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Title: Bordetella pertussis: An Evaluation of Rapid Plasmid Isolation Procedures

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Research was conducted to evaluate the boiling water bath, alkaline lysis, and acid precipitation plasmid isolation techniques on clinical samples of Bordetella pertussis. Variations on these techniques included plasmid purification procedures, experimental temperature differences, cell lysis methods and centrifugation times. The protocols that were examined all require less than two hours preparation time from pelleted, fresh growth of the test organisms to purified plasmid lysate. None of the methods require laboratory equipment more complicated than a microcentrifuge. Agarose gel electrophoresis was used to detect the presence of plasmid DNA in the lysates. A distinctive 21 kb DNA band was produced by each isolation method. The alkaline lysis procedure produced the most intense DNA bands. The boiling water bath and acid precipitation methods were second and third in intensity. Digestion of the isolated plasmid lysates was attempted with the following restriction endonucleases: Bgl I, EcoRl, Hae II, Hind III and Hinc II. None of the restriction enzymes produced a distinctive plasmid profile, however, Bgl I, Hae II, and Hinc II showed promise as possible tools for the "fingerprinting" (profiling) of Bordetella plasmids.

BORDETELLA PERTUSSIS: AN EVALUATION OF RAPID PLASMID ISOLATION PROCEDURES

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INTRODUCTION

The causative agent of pertussis, or whooping cough, is the gram-negative bacterium, Bordetella pertussis. The organism was first isolated from the bronchial exudate of a whooping cough patient and linked to the disease by Bordet and Gengou (1906). The casual name given to Bordetella pertussis is "Bordet-Gengou bacillus" (Freeman, 1985).

Morphologically B. pertussis is a small (0.5 micrometers to 1.0 micrometers by 0.3 micrometers), nonmotile, coccobacillus. It possesses pili, forms a capsule when virulent, and tends to be pleomorphic, particularly in older children (Davis et al., 1980; Freeman, 1985). After prolonged laboratory passage, the organism displays striking pleomorphism and the capsule, virulence, and pili are lost. The change resembles the classic S to R (S: smooth, encapsulated; R: rough, unencapsulated) variation (Davis et al., 1980). The bacteria resemble Haemophilus influenzae, but are strict aerobes, do not grow on peptone agar, and test negative for urease, citrate and nitrate (Freeman, 1985).

Colonies of Bordetella pertussis are smooth, raised, and glistening, with a slightly pearl-like luster. They grow best on Bordet-Gengou (B-G) medium, developed by Bordet and Gengou (1906). The bacteria require 72 hours or more incubation for appearance of colonies. A slight brownish color develops upon further incubation. The cultures produce a mucoid substance and growth is sticky and persistent (Freeman, 1985).

The clinical pathology of pertussis can be seen in any of a spectrum of illnesses collectively called pertussis syndrome.

Classical whooping cough is at one end of the spectrum while the asymptomatic culture positive individual is at the other (Wilhelm, 1986). The disease is of world-wide occurrence and it is believed that, at one time or another, a great number of people suffer from either typical or atypical symptoms (Davis et al., 1980; Freeman, 1985).

Disease progression is as follows. Commencing with a one week incubation period, the disease appears in three stages. The catarrhal stage, of about two weeks duration, begins with a mild cough and symptoms of an ordinary respiratory infection. It progresses to a more severe paroxysmal stage of four to six weeks duration, characterized by rapid consecutive coughs and the deep inspiratory whoop. The whoop is the cardinal sign of this disease. The number and frequency of the paroxysms gradually decrease in the convalescent stage with uneventful recovery. Most cases of pertussis fall in the middle of the spectrum, namely clinical cases characterized by the development of the catarrhal stage with minimal coughing and no paroxysms (Davis et al., 1980; Freeman, 1985; Wilhelm, 1986).

Bordetella pertussis is but one of three commonly recognized species of the genus Bordetella. Bordetella parapertussis and B. bronchiseptica are the other two species. Bordetella parapertussis is a less common cause of whooping cough. It differs from B. pertussis in producing larger colonies, hydrolyzing urea, and utilizing citrate as a sole source of carbon. Bordetella bronchiseptica is the only motile species of the genus and is associated with bronchopneumonia in rodents and dogs. It rarely causes whooping cough in man (Davis et al., 1980; Kloos et al., 1979). A fourth, recently proposed species, B. avium, was found to cause rhinotracheitis in turkeys, but it has

not yet been associated with human infections (Kersters et al., 1984).

As a result of the introduction and use of the whole-cell pertussis vaccine during the late 1940's, a marked reduction in the incidence of whooping cough throughout the developed world has occurred (Weiss & Hewlett, 1986; Davis et al., 1980). Davis et al. reported that there were only 2400 cases in 1974 compared to 120,000 in 1950. Recently, however, concern over adverse reactions to the vaccine has led to lower vaccine acceptance and debate about its continued use (Cherry, 1984; Katz, 1985; Manclark & Cowell, 1984; Robinson et al., 1985; Weiss & Hewlett, 1986). Reported side effects include bronchial and lobar pneumonia, otitis media, encephalopathy (any disease of the brain) and death (Davis et al., 1980; Freeman, 1985; Weiss & Hewlett, 1986). Encephalopathy is most common in children between 15 and 30 months of age (Barnhart & Barnhart, 1987). Pertussis vaccine does not produce permanent immunity nor does it provide protection against B. parapertussis (Davis et al., 1980).

Most of the 14 reported deaths attributed to pertussis infection during 1950 occurred in infants less than 1 year old (Davis et al., 1980; Nelson, 1978). Rather than whooping, infants frequently have choking or apnea (temporary suspension of breathing) and cyanosis (blueness or lividness of the skin and mucus membranes) due to a lack of oxygen (Barnhart & Barnhart, 1987; Field & Parker, 1977; Linnmann, 1979; Nelson, 1978). Bordetella pertussis infection should be suspected in any young child with a severe respiratory infection, in fact the attack rate in this susceptible group may exceed 90 % (Davis et al., 1980; Field & Parker, 1977; Nelson, 1978). It is these

concerns, the problems of serious pertussis vaccine reactions coupled with the high attack rate in susceptible children, that have sparked renewed interest in pertussis, its components, and their mechanisms of action.

Laboratory diagnosis of the disease is based upon the recovery of the causative agent from nasopharyngeal secretions obtained during the catarrhal and early paroxysmal stages (Lautrop & Lacey, 1960). Bordetella pertussis has been recovered from milder forms of pertussis; however, the exact relationship of B. parapertussis and B. bronchiseptica to clinical outbreaks is somewhat unclear. There are no serological procedures available that produce information with diagnostic validity to allow clinicians or researchers the ability to distinguish different strains of Bordetella (Kloos et al., 1979; Wilhelm, 1986), consequently, there is a need to explore other methods as a means of accomplishing that goal.

Analysis of the unique set of extrachromosomal deoxyribonucleic acid (DNA) known as plasmids may be the solution. Plasmids are small, circular forms of DNA found exclusively in bacteria. They were first discovered in 1959 by Japanese scientists who observed multiple drug-resistant bacterial strains that were concomitantly resistant to four or more chemically distinct antibiotics. The agents responsible were determined to be DNA elements other than chromosomal DNA, and were found to be conjugally transferable between different bacteria. They were designated drug-resistant or R plasmids. Since their first discovery, R plasmids have been isolated world-wide and are credited with the majority of bacterial drug resistance encountered today (Freeman, 1985, p. 197; Lacey, 1975).

Most plasmids are relatively stable, are passed to daughter cells during bacterial divisions, and often confer on their host some selective advantage for survival. These advantages include detoxification of antibiotics, production of adhesion factors that allow bacteria to attach to mucosal cells, provisions of additional metabolic enzymes, resistance to heavy metals, ultraviolet light and specific phages, and production of the substances called bacteriocins that kill neighboring bacteria competing for essential nutrients (Tenover, 1984; Zervos et al., 1988; Farrar, 1983; Davis et al., 1980; Freeman, 1985).

Plasmids exist within the bacterial kingdom in literally hundreds of types and sizes. They vary in length from slightly over 1,000 to over 300,000 kilobase pairs (Tenover, 1984; Counturier, et al., 1988). Virtually every genus, although not every species, of bacteria harbors these elements (Tenover, 1984).

A universal property of plasmids is incompatibility. This is the inability of two plasmids to be propagated stably in the same cell line (Couturier et al., 1988; Novick, 1987). This property allows the researcher the ability to classify bacterial strains based on plasmid response. The operational test for incompatibility involves the introduction of a plasmid (by transformation, for example) into a bacterial strain carrying another plasmid. In order to follow their segregation, the two plasmids must have different genetic markers. Selection is usually carried out for the entering plasmid, and the progeny are examined for the continued presence of the resident plasmid. If the resident plasmid is eliminated, the two plasmids are said to be incompatible and are assigned to the same incompatibility

group (Couturier et al., 1988). In their review, Couturier et al. (1988) pointed out that although incompatibility has been generally useful for plasmid classification, it is not without technical and methodological shortcomings. In the former case, the plasmid to be tested may not contain a suitable marker gene or may not be transmissible by the known means of plasmid transfer (conjugation, transduction, or transformation). Surface exclusion may be another technical obstacle wherein the recipient bacteria inhibits entry of the donor plasmid. This may be difficult to distinguish from incompatibility.

The latter methodological limitation primarily arises from replication control systems. The independent replication of plasmids occurs in a controlled manner and results in a defined average number of copies per cell. The plasmids determine the means for correcting deviations from copy numbers. The genes and sites required for autonomous replication and its control constitute the basic replicons of plasmids. The presence of several replicons results in a compatibility complication. When a multireplicon plasmid is used as a resident plasmid and is challenged by an incoming plasmid containing one of its constituent replicons, it is expected not to be displaced, because the presence of a second functional replicon will take over its replication and thus prevent its loss from the cell. In addition to the complications that may present themselves in compatibility determinations, it should be noted that while plasmids are usually stable, the frequent in vitro passage of isolates containing these elements on nonselective media can accelerate their loss (Tenover, 1984).

To obtain the plasmid from bacteria, it is necessary to lyse the cell and separate the plasmid DNA from the chromosomal DNA. Many methods have been devised to isolate plasmids from both Gram-positive and Gram-negative clinical samples (Goering & Ruff, 1983; Parisi & Hecht, 1980; Eckhardt, 1978; Hanson & Olsen, 1978; Farrar, 1983; Kloos et al., 1979; Lacey, 1975; Nelson, 1978; Holmes & Quigley, 1981; Takahashi & Nagano, 1984; Maniatis et al., 1982). Nearly every technique has advantages and disadavantages and the procedure ultimately chosen is dependent upon the organisms to be studied.

