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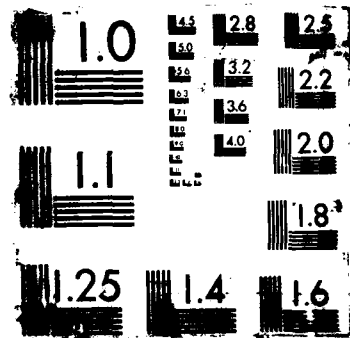
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Infectious Multiple Drug Resistance in the Enterobacteriaceae

Annual Report

Stanley Falkow, Ph.D.

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| 20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A gene encoding a heat-stable enterotoxin (ST) from an <i>Escherichia coli</i> strain isolated from a human with diarrhea has been cloned and characterized by nucleotide sequence analysis. The gene was found to be partially homologous to a previously characterized ST gene from an <i>E. coli</i> strain of bovine origin. Hybridization studies showed that most ST-producing strains of <i>E. coli</i> isolated from humans with diarrhea possess genes highly homologous to either the ST gene from the bovine strain or the ST gene characterized in | | |

20. Abstract (cont)

the present study.

Enterotoxigenic strains of Vibrio cholerae 0-1, biotype El Tor isolated from a case of cholera in Texas in 1973, an outbreak of cholera in Louisiana in 1978 and Louisiana sewage samples in 1980 and 1981 were analyzed for their genetic similarities. A radioactive probe consisting of E. coli heat-labile enterotoxin DNA detected cholera toxin gene sequences in these strains and demonstrated that the toxin gene sequence is identical in these strains and distinctly different from other strains of V. cholerae isolated throughout the world. In addition, two strains of enterotoxigenic V. cholerae non-0-1 isolated from clinical cases, were analyzed and found to possess cholera toxin genes which differed in DNA sequence from the V. cholera 0-1 strains. The data clearly show that a single strain of enterotoxigenic V. cholerae 0-1 is resident in their U.S. Gulf Coast and that a second reservoir of cholera toxin genes exists in V. cholerae non-0-1 strains.

FOREWORD

In conducting this research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



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TABLE OF CONTENTS

| | |
|---|----|
| Introduction | 3 |
| Isolation, Characterization and Use of the ST-H Gene Encoding a Heat Stable Enterotoxin of <i>Escherichia coli</i> | 4 |
| Figure 1 | 5 |
| Table 1. Detection of ETEC by Colony DNA Hybridization | 6 |
| Figure 2. | 7 |
| Molecular Epidemiology of <i>Vibrio cholerae</i> in the U.S. Gulf Coast | 9 |
| Table 2. Summary of DNA Hybridization Results with LT Genes and Enterotoxigenic <i>V. cholerae</i> El Tor Strains | 11 |
| Update of Non-radioactive Assay for DNA-DNA Hybridization - An Update | 12 |

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are important causes of diarrhea in infants, children and adults in developing countries and also in travellers to these nations. The capacity of these strains to produce enterotoxins is plasmid-mediated [1]. Plasmids, called Ent, encode for two general classes of toxin: a heat labile toxin, LT; and a heat stable toxin, ST.

LT is 82,000 daltons in mass and is composed of two subunits; it is functionally, structurally, and genetically related to the classical cholera toxin [2]. Its mechanism of action involves the activation of eukaryotic adenylate cyclase [3]. ST is non-immunogenic, of relatively low molecular weight (<10,000 daltons), and acts by activating guanylate cyclase [4].

Biological, chemical, and genetic evidence suggest that ST from different strains of *E. coli* represent a heterologous group of toxins. There are at least two distinct groups of ST. [5, 6] ST I (also referred to as STa) is methanol soluble and is active in an infant mouse model. ST II (also referred to as STb) is methanol insoluble; it is not active in an infant mouse model but can be detected in ligated pig ileal loops. In large parthuman, ETEC strains encode LT and a form of ST I.

