solution. These variations could perhaps expand the range of pili MW to 12-29 K, but they would not account for the large number of MW species >30 K (of which there are 52).

A multi-step pili purification steps appears necessary if this research path is followed in the future.

Appendix I

Solutions for electrophoresis:

Phosphate buffer stock solution (0.2 M, pH 7.1): 39.0 g NaH_2PO_4 H_2O; 193 g Na_2HPO_4 $7H_2O$; 10 g SDS; make up to 5000 ml with distilled water.

Electrode buffer: 1 part buffer stock plus 1 part distilled H_2O

Phosphate sample buffer (0.01 M, pH 7.1): 5 ml Phosphate buffer stock solution; 1 ml 2-mercaptoethanol; 1 g SDS; qs to 100 ml with distilled H_2O

Acrylamide solution: 22.2 g acrylamide; 0.6 g Bis; qs to 100 ml with distilled water. Store in dark at 4 C.

Ammonium persulfate solution: 150 g Ammonium persulfate; make up to 10 ml with distilled water; store in dark bottle at 4 C.

Bromphenol blue (o.25% w/v): 25 g bromophenol blue; make up to 10 ml with sample buffer.

Detergent solution: Add 0.1 ml Triton-X 100 to 100 ml distilled water.

Fixing Solution: 57 g Trichloroacetic acid; 17 g Sulphosalicylic acid; 150 ml methanol; 350 ml distilled water; add acids to the mixture of methanol and distilled water.

Staining solution: 0.28 g Coomassie brillant blue R-250; 30 g sulphosalicylic acid; 30 g trichloroacitic acid; 186 ml distilled water; 75 ml methanol; add acids to mixture of methanol and water, then add stain.

Destaining solution: 1500 ml ethanol; 500 ml acetic acid; make up to 5000 ml with distilled water.

Preserving solution: 300 ml ethanol; 100 ml acetic acid; 100 ml glycerol; make up to 1000 ml with distilled water.

Appendix II

LKB Instruments and reagents:

LKB 2197 Power Supply
LKB 1860-102 Molecular Weight Markers (range 12,300 - 78,000)
LKB 2117-301 Multiphor unit
LKB 2117-601 Multiphor electrophoresis kit for SDS and PAGE analysis
LKB 1830-101 Sodium dodecyl sulfate (SDS)
LKB 1830-401 2-mercaptoethanol
LKB 1820-101 Acrylamide, Ultrograde
LKB 1820-102 Bis, Ultrograde, methylenebisacrylamide
LKB 1830-901 ammonium persulfate ultrograde
LKB 1840-901 Bromophenol blue
LKB 1830-201 Triton-X 100
LKB 1840-101 Coomassie brillant blue R-250
Trichloroacetic acid, Merck, Germany
Sulphosalicylic acid, Merck, Germany

Checklist - Comprehensive Technical Report

Items from checklist in earlier parts of this report include nos. 1,2,3,4,7,12. Other items are addressed below.

#5 Problems which impeeded the investigation: Producing a quantity of E. coli suitable for pili analysis was time consuming and did not allow for the analysis of 20 isolates from each animal.

#6 Accomplishments: The HA method employed was able to separate animal and environmental isolates into general groups, but was not able to differentiate between isolates from different samples.

#8 Recommendations: The theme of the research in this report is an attempt to discover intrinsic differences in isolates of E. coli. Another way to approach this problem would be to look for host factors which are associated with bacteria excreta from different animals. The diets of carnivores, herbivores and omnivores require different mechanisms of digestion; mechanisms requiring different enzymes would be carried on the excreted microbial biomass. This alternate theme should be explored.

#9 Users: A method to differentiate microbial pollutants associated with different animals will revolutionize sanitary microbiology, and alleviate much of the confusion presently created by standard procedures such as the FC count. Since we are fairly familiar with the disease agents of other animals and the potential disease implications for man, we could perhaps increase the accepted bacterial indicator levels and still retain sanitary quality assurrance. Federal, state and local authorities authorized to protect the public will use such a method.

#10 Dissemination of Results: The results of this study will be the subject of a jouranl article in the area of sanitary microbiology. The Journal of the Water Pollution Control Federation would be a logical place to submitt.

#11 Additional References:

Cravioto, A., R.J. Gross, S.M. Scotland and B. Rowe. 1979. Mannose resistant hemagglutination of human erythocytes by strains of Escherichia coli from extraintestinal sources: lack of correlation with colonisation antigen (CFA/1). FEMS Microbiology Letters 6:41-44.

Salit, I.E., J. Vavougios, and T. Hofmann. 1983. Isolation and characterization of Escherichia coli pili from diverse clinical sources. Infect. Immuno. 42:755-762.

McMichael, J.C. and J.T. Ou. 1979. Structure of common pili from Escherichia coli. J. Bacteriol. 138:969-975,

Orskov, I. and F. Orskov. 1983. Serology of Escherichia coli fimbriae. Prog. Allergy 33:80-105.

Minton, N.P., J. Marsh and T. Atkinson. 1983. The R-factors of multiple antibiotic resistant faecal coliforms isolated from a domestic dog. J. Appl. Bact. 55:445-452.

#13 Publications:

Ellender, R.D., C. Shows, and F.G. Howell. 1984 Comparative hemagglutination patterns of Escherichia coli isolated from animal feces and environmental samples. Abstract (Q 97), 84th Annual Meeting of the Amercian Society for Microbiology, St. Louis, Mo., 4-9 March, 1984. (ISSN 0067-2777).

#14 No. field exercies: 19

No. person Hours expended: 1560

Dollars encumbered (as of May, 1984): $23,384.22

Percent completion of objectives: 95%