There has been just one report of naturally occurring plasmids in B. pertussis (Kloos et al., 1979). In that paper, new basic information on the genetic relationships of various Bordetella was presented. They also characterized the Bordetella plasmid and provided enlightenment on transformation in B. pertussis. They performed DNA-DNA reassociation reactions using radioisotope-labeled B. pertussis DNA with unlabeled B. pertussis, B. parapertussis, or B. bronchiseptica DNA at incubation temperatures of 65 and 80 degrees centigrade. They found that relative binding at the optimal temperature of 65 degrees was 88-94 % for reactions between B. pertussis and B. parapertussis DNA and 72-93 % for reactions between B. pertussis and B. bronchiseptica DNA. Further, there was very little or no reduction in binding at the stringent temperature of 80 degrees. They believe that these preliminary findings provide evidence for one DNA homology group within the genus, strongly suggesting that strains identified as typical B. pertussis, B. parapertussis, and B. bronchiseptica have not diverged enough genetically to be classed as separate species.

A small cryptic plasmid with a mass of about three megadaltons was discovered in most of the B. pertussis and each of the B. parapertussis samples tested. Most of the B. bronchiseptica strains contained one or more medium to large plasmids in addition to a small labile plasmid similar to that found in B. pertussis and B. parapertussis.

Kloos et al. (1979) confirmed the presence of a transformation system in the B. pertussis strains tested. Transformation is a genetic exchange process accomplished by the absorption and uptake of DNA into recipient cells that are in a competent state. Competency in a bacterial cell is a transient physiological state wherein the cell appears to develop protein cell surface receptors for the binding of DNA. Following uptake by the cell, the incoming DNA can genetically recombine with (physically integrate into) the host chromosome and is subsequently expressed (Freeman, 1985, p. 184). Later studies demonstrated that it is possible to introduce plasmid DNA of two incompatibility groups (P and W) into the organism by conjugation or transformation (Weiss et al., 1983; Weiss et al., 1984). Smith et al. (1986) isolated antibiotic-resistant and auxotrophic mutants of B. pertussis and used these as recipients for the uptake from Escherichia coli of broad-host-range R plasmids. Auxotrophs are organisms which through genetic recombination cannot grow on a medium on which its parents could grow because of its requirement for substances the medium does not contain (Barnhart & Barnhart, 1987). These studies further amplify the uniqueness of plasmids and their obvious value in the identification of different strains of B. pertussis.

There is a high probability that the majority of bacteria

isolated in a clinical microbiology laboratory will have unique and strain-specific plasmid profiles or "fingerprints" (Farrar, 1983; Kloos et al., 1979; Portnoy et al., 1981; Shlaes & Currie-McCumber, 1986; Takahashi & Nagano, 1984; Tenover, 1984; Thompson & Falkow, 1983). An examination of the content of several bacterial isolates of the same species should therefore reveal whether or not these organisms are of the same strain.

This thesis is designed to evaluate plasmid isolation procedures for clinical samples of B. pertussis obtained from the Kansas State Health Laboratory. The goal is to accomplish the plasmid isolations using methods that are rapid, and require a minimum of laboratory equipment. Protocols requiring two hours or less for actual isolation, and using no more complicated laboratory equipment than a microcentrifuge are the most desirable. Attainment of the goal would provide researchers with a tool that could allow them to fingerprint suspected Bordetella isolates during epidemics.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The Kansas Department of Health and Environment laboratories provided clinical samples of *Bordetella sp*. consisting of five isolates each of *B. pertussis* and *B. parapertussis*. *Escherichia coli* containing PBR 322 and PBR 329 plasmids were provided by Dr. John J. Iandola, Division of Biology, Kansas State University. An *E. coli* strain containing a plasmid designated J53 was provided by Dr. Glendon Miller, Department of Biological Science, Wichita State University. Dr. Glen Andrews, Division of Biochemistry, University of Kansas Medical Center, provided an *E. coli* strain containing a plasmid designated pIBI 620-Luc.

Lambda DNA

Bacteriophage lambda DNA was used as a marker standard in the agarose gels. Some lambda DNA was graciously donated by Dr. Iandolo's laboratory. Additional quantities, including some digested with EcoR1, were procured from Sigma Chemical Co. (St. Louis, MO).

Restriction Endonucleases and Nucleases

The enzymes EcoR1, HIND III and Hae II were obtained from Sigma. The Bgl 1 and Hinc II were purchased from Promega (Madison, WI). Some ribonuclease (RNase A) was provided by Dr. Iandolo's laboratory. Additional quantities of RNase A and deoxyribonuclease 1 (DNase 1) were purchased from Sigma and stocked at a concentration of 10 mg/ml.

Chemicals and Reagents

A STET buffer containing 8 % sucrose, 0.5 % Triton X-100, 40 mM

EDTA (pH 8.0), and 10 mM Tris-Cl (pH 8.0); and a 7.5 M ammonium acetate buffer were required for the boiling water bath (BWB) plasmid isolation process. The isolated plasmids were stored in a 10 mM Tris, 1 mM EDTA (pH 8.0) buffer (TE) at -20°C to 4°C. Buffer A, the running buffer for electrophoretic separation, consisted of a solution containing 400 mM Tris-acetic acid and 20 mM disodium EDTA (pH 8.0), which was diluted 10 times with deionized distilled water for use.

Solutions I, II and III are required for the alkaline lysis technique. Solution I contained 50 mM glucose, 25 mM Tris (pH 8.0), and 10 mM EDTA. Solution II was prepared by combining 2.6 ml of deionized distilled water, 60 microliters of 10 N NaOH, and 300 microliters of 10 % SDS. Solution III was a 3 M potassium (K), 5 M Acetate (Ac) mixture composed of 60 ml of 5 M K Ac, 11.5 ml of glacial acetic acid, and 28.5 ml of deionized distilled water. The isolated plasmids were stored in a TE buffer containing DNase-free pancreatic RNase (RNase A) at a concentration of 20 micrograms per milliliter.

The stock phenol was stored at -20°C and was thawed in a water bath at 50°C. After 30 min, 10 ml of phenol was placed in a 50 ml beaker and 5 ml of TE buffer was added. The pH of the solution was adjusted to 8.0. It was allowed to stand at room temperature (RT) for 20 min, and the pH was again adjusted to 8.0. The phenol/TE was then placed in a sterile bottle and wrapped in aluminum foil for storage at room temperature. The chloroform was mixed with isoamyl alcohol at a ratio of 24:1, and was stored at RT in a sterile bottle covered with aluminum foil.

The New Wash was used in the Geneclean (Bio 101, Inc.) procedure. It consisted of a mixture of Tris-acid and Tris-base at a pH range of

7.0 to 8.5, and was prepared by adding the contents of a 7 ml screwcap plastic vial provided by the manufacturer to 140 ml of deionized distilled water in a 500 ml flask. The solution was mixed to dissolve all salts. Then 155 ml of 100 % ethanol was added, and the solution was again mixed thoroughly. The New Wash was stored at -20^{o}C.

High ionic strength activity buffers were used for the DNA digestion procedures. The activity buffer for digestion by Hind III contained 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl, and 1 mM dithiothreitol, concentrated 10 times and diluted for use. The EcoRl activity buffer consisted of 10 mM MgCl, 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 100 mg/l of bovine serum albumin (BSA), concentrated 10 times and diluted for use. The Hae II activity buffer contained 50 mM Tris-HCl (pH 8.4), 10 mM MgCl, and 50 mM NaCl. The Hinc II activity buffer had 10 mM TrisCl (pH 7.5),100 mM NaCl, 7 mM MgCl, and 0.1 mg/ml BSA. The activity buffer for Bgl I contained 100 mM Tris-Cl (pH 8.0), 60 mM NaCl, 5 mM MgCl, and 0.1 mg/ml BSA. Both the Hinc II and Bgl I activity buffers were provided by the manufacturer (Promega). The stop buffer, used to stop the digestion process, contained 125 mM EDTA (pH 7.5).

Isopropylnol, isoamyl alcohol, and ethanol were obtained from stock. Lysozyme (Sigma) was used in the isolation procedures at a concentration of 10 mg/ml in water. Sodium dodecyl sulfate (SDS) and lithium dodecyl sulfate (LDS) were obtained from Sigma.

The acid precipitation procedure employs a TEL buffer consisting of 10 mM Tris HCL, 10 mM EDTA, and 0.1 % LDS (pH 8.0); 0.2 % v/v acetic acid in deionized distilled water; 10 M ammonium acetate; and RNase A solution (10 milligrams per milliliter).

Except as indicated, all reagents were prepared from stock.

Growth Media

Mueller Hinton (M-H) broth (BBL, Cockeysville, MD) was used for the growth of E. coli and to make cell suspensions for plasmid isolation. The agar was removed from the stock M-H media by filtration through Whatman disc filter papers. Nutrient agar (NA) slants were prepared by adding Noble agar to nutrient broth in distilled water, autoclaving, and adding ampicillin at a concentration of 50 micrograms per ml. The Bordet-Gengou (B-G) agar contained B-G agar base, glycerol, distilled water, and 10 % sterile defibrinated sheep blood (The Brown Laboratory, Topeka, KS). This media was required for the growth of Bordetella sp. Chocolate agar, used for Bordetella parapertussis growth, was prepared by dissolving dehydrated tryptose blood agar base in distilled water and adding 5 % sterile defibrinated sheep blood . This mixture was then heated to 55 degrees centigrade to convert the blood to chocolate blood agar. Luria-Bertani (LB) media, for growth of the 620-Luc-containing bacteria, was prepared by dissolving Bacto-tryptone, Bacto-yeast extract, NaCl, and Nobel agar in deionized distilled water (pH adjusted to 7.5 with 1N NaOH). The mixture was autoclaved and cooled to 50^{o}C. Ampicillin was then added at a concentration of 50 micrograms per ml.