Plasmid genes encoding ST I have been isolated by recombinant DNA methods [7, 8]. One of these, which we call ST-P for convenience, is part of a bacterial transposon [9] and its nucleotide sequence has been determined [10]. Last year, we reported the use of an isolated DNA fragment of LT and of ST-P as genetic probes to identify ETEC from patients with diarrhea by DNA hybridization [11]. While all LT producing strains were identifiable by the LT DNA sequence, many ST producing strains (as determined by the infant mouse assay) did not react with the ST-P probe. On that basis, we isolated and cloned a new ST gene which we designate ST-H for convenience. This gene has now been characterized at the nucleotide sequence level and utilized in further field studies in Bangladesh to understand better the epidemiology of *E. coli* ST infection.

We also reported last year that the LT genetic probe could be used to detect toxigenic strains of *V. cholerae* 0-1, non-0-1, and environmental isolates [12]. At that time, we proposed that it might be possible to develop a "molecular fingerprint" of the cholera toxin gene that could serve as an epidemiological marker. We report here our successful use of this fingerprinting method to define the nature of cholera strains in the U.S. Gulf States and their unique distinction from strains found elsewhere in the world.

Since we have concentrated upon DNA hybridization as a tool for our studies, it seemed worthwhile to attempt to develop a non-radioactive means to identify nucleic acid hybrids. We hoped this would simplify our current method using radioactive DNA for hybridization, and thus increase the utility of the general methodology for field use. We report here our first efforts to develop such methods. While we have to some extent been successful, we still lack sufficient sensitivity to supplant our radioactive methods. The results are promising, however.

PROGRESS REPORT

I. Isolation, Characterization and Use of the ST-H Gene Encoding a Heat Stable Enterotoxin of *Escherichia coli*.

A. Plan of Study.

Purified plasmid DNA from *E. coli* strain 153837-2 isolated from a patient in Bangladesh was cleaved with restriction endonucleases and the gene encoding ST was cloned into the vector plasmid pBR322. Analysis of the resulting hybrid plasmid pSLM004 by nucleotide sequence analysis revealed (Figure 1) that the structural gene was preceded by a ribosomal binding site (GGAGG), followed by a translational start codon (ATG), and ending some 216 nucleotides later with the translational stop codon TAA. This termination signal is followed by a 15 base pair region of dyad symmetry suggestive of a transcriptional termination structure. A 215 base pair *Hpa* *II* fragment of pSLM004 was selected for further use as a means of identifying ETEC in patients with diarrheal disease.

Diarrheal stools from 108 patients consecutively admitted to the intravenous rehydration ward of the International Center for Diarrheal Control in Dacca, Bangladesh were screened for the presence of ETEC by standard assays (CHO tissue culture assay for LT, infant mouse assay for ST) and by hybridization with LT, ST-H and ST-P as we described previously [11, 13].

Stools were directly spotted in triplicate on nitrocellulose filters, placed on MacConkey's agar for colony hybridization [11], and streaked onto MacConkey's agar for isolation of colonies. Two colonies and a pool of five colonies were cultured for the standard enterotoxin tests.

B. Use of an ST Gene Probe for Detection of ETEC in Patients with Diarrhea.

Diarrheal stools from 108 patients consecutively admitted to the intravenous rehydration ward of the ICDDR, Bangladesh, Dacca were screened for the presence of ETEC by standard assays and by hybridization with LT, ST-P and ST-H gene probes. The LT gene probe was a 1,200 bp fragment obtained from a *Hinc* *II* digest of plasmid EWD299 as described previously [14]. The ST-P probe consisted of the 157 bp fragment used in our earlier studies [11, 13] and the ST-H probe consisted of a 215 base pair *HPA* *II* fragment of pSLM004 (sequence position 38-254 in Figure 1). The results are shown in Table 1.

The LT probe detected ETEC in all stools from which LT-producing colonies were isolated as well as from five additional stools that failed to yield LT-producing *E. coli* as characterized by standard techniques. Thirty-six patients were found to be infected with ST-producing ETEC by the infant mouse assay. Stools from 34 of these patients yielded bacterial growth possessing DNA sequences homologous to one or both of the ST I DNA probes. The probes detected homologous DNA in bacterial growth from stools of four patients from which ST-producing ETEC were not detected by the infant mouse assay. Of the 38 stools that were positive for ST by colony hybridization, three were detected with the ST-P probe, 33 were detected with the ST-H probe, and two stools yielded bacterial growth with DNA sequences homologous to both ST probes.