Plasmid Isolation Procedures

Boiling Water Bath (BWB)

Modified procedures of Holmes and Quigley (1981) were used to isolate plasmids using the BWB technique. For the isolation of plasmids from E. coli, cells were inoculated onto a NA slant

containing 50 micrograms per ml of ampicillin and were incubated at 37°C for 24 hours. A loopful of this growth was then transferred to either 3 ml of M-H broth with ampicillin, or to a petri dish containing NA and ampicillin, followed by incubation for 24 hours. The Bordetella pertussis were grown on B-G agar for 4 to 8 days. Then 1.5 ml of the cell suspension, or 10 loopfuls of the growth on agar plus 500 microliters of M-H broth, were transferred to a 1.5 ml polypropylene (Eppendorf) microcentrifuge tube. The cells were centrifuged in a Brinkmann Model 5414 microcentrifuge at maximum speed for 5 min at room temperature (RT). The pellet was resuspended in 250 microliters of STET at RT. A sterile, flat toothpick was used to loosen the pellet from the sides of the tube. The tube was then vortexed to produce a homogeneous suspension. Five microliters of fresh lysozyme (10 mg/ml) were added to the tube and it was again vortexed. The tube was then incubated at RT for 2 min followed by an exposure for 45 sec to a boiling water bath (BWB). After gently tipping back and forth to mix the tube, it was centrifuged for 10 min in the microcentrifuge. The pellet was removed with a sterile toothpick or the supernatant was transferred to another Eppendorf tube. To the supernatant was added 100 microliters of 7.5 M ammonium acetate and 500 microliters of isopropylnol at RT. After mixing by hand, the tube was allowed to stand for 15 min at RT. Then 500 microliters of 95 % ethanol was added to the tube followed by a 30 sec centrifugation. The supernatant was decanted and the inverted tubes were drained on a paper towel. The tube was kept inverted on a paper towel until dry (at least 20 min). From 10 to 25 microliters of TE buffer were added to the tube. The buffer was run down along

the sides of the tube to resuspend all pelleted material. The lysate was then treated with a restriction enzyme, treated with RNase to digest the RNA that is co-purified with the plasmid DNA, run on an agarose gel, and/or placed at -20°C to 4°C for later analysis.

Sonication

A variation of the BWB method was performed to determine the effect of sonication on plasmid yield. To 500 microliters of M-H broth was added 10 loopfuls of an 8-day growth of B. pertussis. The mixture was centrifuged for 5 min. The pellet was resuspended in 500 microliters of STET, to which 20 microliters of lysozyme was added. After a 10 min sonication in a Heat Systems-Ultrasonics Sonicater (Ultrasonic Processor Model 385, Heat Systems-Ultrasonics, Inc., Farmingdale, NY), the tube was placed in boiling water for 45 sec. The BWB method was then completed on the sample.

GENECLEAN

The GENECLEAN method (BIO 101, Inc., La Jolla, CA) is a DNA purification process that eliminates the need for RNase and removes co-contaminating protein. This method also provides an isolation protocol that is quite effective and that requires no alcohol precipitation. With this technique, a 1.4 ml suspension of cells in M-H broth was centrifuged in a microcentrifuge for 30 sec. The pellet was resuspended in 100 microliters of STET and vortexed. Ten microliters of lysozyme (10 mg/ml) were added, vortexed and the Eppendorf tube was placed in an ice bath for 10 min. A 2 min BWB was followed by a 15 min spin (microcentrifuge) at 4°C. The supernatant was transferred to another Eppendorf tube for purification. Next, 100 microliters of NaI and 5 microliters of "Glassmilk" (supplied by Bio

The supernatant was transferred to another Eppendorf tube for purification. Next, 100 microliters of NaI and 5 microliters of "Glassmilk" (supplied by Bio 101, Inc.) were added to the lysate that was produced during the plasmid isolation protocol. After 5 min incubation at RT, the tube was centrifuged for 5 sec. The pellet was washed 3 times with 400 microliters of New Wash. The tube was then inverted on a paper towel to remove excess fluid. Ten microliters of sterile, deionized distilled water were added to the pellet, and it was placed in a water bath at 55°C for 3 min. After 30 sec centrifugation, the supernatant was transferred to a clean Eppendorf tube. This procedure was then repeated from the point where ten microliters of sterile water was added to the pellet, and the supernatant was added to the same clean Eppendorf tube. The tube containing the purified plasmid was kept at 4°C or colder while awaiting further treatment/analysis.

Alkaline Lysis

A rapid plasmid preparation that proved extremely powerful was performed by pelleting 3 ml of a cell suspension in an Eppendorf tube. This was done by placing 1.5 ml of suspended cells in a tube followed by a 10 min centrifugation at 4°C. The step was repeated for the remaining 1.5 ml of cells in the same tube. The tube was well drained on a paper towel and placed on ice. The cell pellet was resuspended in 100 microliters of solution I. A sterile toothpick was used as a stirring rod to suspend the pellet. Ten microliters of solution I containing 10 mg/ml of lysozyme was added. The tube was then allowed to incubate for 5 min. at RT. At this point, 200 microliters of freshly prepared solution II was added. The tube was mixed by

inversion and placed on ice for 5 min. Then 150 microliters of solution III were added and the tube was finger vortexed. The sample was placed on ice for 5 min followed by centrifugation for 5 min at 4°C. The supernatant was collected with a Pasteur pipet (care was taken to avoid all precipitate), and was immediately placed on ice (Griffith, 1988). This lysate was then digested with an enzyme, or was GENECLEAN-treated prior to enzyme treatment, followed by electrophoresis.

Phenol/Chloroform Extraction

The alkaline lysis procedure is enhanced when the lysate produced is treated with equal volumes of phenol and chloroform (Griffith, 1988; Ziai et al., 1989) prior to Geneclean treatment or any other analysis. To accomplish the extraction, an equal volume of phenol/TE was added to the lysate in an Eppendorf tube. After 2 min vortex at RT, the tube was centrifuged at 4°C for 5 min. The aqueous phase was collected with a pipet and transferred to a clean tube. An equal volume of chloroform/isoamyl alcohol was added to the aqueous phase, and the tube was mixed by hand for one minute, then vortexed for one minute. The tube was centrifuged for 5 min at 4°C. The aqueous phase was collected and transferred to a clean Eppendorf tube. The lysate was then placed in an ice bath or stored at 4°C or colder until further analysis.

Acid Precipitation

This technique (Ziai et al., 1989), requiring neither lysozyme, phenol nor chloroform, is a rapid isolation method following the precipitation of bacterial proteins with dilute acetic acid. Five

of M-H broth in Eppendorf tubes. After 30 sec centrifugation, all fluid was removed and the pellet was suspended in 300 microliters of TEL. The tubes were placed in a boiling water bath for 2 min followed by incubation at RT for 2 min. Then 10 microliters of RNase A was added and the tubes were vortexed. After a 15 min incubation, 300 microliters of 0.2 % acetic acid was added. The tubes were shaken gently to mix, 100 microliters of ammonium acetate was added, and they were shaken again. After a 10 min centrifugation, 650 microliters of the supernatant was placed in a clean Eppendorf tube and 650 microliters of isopropylnol was added. The tubes were mixed by hand and were immediately centrifuged for 20 min at RT. All but 20 microliters of the supernatant was removed and discarded. The pellet was rinsed twice with cold 80 % ethanol. All fluid was removed and the tube was inverted on a paper towel for 20 min to allow the plasmid to dry. The plasmid was suspended in 50 microliters of TE buffer and was immediately placed in an ice bath or stored at -20°C.

Restriction Endonuclease Digestion

The standard procedure used to digest DNA lysates with restriction endonucleases was to place 5 microliters of the DNA in a clean Eppendorf tube, add 15 microliters of (1X) activity buffer, and mix by gently tapping the tube with the fingers. Then one unit of the RE was added to the mixture and it was again mixed by tapping. The tube was placed in an incubator at 37°C for one hour. Every 15 minutes of incubation, the tube was gently mixed by tapping. The tube was removed from the incubator at the end of one hour and 2 microliters of stop buffer (125 mM EDTA, pH 7.5) was added. The RE-treated lysate was either stored at -20°C, or subjected to

electrophoresis.

Gel electrophoresis

A 1 % agarose gel was used for the separation of chromosomal and nuclease-treated plasmid DNA. The gel was prepared by adding 0.33 g of DNA grade agarose (Sigma) to 30 ml of Buffer A. This mixture was heated to boiling until all the agarose granules were dissolved. It was cooled to the touch and poured into a plate. A mold for the sample wells was placed in the soft gel and it was allowed to polymerize for at least 60 min. After the mold was removed, the sample wells were washed with Buffer A. A Kimwipe (Kimberly-Clark Corperation, Neenah, WI) was used to absorb the fluids from the wells. One microliter of 2 % bromocresol blue tracking dye (Sigma) was placed into each well prior to the loading of the samples. The lysate samples were loaded into the wells. The running buffer (Buffer A) was added until it reached the top of the gel without covering it. An electrical charge of 6 volts/cm was applied to the gel for 5 min. The gel was then covered with Buffer A to a level of 2 mm. A 6 V/cm charge was applied to the gel for 100 to 120 min. At the end of this time, the gel was stained for 60 min with ethidium bromide (EB, Sigma). The EB (0.5 micrograms per ml) was stored at 200X concentration and was diluted by adding 99.5 ml of deionized distilled water to 0.5 ml of (200X) EB. The gel was destained twice with 30 ml of deionized distilled water, placed under UV illumination, and photographed with a Polaroid camera using Polaroid 107 (3000 speed) Black and White Instant Film Packs.

RESULTS

Plasmid Isolation Procedures

In this study, variations on the boiling water bath method, the alkaline lysis procedure, and the acid precipitation protocol for plasmid isolation were conducted. The procedures utilized E. coli containing known plasmids as controls. The experimental organisms were strains of Bordetella. The results were based on the interpretation of photographs taken of samples after treatment as outlined in Materials and Methods, and as described below.

Boiling Water Bath

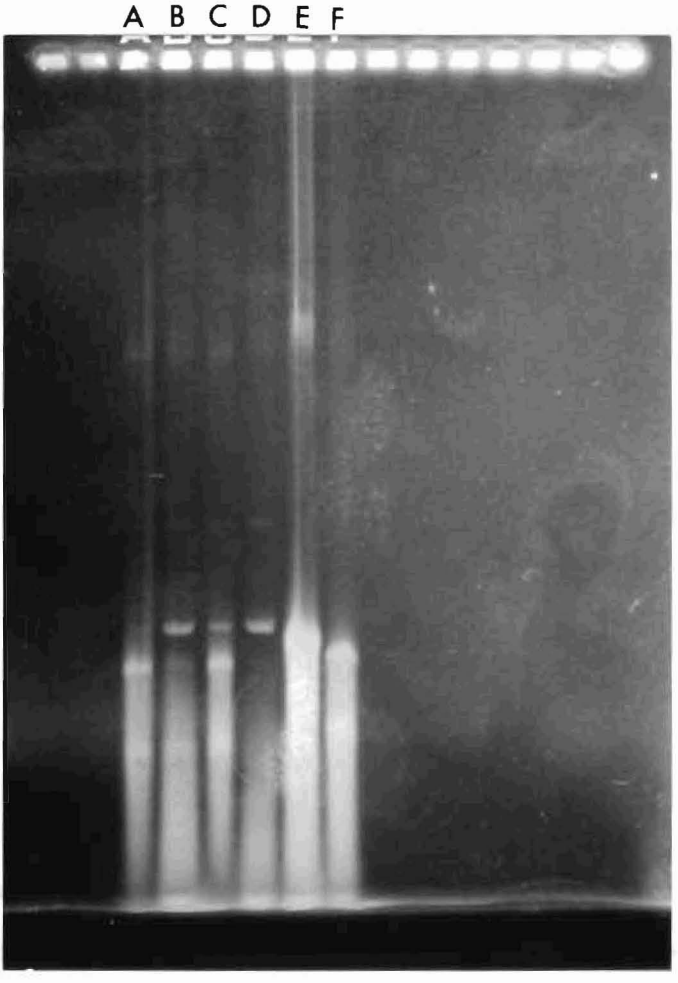
One loopful of organisms (E. coli) containing the plasmids J53, pBR 322 and pBR 329 was streaked onto nutrient agar slants and incubated for 24 hours at 37°C. From each slant, one loopful of organisms was removed and inoculated into separate, screw-cap test tubes containing 3 ml of M-H broth. The test tubes were incubated for 15 hours at 37°C followed by a 10 min centrifugation (4000rpm) at room temperature (RT). The pellets were resuspended (vortexed) in 3 ml of cold (ice bath), freshly prepared STET buffer. Then 0.5 ml of each mixture was transferred by pipet to separate, clean Eppendorf tubes. The tubes were centrifuged for 18 sec at 4°C (cold room, CR), and the pellets were resuspended in 50 microliters of STET buffer. To each tube was added 5 microliters of freshly prepared lysozyme (10 mg/ml). The tubes were placed in a boiling water bath (BWB) for 50 sec, vortexed for 10 sec, and centrifuged for 5 min (CR). The pellets were removed by a flat toothpick and discarded. An equal volume (55