C. Interpretation of the Results.

FIGURE 1

5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
 TTCTG GTTT GATTG AATG AACG TTCGT GGATG CCATG TCCGG AGGTA ATATG AAGAA ATCAA TATTA TTTAT TTTTC
HpaII

80 85 90 95 100 105 110 115 120 125 130 135 140 145 150
 TTTCT GTATT GTCTT TTTCA CCTTT CCCTC AGGAT GCTAA ACCAG TAGAG TCCTC AAAAG AAAAA ATCAC ACTAG
DdeI

155 160 165 170 175 180 185 190 195 200 205 210 215 220 225
 AATCA AAAAA ATGTA ACATT GCAAA AAAAA GTAAT AAAAG TGGTC CTGAA AGCAT GAATA GTAGC AATTA CTGCT
AvaII

230 235 240 245 250 255 260 265 270 275 280 285 290 295 300
 GIGAA TTGTG TTGTA ATCCT GCTTG TACCG GGTGC TATTA ATAT ATAAA GGGAA CTAAA CAGTT CCCII TATAT
HpaII

305 310 315 320 325 330 335 340 345 350 355 360
 TTTGTT CTGAT TCTGA TGATG TCTGT AACGT ATGTA CCTGT TGCTT TGCTT TGTTG AATAA ATCGA
TaqI

Table 1.

Detection of ETEC by colony DNA hybridization.

| Toxins Produced* | No. | Detected by Probe [#] | | | |
|------------------|-----|--------------------------------|-------|-------|----|
| | | LT | ST Ia | ST Ib | 0 |
| LT | 3 | 3 | 0 | 1 | 0 |
| ST | 24 | 6 | 3 | 20 | 2 |
| LT+ST | 12 | 12 | 2 | 11 | 0 |
| 0 | 69 | 5 | 0 | 3 | 61 |

*Two colonies and a pool of five colonies from each stool were tested for LT production by the CHO cell assay and for ST by the infant mouse assay.

[#]A loopful of each stool was spotted on three nitrocellulose filters overlaid on MacConkey's agar for colony hybridizations with each of the three probes.

FIGURE 2

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
 ATG AAA AAG CTA ATG TTG GCA ATT TTT ATT TCT GTA TTA TCT TTC CCC TCT TTT AGT CAG
 met lys lys leu met leu ala ile phe phe leu val leu ser phe pro ser phe ser pro gln
 met lys lys ser ile leu phe phe leu ser val leu ser phe ser pro phe pro gln
 ATG AAG AAA TCA ATA TTA TTT ATT TTT CTT TCT GTA TTG TCT TTT TCA CCT TTC CCT CAG

ST Ia:
 ST Ib:
 (ST-H)

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
 TCA ACT GAA TCA CTT GAC TCT TCA AAA GAG AAA ATT ACA TTA GAG ACT AAA AAG TGT GAT
 ser thr glu ser leu asp ser ser lys glu lys ile thr leu glu thr lys lys cys asp
 asp ala lys pro val glu ser ser lys glu lys ile thr leu glu ser lys lys cys asn
 GAT GCT AAA CCA GTA GAG TCT TCA AAA GAA AAA ATC ACA CTA GAA TCA AAA AAA TGT AAC

ST Ia:
 ST Ib:
 (ST-H)

41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
 GTT GTA ANA AAC AAC AGT GAA AAA AAA TCA GAA AAT ATG AAC AAC ACA TTT TAC TGC TGI
 val val lys asn asn ser glu lys lys ser glu asn met asn asn thr phe tyr cys cys
 ile ala lys ser asn lys ser gly ser pro glu ser met asn ser ser asn tyr cys cys
 ATT GCA AAA AAA AGT AAT AAA AGT GGT CCT GAA AGC ATG AAT AGT AGC AAT TAC TGC TGT

ST Ia:
 ST I*:
 ST Ib:
 (ST-H)

61 62 63 64 65 66 67 68 69 70 71 72 73
 GAA CTT TGT TGT AAT CCT GCC TGT GCT GGA TGT TAT TAA
 glu leu cys cys asn pro ala cys ala gly cys tyr trm
 glu leu cys cys tyr pro ala cys ala gly cys asn
 glu leu cys cys asn pro ala cys thr gly cys tyr trm
 GAA TTG TGT TGT AAT CCT GCT TGT ACC GGG TGC TAT TAA

ST Ia:
 ST I*:
 ST Ib:
 (ST-H)

Our study has confirmed the existence of heterologous genes encoding ST I, and reports the isolation and nucleotide sequence determination of one of these genes from a strain of *E. coli* of human origin. So and McCarthy [10] have reported the isolation and sequence determination of a gene encoding ST I from a strain of bovine origin, and have designated the toxin encoded by this gene ST-P. An apparently homologous ST I gene from an *E. coli* strain of porcine origin has also been isolated, and the product of that gene was characterized using an *in vitro* transcription-translation system [15]. All nucleotide sequence and gene product analyses suggest that the ST molecule is initially synthesized as a 72 amino acid precursor of a molecular weight of 5,000 [9, 15, this study]. However, biochemical studies on ST preparations have resulted in reports of an ST I from a porcine strain consisting of 33 amino acids with a molecular weight of 3,580 and an ST I from a human strain consisting of 18 amino acids of a molecular weight of 1,972 [16]. Recent studies utilizing isolate fragments of DNA encoding ST-P as genetic hybridization probes have demonstrated that ST I and ST II are genetically distinct, and, furthermore, that there appear to be at least two genetically distinct forms of ST I [11, 17]. The toxin(s) encoded by genes heterologous to the ST-P gene have been designated ST-H [17]. The sequence of the ST-H gene isolated in the present study is shown in Figure 2 with the predicted amino acid sequence of its product in comparison with the nucleotide sequence of the ST-P gene and the amino acid sequence of its product as reported by So and McCarthy [10], and the amino acid sequence of an ST I from a human strain determined by Chan and Gianella [18]. The two nucleotide sequences appear to share a common evolutionary origin. There are no insertions or deletions; all changes are substitutions. Among the 216 nucleotides from codon 1 to 72, there are a total of 67 nucleotide differences between the ST-P and ST-H genes, resulting in a sequence divergence of 31%. These changes result in a total of 29 amino acid changes, with most changes (18/29) being nonconservative.

The enterotoxin gene probes detected ETEC in stools of 37/39 patients (95%) from whom ETEC was isolated, as well as in eight stools from which no ETEC was isolated by standard techniques. Reconstruction experiments suggest that ETEC/normal flora *E. coli* ratios as low as 1/100 may be detected by the colony hybridization technique described here [unpublished observations], thereby resulting in a somewhat higher sensitivity than the standard procedure at the ICDDR,B (assaying two colonies and a pool culture of five colonies from each stool). Also, a number of stools found to contain ETEC producing only ST by standard assays were found by the colony hybridizations to contain *E. coli* with DNA sequences homologous to the ST and LT gene probes. Our past experience with isolated strains of *E. coli* suggest that these results are most likely due to false negative results with the CHO cell assay. In a previous study [11], such discrepancies were always resolved on retesting in the CHO assay. We have not isolated an *E. coli* which possessed DNA sequences homologous to the LT probe yet did not produce LT. ST producing *E. coli* were isolated from two stools in which no homologous sequences to either ST I probe was detected. Whether these results are due to false positive infant mouse assays, or, in fact, suggest the existence of yet another class of ST genes is not yet known.

The data reported here regarding use of the ST-H gene as a probe for the detection of ETEC suggest that most ST-producing *E. coli* which are not detected by the ST-P gene probe are, in fact, detected by the ST-H gene probe. The proportion of strains hybridizing with the ST-P gene probe differs significantly from the results of a similar study done in the same location one year

previously, and also from a proportion of strains detected by this probe among a number of isolates from Morocco [11]. The previous study found that approximately two-thirds of strains producing only ST possessed sequences homologous to the ST-P probe, while very few strains producing ST and LT hybridized with the probe. The present study indicates that the majority of all ST producing strains of human origin in Bangladesh possess DNA sequences homologous to the ST-H gene probe. These results and a further investigation of the use of enterotoxin gene probes for the detection of ETEC [13] suggest that distribution of the two genes may differ in different geographical locations and may change with time. This emphasizes the potential usefulness of the enterotoxin gene probes in the study of the epidemiology of enterotoxin encoding genes, plasmids, and strains.