microliters) of dry-ice-cooled isopropynol was added to the supernatant in each tube. The tubes were placed in an ice bath for 5 min. If a precipitant failed to occur, another 55 microliters of dry-ice-cooled isopropynol was added to the tube and it was again incubated in an ice bath for 5 min. The tubes were centrifuged for 5 min (CR) and the supernatant was removed by a pipet. To each tube was added 20 microliters of sterile, deionized distilled water. The tubes were placed in an ice bath for 60 min to solubilize the DNA. Each tube was mixed gently with a pipet followed by gel electrophoresis for 90 min. No evidence of DNA was detected in the gel (photographs not available).

A BWB experiment was conducted using reagents that were not prepared fresh (not prepared on the day of the experiment), wherein all the protocol was performed at RT. Test organisms containing pBR 322, pBR 329, and J53 plasmids were grown for 24-hours in 3 ml of M-H broth and on Nutrient Agar plates. The results of that experiment are provided in Figure 1. The organisms grown on broth (lanes A, D and F) were found to produce less DNA than those grown on plates (lanes B, C and E). Six bands are visible in the pBR 322 plate lysate (lane B), while only 4 DNA bands can be seen in the broth lysate (lane A). Five DNA bands are evident in the pBR 329 plate lysate (lane C), but just 2 bands are detectable in the broth lysate (lane D). The lower J53 bands are badly smeared, but a more intense upper band is visible in the plate lysate (lane E).

Each of the Bordetella samples provided by the Kansas State Health Laboratory were grown for 4 days on B-G agar. They were subjected to the BWB method, digested with EcoR1 for 2 hrs at 37^{o}C,

Figure 1. Agarose gel electrophoretogram of pBR 322, pBR 329 and J53 plasmids extracted from E. coli with the BWB method at RT using stored reagents (see Materials and Methods). Organisms were grown on M-H broth (lanes A, D and F) and Nutrient Agar plates (lanes B, C and E). Lanes A and B contain pBR 322, while pBR 329 is in lanes C and D, and J53 is in lanes E and F. An .8 % agarose gel was used. Gel run time: 90 min. Stained 30 min with EB. Film: Polaroid 107, 3000 (speed); 10 sec. exposure, IWF (indoors without flash); 60 sec develop.


A B C D E F

and run on an agarose gel for 100 min. The controls for this experiment were B. pertussis and B. parapertussis lysates that had neither received heat nor restriction endonuclease (RE) treatment. Figure 2 shows evidence of DNA in all control lanes (lanes B, D, F, H, J, L, N, P and R). The bands are most intense in the B. parapertussis control lanes (lanes L, N, P and R). All detectable Bordetella DNA bands are in the 21 kb (21,000 bp) range. The experimental (EcoR1-treated) lanes (Lanes C, E, G, I, K, M, O, Q and S) also show DNA bands that are much less intense than the controls. Again, the strongest experimental bands are in the B. parapertussis samples (lanes M, O, Q and S). All experimental bands appear to be in the 21 kb range; therefore, the EcoR1 failed to digest any of the Bordetella lysates.

A 7-day growth of B. pertussis (P1) on B-G agar was subjected to the BWB method. To determine whether chromosomal DNA was a component in the 21 kb DNA band of B. pertussis, a reextraction was applied to the pellet that resulted after the boiling step (see Materials and Methods). The pellet was treated as if it were a normal growth of Bordetella organisms, and was subjected to the complete BWB protocol. Figure 3 shows that the chromosomal DNA is completely lost (lane C), and provides evidence that the control DNA band (produced by the normal BWB method, lane B) is indeed all extrachromosomal, plasmid DNA with approximately 21,000 bps.

In an effort to obtain a more intense DNA band, and to determine the effects of ribonuclease A (RNase A) treatment on the lysates, a variation to the protocol was to sonicate the organisms prior to the boiling step followed by RNase A treatment. The control for this

Figure 2. Agarose gel electrophoretogram of plasmids extracted from clinical isolates of Bordetella pertussis and B. parapertussis with the BWB method. The lysates were digested with EcoR1 for 2 hrs at 37°C (see Materials and Methods). The molecular weight marker, EcoR1-digested bacteriophage lambda DNA, is in lane A. Lanes B-K contain the B. pertussis lysates P1 through P5, in order (see Table 1). The B. parapertussis lysates (PP1 through PP4, in order) are in lanes L-S. The controls were Bordetella lysates that had received neither heat nor RE treatment. All control lanes show evidence of DNA (lanes B, D, F, H, J, L, N, P and R). The RE-treated lysates are in lanes C, E, G, I, K, M, O, Q and S. Lanes L-S (B. parapertussis) contained the most intense bands. All detected DNA is in the 21 kb (21,000 bp) range (see Results). A 1 % agarose gel was used. Wells were washed with running buffer (pH 7.9) after polymerization. Eight microliters were loaded into each Bordetella well; 10 microliters in the marker well. Condition run: 5 min. Gel run: 100 min. Stained 60 min with EB. Film: Polaroid 107, 3000 (speed); 60 sec exposure, IWF; 60 sec develop.

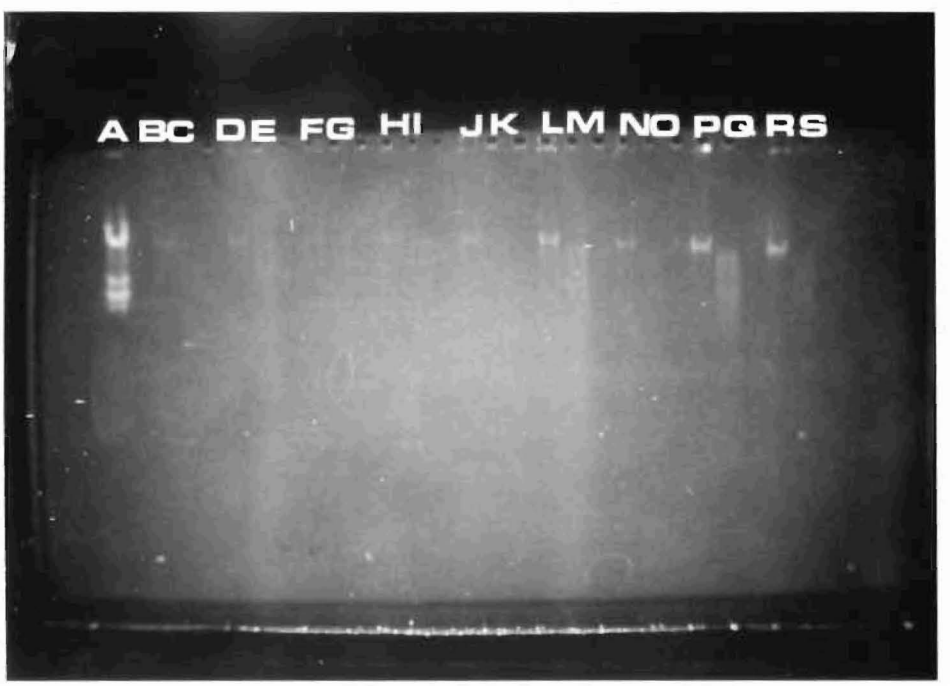

A BC DE FG HI JK LM NO PQ RS

Figure 3. Agarose gel electrophoretogram of plasmids extracted from a clinical isolate of B. pertussis (P1) using the BWB method (see Materials and Methods). Lane A contains the molecular weight marker (EcoR1-digested bacteriophage lambda DNA). The control, the standard BWB method, is in lane B. A reextraction was applied to the pellet that results after the boiling step (see Materials and Methods) in an attempt to determine if chromosomal DNA is a component of the 21 kb band (see Results). No DNA bands were detected in the reextracted lysate (lane C). A 5-days old, 1 % agarose gel was used. Wells were washed with running buffer (pH 8.0) after polymerization of the gel. One microliter of marker, and 10 microliters of sample lysate were loaded into respective wells. Condition run: 5 min. Gel run: 100 min. Stained 60 min (EB). Film: Polaroid 107, 3000 (speed); 30 sec exposure, IWF; 40 sec develop.


A B C

experiment employed the BWB method on the same strain of Bordetella (P2) without sonication (see Figure 4a, lane B). The result was a band that was less intense than the control (lanes C and D). There was a large smear of nucleic acid produced with this method. Treatment of the sonicated sample with 1 microliter of RNase A failed to eliminate the smear (lane D). Evidence of a second band below the 3530 bp marker is also prevalent in the control and the sonicated samples (see lanes B, C and D).