II. Molecular Epidemiology of *Vibrio cholerae* in the U.S. Gulf Coast.

A. Plan of Study.

In September of 1978, an outbreak of cholera occurred in Louisiana which involved 11 people. The vehicle of transmission was shown to be inadequately cooked crabs, and strains of *Vibrio cholerae* 0-1 isolated from patients bore a striking resemblance to a strain isolated from a case of cholera in Port Lavaca, Texas in 1973. Because of the similarities in hemolytic activities and plaque type of the Louisiana and Texas strains, the possibility of an endemic focus of *V. cholera* in the U.S. Gulf Coast was raised [19]. Subsequently, in 1980 and in the summer of 1981, enterotoxigenic strains of *V. cholerae* 0-1, Inaba, were isolated from sewage samples in Louisiana.

The question that arises is whether the cholera strains isolated in the U.S. are indeed related to each other and/or to the other strains of *V. cholerae* isolated throughout the world. Because of the DNA sequence homology between cholera toxin (CT) and the heat-labile enterotoxin (LT) of *E. coli*, a molecular probe can be made of LT genes which hybridizes with DNA extracted from *V. cholerae* [12, 14]. We decided to use a refinement of this technique to gain further information about *V. cholerae* in the U.S. Gulf Coast and their relationship to cholera strains from other parts of the world.

Several strains of *V. cholerae* 0-1 were chosen for study. These included the 1973 Texas isolate (E506) and stool and sewage isolates from Louisiana from the past three years. In addition, strains of *V. cholerae* isolated early in the current pandemic (E8439, E9120) were examined because of their similarity to Gulf Coast strains in hemolytic reactions. The well studied toxigenic classical strain 569B was also included.

Whole cell DNA from each of these strains was extracted by the method of Brenner *et al.* [20]. One microgram amounts of the DNA preparations were digested with *Eco RI* and *Hind III* restriction endonucleases. The resulting DNA fragments were separated by agarose gel electrophoresis and then transferred from the gel to nitrocellulose strips by the method of Southern [21].

A molecular probe consisting of the gene encoding the *E. coli* LTA subunit was prepared. This 1,200 bp fragment was labeled with radioactive phosphorous. The probe was hybridized to the filters for 16 hours. The filters were washed, dried, and autoradiographed for 24 hours at -70° and then developed. The results of the hybridization are visualized by the radioactive probe binding to

the homologous filter-bound *Vibrio* DNA, thereby producing a darkening of the X-ray film.

B. Results.

When chromosome from the strains is digested with restriction endonucleases, separated by gel electrophoresis, and hybridized to the LT probe, a distinct autoradiographic pattern, "molecular fingerprint", emerges, depending upon which enzyme and strain are employed. For example, with the *Hind III* enzyme, the classical *V. cholerae* biotype exhibits at least two large restriction fragments with homology to the LT probe. The strains of the El Tor biotype were found to possess only a single large *Hind III* fragment with the exception of El Tor strains isolated from the 1978 Louisiana outbreak.

Two non-0-1 strains (2002H and 2011H) were isolated from patients with severe diarrhea in Louisiana. Both produced cholera toxin. Both strains hybridized with the LT probe, yielding fragments similar, but not identical, to the Louisiana strains. The results of the hybridization studies are summarized in Table 2.

C. Interpretation of Results.

Most El Tor strains examined possessed only a single large *Hind III* fragment ranging in size from 13 to 23 kilobases. The exceptions to this pattern were all isolated from the U.S. Gulf Coast. All possessed a unique *Hind III* site within the toxin gene, which caused two small fragments to hybridize with the LT probe rather than a single large fragment. This indicates that the toxin gene base sequence has undergone evolutionary divergence and that slightly different base sequences are to be found among different strains. The differing size among El Tor biotypes for a single fragment suggests that they were also derived from evolutionarily distinct strains. It could be of considerable interest to map in some detail isolates from different parts of the world in the chronological order of their appearance since the current pandemic. These studies could be useful in following the "flow" of distinct El Tor types during the past two decades.