To re-examine the effect of RNase and to determine the effect of DNase on the sonicated and non-sonicated samples, another ribonuclease treatment experiment was conducted. This experiment used two controls: the sonicated and non-sonicated lysates from the previous experiment. The non-sonicated, non-nucleased lysate control is in Figure 4b, lane B. An intense 21 kb DNA band is apparent. When this lysate was treated with one microliter of DNase, the upper (21 kb) band was lost (lane C). The non-nucleased, sonicated control shows evidence of the 21 kb band (lane D). When the sonicated lysate was treated with one microliter of RNase A, the 21 kb band remained (lane E). When this lysate was treated with one microliter of DNase, the 21 kb band was lost (lane F). This provided evidence that the 21 kb band is DNA. However, when the sonicated, RNase-treated lysate was retreated with 3 microliters of RNase A (three times the amount normally used), the 21 kb band was lost (lane G). This was probably due to artifact (heat or DNase contamination) rather than solely attributed to the effects of RNase action. While the nucleic acid smear remained in the sonicated lysates, it was less intense in the four-times-RNase-treated lysate (lane G). This provided more evidence

Figure 4a. Agarose gel electrophoretogram of plasmids extracted from a clinical isolate of B. pertussis (P2) using the BWB method supplemented with sonication of the organisms prior to the boiling step, followed by RNase A treatment (see Materials and Methods). The molecular weight marker, bacteriophage lambda DNA digested with EcoRl, is in lane A. The control, P2 subjected to the BWB method without sonication, is in lane B. Lane C contains the sonicated sample without RNase, while the sonicated, RNase-treated lysate is in lane D. All B. pertussis lysates show evidence of a 21 kb DNA band and a second band below the 3530 bp marker. The sonicated lysates produced large nucleic acid smears (lanes C and D) which were not eliminated by the RNase treatment (see Results). A 1 % agarose gel, aged for 24 hrs, was used. The wells were washed with running buffer (pH 8.0) after gel polymerization. One microliter was loaded into the marker well, 5 microliters were loaded into the control well, and 3 microliters were loaded into each of the sonicated wells. Condition run: 6 min. Gel run: 100 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 30 sec. exposure, IWF; 40 sec develop.

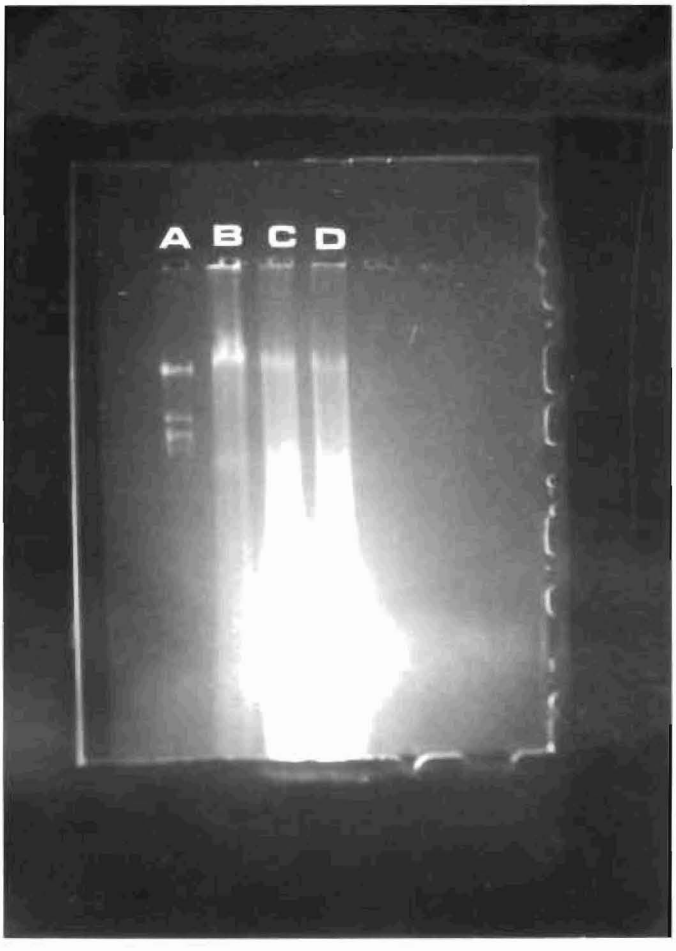

A B C D

Figure 4b. Agarose gel electrophoretogram of plasmids stored for 24 hrs at -20°C. The plasmids were extracted from a clinical isolate of B. pertussis (P2) using the BWB method supplemented with sonication of the organisms prior to the boiling step, followed by RNase treatment (see Materials and Methods). The molecular weight marker, bacteriophage lambda DNA digested with EcoR1, is in lane A. The control, P2 extracted with the BWB method without sonication, is in lane B. Lane C contains some of the same lysate used in the control lane treated with one microliter of DNase. The sonicated lysate without RNase or DNase (the sonicated control) is in lane D. The sonicated lysate treated with one microliter of RNase is in lane E. The sonicated lysate treated with one microliter of DNase is in lane F. Lane G contains some of the same lysate used in lane E, but treated with an additional 3 microliters of RNase. The 21 kb band was lost after treatment with DNase and 4 microliters of RNase (see Results). A 1 % agarose gel was used. The wells were washed with running buffer (pH 7.9) after gel polymerization. Two microliters were loaded into lane A, 2 microliters into lane B, 5 microliters were loaded into lanes C, E, F and G, and 3 microliters were placed into lane D. Condition run: 5 min. Gel run: 100 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 30 sec exposure, IWF; 40 sec develop.


A B C D E F G

that the nucleic acid smear was RNA. The detectable 21 kb band from the non-nucleased, sonicated lysate (lane D) was not as intense as that from the non-nucleased, non-sonicated lysate (lane B). This would indicate that the BWB method without sonication is better than the BWB method with sonication.

Another variation to the BWB method in the treatment of Bordetella samples involved increasing the lysozyme concentration to 2, 3 and 4 times (2X, 3X and 4X) that recommended (Figures 5a, b and c respectively). The tube that received 2X the lysozyme concentration was allowed to stand at room temperature for 3 min. The 3X and 4X lysozyme concentrations were kept at room temperature for 8 min each (see Materials and Methods). Each lysate produced by these variations was treated with Hind III and EcoR1. The lysates produced by the 2X and 4X lysozyme concentrations were incubated for 1 hour after RE treatment. A 10 min incubation was allowed for the 3X lysozyme concentration lysate. In the 2X lysozyme treatment (Figure 5a), two strains of B. pertussis were used (P2 and P4). The controls, lysates produced by the BWB technique without RE treatment, are in lanes B and D. The P2 was treated with two units of Hind III (lane C) while the P4 was EcoR1-treated (two units). The samples appeared to be lost in lanes D and E due to possible thin bottoms in wells. Two DNA bands are evident in the P2 samples (lanes B and C). The Hind III failed to digest the Bordetella (lane C). Three strains of Bordetella (P2, P3 and P5) were used in the 3X treatment. The controls were lysates obtained with the BWB method without RE treatment (lanes C, E, G, I and K). Two DNA bands can be detected in lanes C, E and G. One weak upper (21 kb) band appears in lanes I and K. Four units of Hind III failed to digest the Bordetella (lanes B, D, F and J). Two units of

Figure 5a. Agarose gel electrophoretogram of plasmids extracted from clinical isolates of B. pertussis (P2 and P4) using the BWB method with two times the recommended lysozyme, digested with Hind III and EcoR1 (see Materials and Methods). The molecular weight marker, bacteriophage lambda DNA digested with EcoR1, is in lane A. The P2 and P4 controls, untreated lysates, are in lanes B and D respectively. Two units of Hind III was used to treat the P2 lysate which was placed in lane C, while lane E contains the P4 lysate that was treated with two units of EcoR1 (in kilobase, Kb, units). The samples appear to have been lost in lanes D and E, attributed to thin bottoms in wells, and the Hind III did not digest the P2 lysate (see Results). A 1 % agarose gel, aged for 4 days, was used. The wells were washed with running buffer (pH 8.0) after polymerization. Three microliters of marker was loaded into lane A and 5 microliters of the appropriate samples were loaded into lanes B-E. Condition run: 5 min. Gel run: 111 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 30 sec exposure, IWF; 40 sec develop.

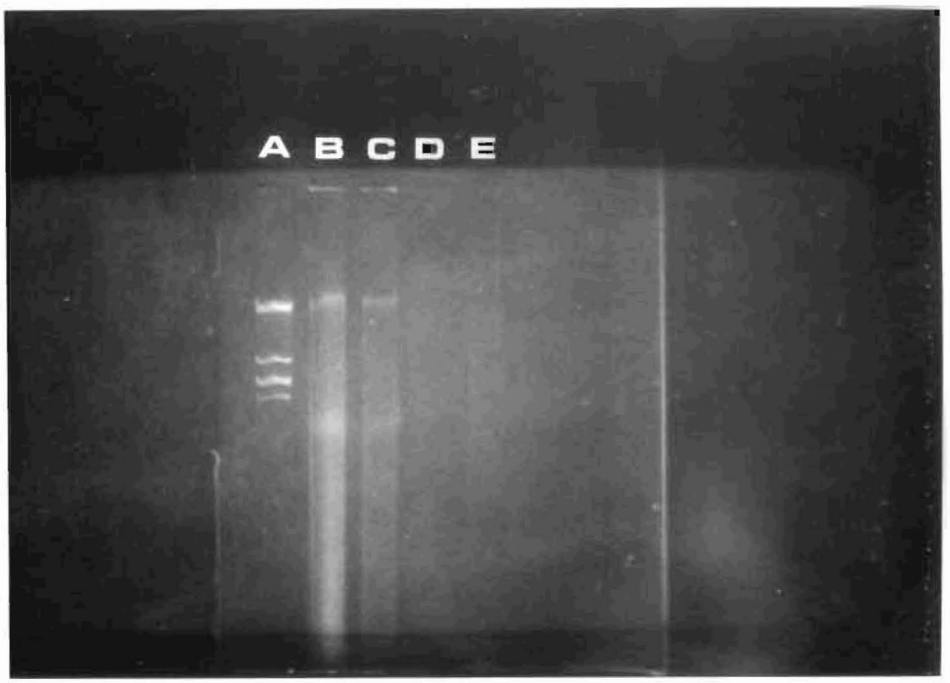

A B C D E

Figure 5b. Agarose gel electrophoretogram of plasmids extracted from clinical samples of B. pertussis (P2, P3 and P5) using the BWB method with three times the recommended lysozyme, digested with Hind III and EcoR1 (see Materials and Methods). Lane L contains the molecular weight marker, EcoR1-digested bacteriophage lambda DNA. The controls, untreated, BWB-derived lysates are in lanes C (P2, 7-days growth), E (P2, 9-days growth), G (P3, 7-days growth, 5 % SRBC: sheep red blood cells), I (P3, 7-days growth, 10% SRBC), and K (P5, 7-days growth, 10% SRBC). The Hind III-treated lysates are in lanes B (P2, 7-days), D (P2, 9-days), F (P3, 5 %), and J (P5, 10 %). The EcoR1-treated lysates are in lanes A (P2, 7-days), and H (P3, 10 %). While the 21 kb band is evident in most samples, two DNA bands can be detected in lanes C, E and G (see Results). A 1 % agarose gel was used. The wells were washed in running buffer (pH 8.0) after polymerization of the gel. Three microliters were loaded into the marker lane, 7 microliters were placed in each of lanes A-C, and 5 microliters were loaded into each of lanes D-K. Condition run: 5 min. Gel run: 110 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 30 sec exposure, IWF; 40 sec develop.