The toxin gene in the two non-0-1 types from Louisiana has diverged still further than the El Tor strains, since they also possess a unique *Eco RI* site within the toxin gene. These strains are also negative for sucrose fermentation and would probably more correctly be identified as *Vibrio mimicus* rather than *V. cholerae*.

In Louisiana, four different classes of *V. cholerae* have been isolated: (1) *V. cholerae* non-0-1, which do not produce cholera toxin (CT); (2) *V. cholerae* non-0-1 which do produce CT; (3) *V. cholerae* 0-1 which do not produce CT, and (4) *V. cholerae* 0-1 which produce CT. Strains of the first class, *V. cholerae* non-0-1 which do not produce CT have been repeatedly isolated from both environmental and clinical samples. Clearly, the important virulence determinants for this class have not yet been described, since they obviously cause disease without elaborating CT.

In recent years, a great deal of attention has been focused on strains of *V. cholerae* 0-1 which do not produce CT. These strains have been chiefly isolated from water and seafood all over the world, usually in the absence of cholera in the nearby community. The role of these isolates in the epidemiology

Table 2
 Summary of DNA Hybridization Results with LT Genes
 and Enterotoxigenic V. cholerae El Tor Strains

| Strain | Source (yr) | Restriction Pattern | |
|----------------------------|---------------------------|---------------------|-------------------|
| | | <u>Hind</u> III(kb) | <u>Eco</u> RI(kb) |
| <u>V. cholerae</u> 0-1 | | | |
| E506 | Stool, Texas (1973) | 6,7 | 26 |
| 4808 | Stool, Texas (1978) | 6,7 | 26 |
| SGN 7277 | Sewage, Louisiana (1980) | 6,7 | 26 |
| SGN 7700 | Sewage, Louisiana (1981) | 6,7 | 26 |
| 30167 | Stool, Bangladesh (1976) | 19 | |
| 62640 | Stool, Bangladesh (1976) | 19 | |
| E8439 | Stool, Hong Kong (1961) | 24 | |
| E9120 | Stool, Indonesia (1961) | 24 | |
| E9950 | Stool, Phillipines (1980) | 13 | |
| Stokes 1 | Stool, Australia (1977) | 20 | |
| <u>V. cholerae</u> non-0-1 | | | |
| 2002H | Stool, Louisiana (1979) | 6,8 | 6,7.5 |
| 2011H | Stool, Louisiana (1979) | 6,8 | 6,7.5 |

of cholera as a possible reservoir was uncertain until molecular and genetic studies performed by us [12] several years ago under contract support demonstrated that these strains did not possess any genetic material capable of CT production.

The *V. cholerae* non-O-1 which do produce CT have been shown by us to be quite distinct and possibly not strictly *V. cholerae*. Such strains surely cause sporadic human disease but no human disease to our knowledge. There is, however, a single enterotoxigenic strain of *V. cholerae* definitely resident in the U.S. Gulf Coast and apparently unique to this area. It continues to cause disease [22].

III. Development of a Non-Radioactive Assay for DNA-DNA Hybridization - An Update

Last year, we proposed to chemically modify DNA by biotin linking so that we might detect biotinylated DNA probes by the protein avidin linked to horseradish peroxidase or by anti-avidin antibody. We have, in fact, recently succeeded in preparing biotinylated DNA probe more or less at will. However, the use of avidin-coupled horseradish peroxidase as a detection system has been disappointing because of non-specific binding of avidin to the surface matrix we employ for hybridization.

If one foregoes the avidin-enzyme linked step and substitutes merely anti-biotin antibody, the procedure works successfully (using a fluorescence assay), but at present suffices to detect only 50 pg of hybrid. We require a sensitivity of at least 1 pg of DNA for our work. Yet this is not such a difficult technical task to overcome and we are currently continuing to move ahead on refining this method.

It did not seem worth detailing our negative efforts here. We are not any less keen about this aspect of our contract work and we believe that we will be successful in establishing a colony hybridization method during 1982 - 1983.

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