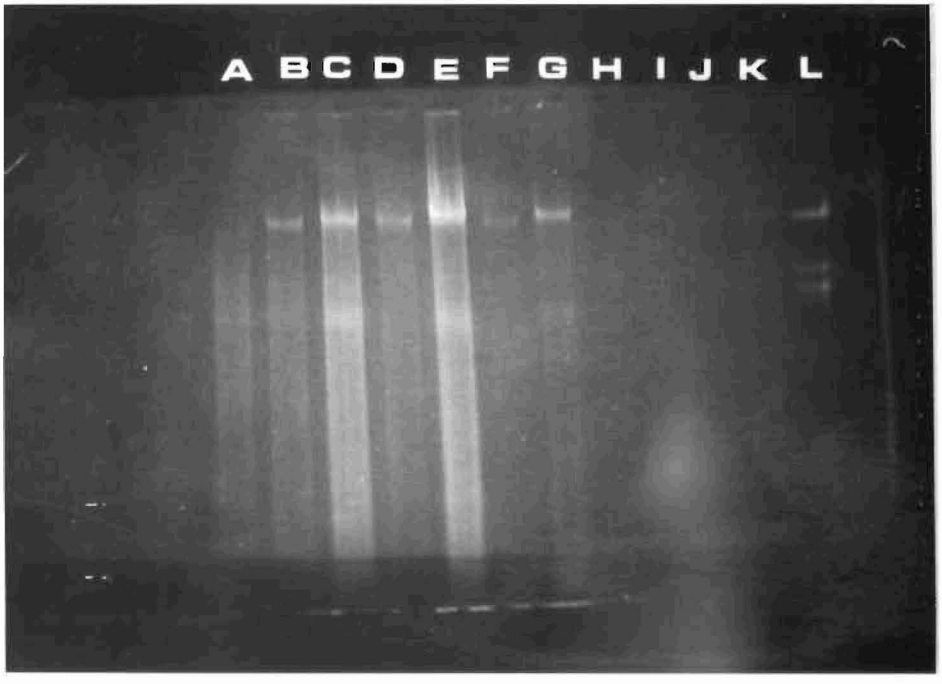

A B C D E F G H I J K L

Figure 5c. Agarose gel electrophoretogram of plasmids extracted from clinical isolates of B. pertussis (P2) using the BWB method with three times the recommended lysozyme, digested with Hind III and EcoR1 (see Materials and Methods). The molecular weight marker, EcoR1-digested bacteriophage lambda DNA, is in lane A. The control, P2 lysate without RE treatment, is in lane B. Lane C contains the Hind III-treated lysate, and lane D contains the EcoR1-treated lysate. While the 21 kb band in the control was more intense than the 1X or 2X lysozyme treatment, no banding was detectable in the RE-treated lysates (see Results). A 1 % agarose gel, aged 6 days, was used. The wells were washed with running buffer (pH 8.0) after polymerization of the gel. Three microliters of marker was placed into lane A, and 5 microliters of sample was loaded into each of lanes B-D. Condition run: 5 min. Gel run: 100 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 30 sec exposure, IWF; 40 sec develop.


A B C D

EcoR1 appeared to either marginally digest (lane A), or over digest (lane H) the Bordetella lysates. One Bordetella strain (P2) was used in the 4X treatment. The control, BWB technique without RE treatment, shows an intense upper band and a weak lower band of DNA (lane B). Neither the Hind III-treated (four units), nor the EcoR1-treated (two units) lysates (lanes C and D respectively) show evidence of any DNA. This too could be due to thin well bottoms. The DNA bands progressively increased in intensity as the lysozyme concentration increased. There was also an increase in the amount of nucleic acid smear as a function of the lysozyme concentration. It was desirable to reduce the nucleic acid smear while enhancing the intensity of the DNA band produced. Two times the lysozyme concentration (10 microliters) was therefore used in subsequent protocols.

Geneclean

In an attempt to obtain a purer, RNA-free lysate, the Geneclean technique was employed. When the Geneclean method was used on E. coli containing pBR 322, however, the intensity of the 21 kb band ranged from barely detectable (Figure 6a, lane E) to weakly visible (Figure 6b, lanes B and D).

The experiment represented by Figure 6a involved E. coli grown for 24 hours on 2 nutrient agar plates. Ten loopfuls of organisms from one plate were subjected to the BWB method (see Materials and Methods), and represented the control for the experiment (lane B). The Geneclean protocol, as outlined in Materials and Methods, was applied to 10 loopfuls of organisms from the second plate (experimental, lane D). Three distinct DNA bands resulted with the BWB method (lane B). Both the control and the experimental were

Figure 6a. Agarose gel electrophoretogram of pBR 322 plasmids extracted from *E. coli* using the BWB method and the Geneclean procedure followed by digestion with Hinc II (see Materials and Methods). Lanes A and F contain the molecular weight marker, EcoR1-digested bacteriophage lambda DNA. Lane B contains the BWB control, untreated lysate. The BWB lysate treated with Hinc II is in lane C. Lane D contains the Geneclean-treated control (no RE treatment). The RE-treated, Geneclean-extracted lysate is in lane E. The BWB lysate was digested by Hinc II, but no bands are evident in the Hinc II-treated, Geneclean lysate (see Results). A 1 % agarose gel, aged 19 days, was used. The wells were washed with running buffer (pH 7.8) after polymerization. Three microliters were loaded into each of the marker wells, and 6 microliters were loaded into each of the sample lanes (B-E). Condition run: 5 min. Gel run: 103 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 30 sec exposure, IWF; 40 sec develop.

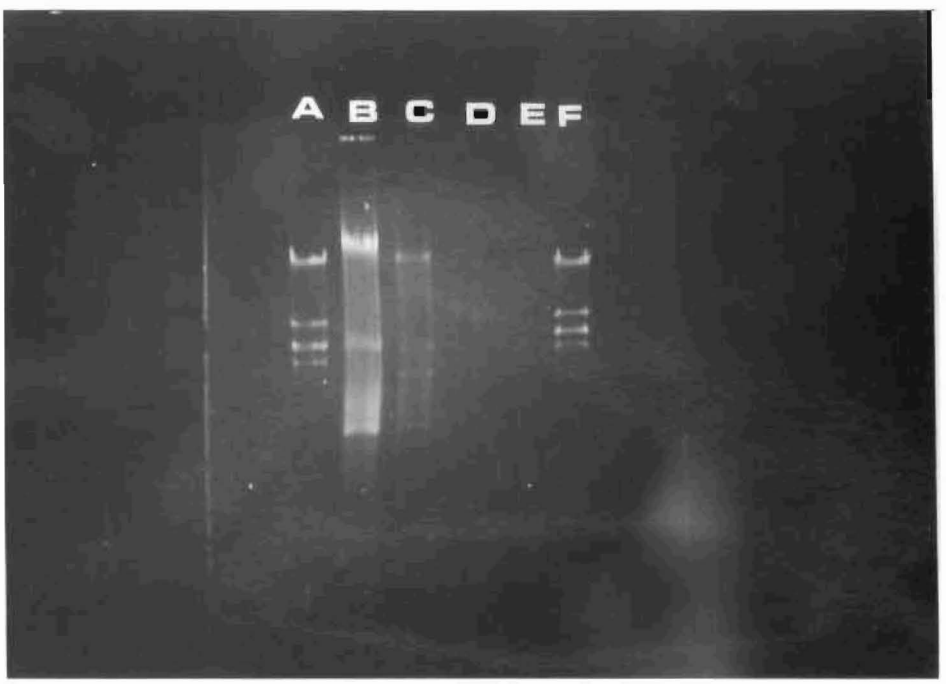
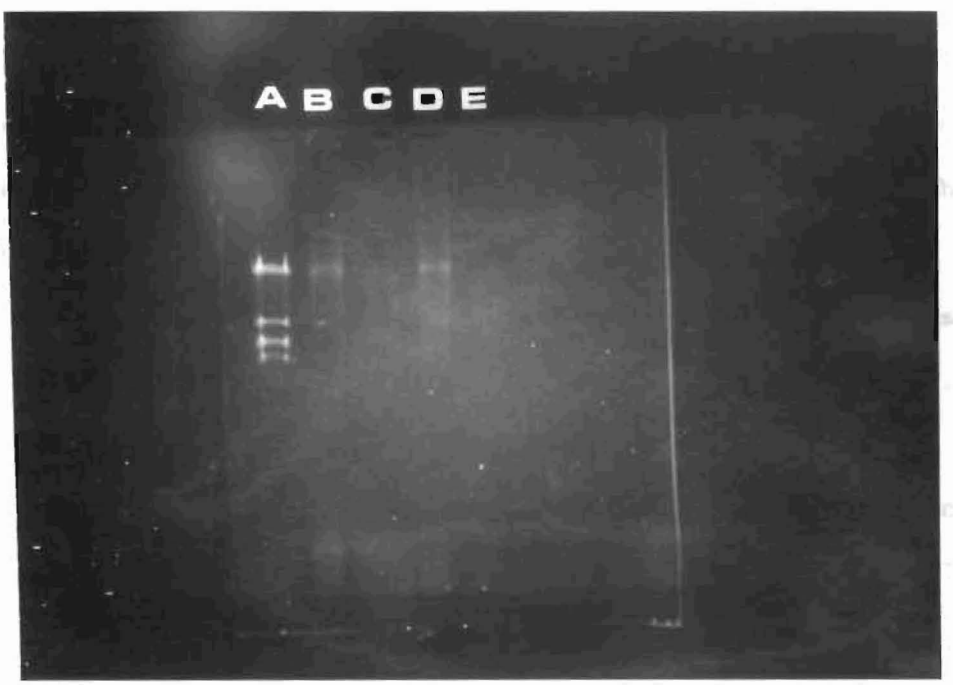

A B C D E F

Figure 6b. Agarose gel electrophoretogram of pBR 322 plasmids extracted from E. coli using the Geneclean procedure followed by digestion with Bgl I (see Materials and Methods). Lane A contains the molecular weight marker, EcoRl-digested bacteriophage lambda DNA. The controls, Geneclean-derived lysates with no RE treatment, are in lanes B and D. The lane B control was allowed to incubate at RT for 5 min after the addition of the NaI and "Glassmilk", while the lane D control incubated in an ice bath for 5 min (see Materials and Methods). Lane C contains the RT-incubated lysate that was treated with Bgl I. Lane E contains the lysate incubated in an ice bath that was treated with Bgl I. Weak bands occurred with this technique (see Results). A 1 % agarose gel was used. The wells were washed with running buffer (pH 7.8) after polymerization. One microliter of tracking dye was placed into each well, then 3 microliters of marker was loaded into lane A, and 6 microliters of sample was placed into each of lanes B-E. Condition run: 5 min. Gel run: 100 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 30 sec exposure, IWF; 40 sec develop.


A B C D E

treated with one unit of Hinc II for 15 min. The Hinc II digested the middle band in the BWB lysate producing 2 bands (lane C). No bands could be detected in the Hinc II-treated Geneclean lysate (lane E).

Figure 6b represents an experiment wherein E. coli harboring pBR 322 was incubated for 24 hours in 3 ml of M-H broth. Two Eppendorf tubes then received 1.4 ml of the growth which was subjected to the Geneclean protocol (see Materials and Methods). One lysate was allowed to incubate in an ice bath for 5 min after the addition of the NaI and "Glassmilk", while the other incubated at RT for 5 min. Both lysates then completed the standard Geneclean protocol as shown in Materials and Methods. Following the Geneclean treatment, the lysates were treated with one unit of Bgl I for 15 min. The control lysates (Genecleaned without RE, lanes B and D) show weak 21 kb bands. No banding is evident in the experimental lanes (lanes C and E). All Geneclean-treated lanes show much reduced RNA contamination.

When two strains of B. pertussis (P2 and P4) were subjected to the complete Geneclean protocol, no DNA was evident (Figure 7, lanes D, E, F and G). A weak band was detected (Figure 7, lane H) when a previously isolated and frozen lysate (P2, obtained using the BWB method) was subjected to the Geneclean washes. This was accomplished by the addition of the sodium iodine and "Glassmilk" to the lysate followed by the completion of the Geneclean process (see Materials and Methods). When the same lysate was treated with one unit of Hinc II for 15 min, no bands were detected (lane I). Again, however, reduced RNA contamination, one of the goals of the Geneclean process, was achieved. It was evident that the Bordetella lysates that had received no RE treatment used in experiments to this point, were producing the same DNA band (a 21 kb band). Sample numbers were

Figure 7. Agarose gel electrophoretogram of pBR 322 and plasmids extracted from clinical isolates of B. pertussis (P2 and P4) using the BWB and Geneclean protocols, digested with Hinc II (see Materials and Methods). Lane A contains the molecular weight marker, EcoR1-digested bacteriophage lambda DNA. The controls for the untreated (no RE) lysates, produced with the Geneclean protocol, are in lanes B (pBR 322), D (the P2 control, an isolate under continuous incubation for 46 days (13 days at 37 degrees centigrade, and 33 days at RT), and F (the P4 control). Lane H contains the control for a P2 lysate that was extracted with the BWB method, stored at -20°C for 10 days, subjected to the Geneclean washes (see Materials and Methods), and received no RE treatment. The Hinc II-treated, pBR 322 lysate is in lane C. The 45-days incubation lysate treated with Hinc II is in lane E. Lane G contains the Hinc II-treated, P4 lysate. In lane I is the Hinc II-treated, BWB/Geneclean washed lysate. Only the previously frozen lysate produced a detectable DNA band (see Results). A 1 % agarose gel, aged for 3 days, was used. The wells were washed with running buffer (pH 7.9) after polymerization. Samples loaded into wells: lane A, 4 microliters; lane B, 8 microliters; and lanes C-I, 7 microliters each. Condition run: 5 min. Gel run: 105 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 30 sec exposure, IWF; 40 sec develop.


A B C D E F G H I

therefore not referenced in further experiments.

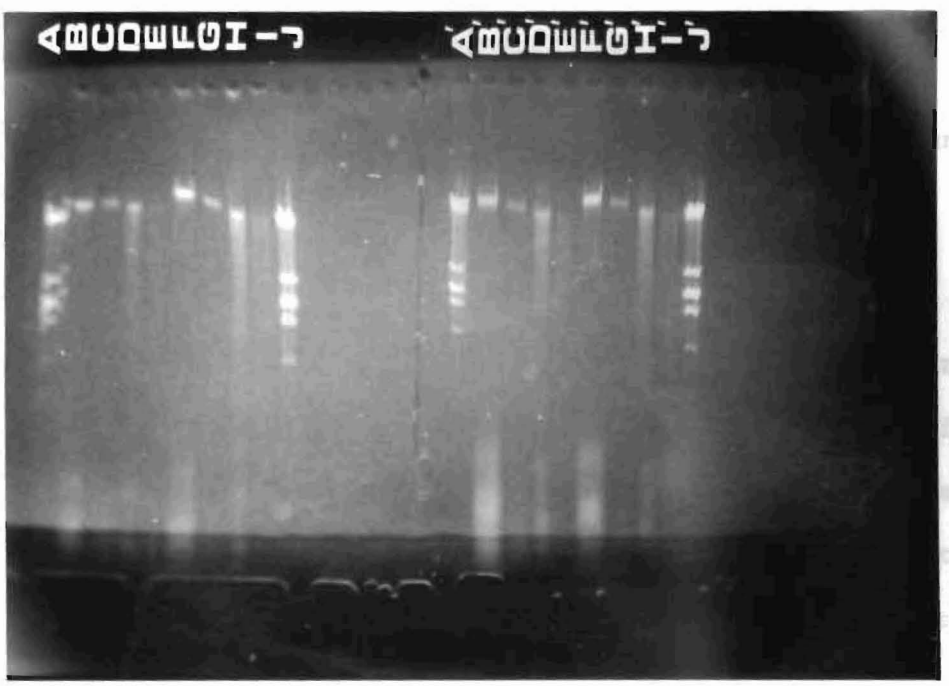
Alkaline Lysis With Lysozyme/Geneclean

Single large and medium colonies of E. coli containing the 620-LUC plasmid, grown on L-B media were treated with the alkaline lysis procedure followed by Geneclean treatment and EcoR1 digestion (two units) for 15 min. Figure 8 shows the results of that experiment. The controls were a large sized colony, no Geneclean, no RE (lanes B and B'), and a medium sized colony, no Geneclean, no RE (lanes F and F'). Only the 21 kb DNA band can be seen in all lanes except lanes E, E', I and I' (large colony + Geneclean + EcoR1, and medium colony + Geneclean + EcoR1 respectively). As evidenced in lanes C and C' (large sized colony, no Geneclean, + EcoR1) and G and G' (medium sized colony, no Geneclean, + EcoR1), the EcoR1 failed to digest the DNA bands. The large and medium sized, Genecleaned colonies (lanes D and D', and H and H' respectively) did produce intense banding in the 21 kb area; however, there is also evidence of nucleic acid smearing in both lanes. The lambda marker (lanes A, A', J and J') was heated for 5 min at 90°C. This resulted in 5 distinct DNA bands.

A thicker gel was used in this experiment as well. The usual thickness was 30 ml of 1 % agarose (see materials and methods), while the experiment used a thickness obtained with 50 ml of 1 % agarose. The 30 ml gel is the right half of Figure 8 (lanes A' through J'); the 50 ml gel is on the left. The 30 ml gel thickness produced clearer, less distorted bands.

Two loopfuls of a 24-hour, L-B media grown 620-LUC were transferred to each of 4 Eppendorf tubes containing 500 microliters of

Figure 8. Agarose gel electrophoretogram of 620-LUC plasmids extracted from large and medium colonies of E. coli with the Geneclean protocol, and digested with EcoR1 (see Materials and Methods). The marker, bacteriophage lambda DNA digested with EcoR1 and heated at 90°C for 5 min, is in lanes A, A', J and J'. The controls are a large colony, no Geneclean washes (LN), no RE (lanes B and B'), and a medium colony, no Geneclean washes (MN), no RE (lanes F and F'). The EcoR1-treated LN lysate is in lanes C and C'. The large colony, treated with Geneclean (LG), no RE, is in lanes D and D'. The EcoR1-treated LG lysate is in lanes E and E'. The EcoR1-treated MN lysate is in lanes G and G'. The medium colony, treated with Geneclean (MG), no RE, is in lanes H and H'. The EcoR1-treated MG lysate is in lanes I and I'. While intense DNA bands were detected, no RE digestion of Bordetella occurred (see Results). Used in this experiment were gels made with 30- and 50 ml of 1 % agarose (lanes A-J and A'-J' respectively). The 50 ml gel was aged 4 days. The 30 ml gel was aged 18 days. Wells were washed with running buffer (pH 7.8) after polymerizations. Lanes A and A' were loaded with 6 microliters of marker, while 10 microliters were placed in each of lanes B-I. Condition run: 5 min. Gel run: 120 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 90 sec exposure, IWF; 40 sec develop.


ABCDEFGHI-J
A'B'C'D'E'F'G'H'I'-J'

M-H broth. After subjecting each tube to the alkaline lysis procedure, the final supernatant was pooled into one tube. The concentrated lysate was treated with one or two units of EcoR1 for 60 min followed by gel electrophoresis. As can be seen in Figure 9, the pooled, unheated lysate that had received no RE treatment produced 3 distinct bands (lane F). The lower band (about 4 kb) was lost once the lysate was heated (lanes B, C and D). The RE control (lane B) consisted of pooled lysate heated to 37°C for 60 min. Neither the one unit nor the two units of EcoR1 (lanes C and D respectively) appeared to digest either the upper or the middle band. All Geneclean-treated lysates show reduced RNA contamination.

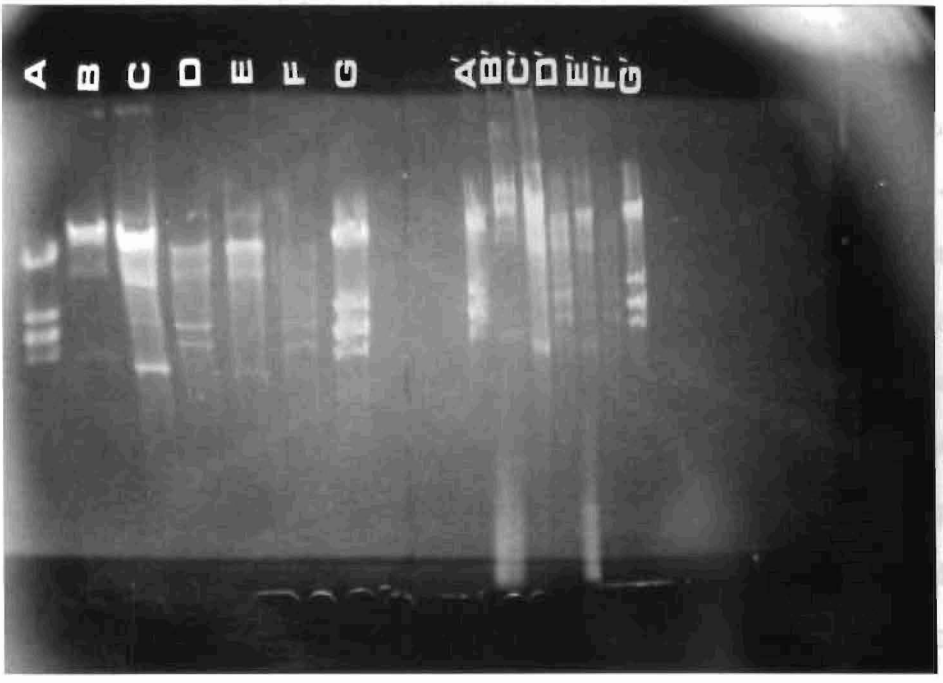
Phenol/chloroform Extraction

The alkaline-treated, pooled lysate (referenced in Figure 9, lane F) was then subjected to phenol/chloroform (P/C) extraction followed by Geneclean treatment (Figure 10). The 4 DNA bands that resulted from this treatment were more intense (lanes C and C') than the 3 bands that are evident in the control: alkaline-treated, pooled lysate that had received no P/C or Geneclean treatment (lane B and B'). The P/C-treated lysate was digested with two units of EcoR1 for 60 min producing a distinctive profile of 2 upper and 2 lower bands (lanes D and D'). Three bands resulted from the lysate that was Genecleaned, but received no P/C treatment (lanes E and E'). When this Genecleaned, no P/C lysate was treated with two units of EcoR1 for 60 min, only the lower 2 bands were clearly (but weakly) visible (lanes F and F'). The upper bands could barely be detected. Lanes A, A', G and G' contain the DNA marker. The lysates treated with Geneclean were generally free of RNA contamination, and the DNA bands

Figure 9. Agarose gel electrophoretogram of pooled 620-LUC plasmids extracted from E. coli using the alkaline lysis procedure, followed by EcoR1 digestion (see Materials and Methods, and Results). Lanes A and E contain the marker DNA, bacteriophage lambda DNA digested with EcoR1, heated for 5 min. at 90°C, and stored for 3 days at -20°C. Lane B contains one of the two controls, pooled lysate heated for 60 min at 37°C, no RE treatment. The second control is in lane F, pooled unheated lysate that had received no RE treatment. In lane C is the pooled lysate treated with one unit of EcoR1. Lane D contains the pooled lysate that was treated with two units of EcoR1. Three distinct bands are detectable in lane F, but the EcoR1 failed to digest the bands (see Results). A 1 % agarose gel, aged for 38 days, was used. The wells were washed with running buffer (pH 7.8) after polymerization. Six microliters were loaded into each of the marker wells, and 8 microliters of samples were placed into each of lanes B, C, D and F. Condition run: 5 min. Gel run: 120 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 90 sec exposure, IWF; 40 sec develop.


A B C D E F

Figure 10. Agarose gel electrophoretogram of pooled 620-LUC plasmids extracted from E. coli with the alkaline lysis procedure, stored for 4 days at -20°C, purified with phenol/chloroform (P/C), treated with Geneclean washes, and digested with EcoR1 (see Materials and Methods, and Results). The marker, bacteriophage lambda DNA digested with EcoR1, is in lanes A, A', G and G'. The control, lysate that had received neither P/C, Geneclean, nor RE treatments, is in lanes B and B'. The lysate treated with P/C and Geneclean, no RE, is in lanes C and C'. Lanes D and D' contain the P/C-Geneclean-RE-treated lysate. Lanes E and E' contain the lysate treated with Geneclean washes only, while this same lysate treated with EcoR1 is in lanes F and F'. Four DNA bands are detectable in control lane C. A distinctive profile was produced by the RE digestion shown in lane D (see Results). Used in this experiment were 1 % agarose gels with two width sizes of wells: 5mm and 3mm (lanes A-G and A'-G' respectively). The gel with 5mm wells was aged 35 days, while that with 3mm wells was prepared on the day of the experiment. Wells were washed with running buffer (pH 7.9) after polymerization. Six microliters were loaded into each of lanes A-G, while 10 microliters were placed into lanes A'-G'. Condition run: 6 min. Gel run: 120 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 90 sec exposure, IWF; 40 sec develop.

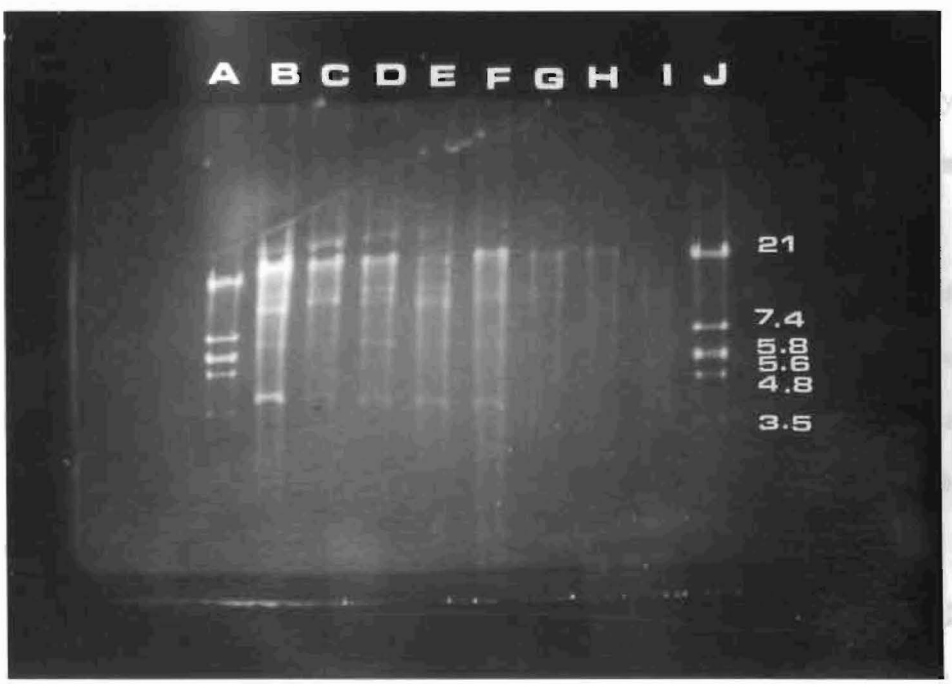

A B C D E F G
A B C D E F G

were more intense than those of the control.

Two width sizes of wells (5mm and 3mm) were used in this experiment also. The gel with 5mm wells (left gel, Figure 10) received 6 microliters of sample, while 10 microliters were loaded into the 3mm wells (right gel, Figure 10, lanes A' through G'). The wider wells produced the clearest, less distorted bands.

Two loopfuls of a 24-hour growth of bacteria containing 620-LUC were transferred to each of 2 Eppendorf tubes containing 500 microliters of M-H broth. The tubes were treated with the alkaline lysis procedure. The pooled lysate (400 microliters) was extracted with P/C. In a separate tube, 400 microliters of an alkaline-lysis-extracted lysate that had been stored at -20°C (the same lysate that was referenced in Figure 9, lane F) was also extracted with P/C. Both lysates were divided in half, producing 4 tubes containing about 200 microliters in each. All tubes were then treated with Geneclean, and the resultant lysates were pooled separately. Each of the 2 pooled lysates were digested with two units each of EcoRl and Bgl I for 60 min. The results of this experiment are provided in Figure 11. Lanes B-E represent the lysate that was stored at -20°C, and the freshly prepared lysate was placed in lanes F-I. The most intense bands appear in the lysate that was stored at -20°C. The intensity of the bands in the freshly prepared lysate ranged from fair (lanes F and G) to poor (lanes H and I). All Geneclean-treated lanes are free of RNA contamination (lanes B-I). Six distinct DNA bands are visible in control lanes B and C (pooled lysate + P/C, and pooled lysate + P/C + 37°C for 60 min respectively). In the heat-treated control, the two lowest bands (about 4 kb and 6.2 kb), and the middle upper band (about

Figure 11. Agarose gel electrophoretogram of pooled 620-LUC plasmids extracted from E. coli with the alkaline lysis procedure, stored at -20°C for 7 days, or pooled, 620-LUC lysates freshly extracted with the alkaline lysis method on the day of the experiment. These lysates were phenol/chloroform (P/C)-purified, Geneclean-treated, and digested with EcoR1 or Bgl I (see Materials and Methods, and Results). Lanes A and J contain the EcoR1-digested, bacteriophage lambda (marker) DNA (Kb). The control for the stored lysate, P/C and Geneclean-treated, no RE, is in lane B. Lane C contains the stored lysate that was heated for one hour at 37°C. Lanes D and E contain the EcoR1- and Bgl I-treated, stored lysates, respectively. The control for the freshly prepared lysate, P/C and Geneclean-treated, no RE, is in lane F. Lane G contains the fresh lysate that was heated for one hour at 37°C. Lanes H and I contain the EcoR1- and Bgl I-treated, fresh lysates, respectively. More intense banding occurred with the stored lysates (see Results). A 1 % agarose gel was used. The wells were washed with running buffer (pH 7.9) after polymerization. Six microliters were loaded into each marker well, while 10 microliters were placed into each of lanes B-I. Condition run: 5 min. Gel run: 124 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 45 sec exposure, IWF; 40 sec develop.


A B C D E F G H I J
21
7.4
5.8
5.6
4.8
3.5

19 kb) are very weak in intensity (lane C). Neither the EcoR1 nor the Bgl I (lanes D and E respectively) produced distinctive profiles. As was evident in a previous experiment (Figure 9), the plasmid bands became weaker after the lysate shown in lane B was heated (see lane C). The lambda marker was heated 5 min at 90°C. All 6 DNA bands are detectable (lanes A and J).

Alkaline Lysis Without Lysozyme

An alkaline lysis procedure using a 30-second centrifugation of the test organisms suspended in M-H broth, and employing no lysozyme was performed on bacteria containing 620-LUC and pBR 322 plasmids. Very intense DNA bands were produced with this method (Figure 12, lanes B and C). The lambda marker in lanes A and E was heated 5 min at 80°C. Five distorted bands were produced with this procedure. A lambda marker sample was previously heated for 5 min at 90°C and was reheated in the same manner after storage at -20°C. This lambda sample was loaded into lane D and produced 5 distinct bands.

When the same alkaline lysis method was applied to Bordetella samples, bands were clearly visible in the 21 kb range (Figure 13, lanes D, E, I and J). The most intense, heavily concentrated DNA bands were in the B. parapertussis lanes (lanes E and J).

In the same gel (Figure 13), a lambda temperature treatment experiment was conducted wherein unheated DNA (control) was loaded into lanes A and F. Lanes B and G contain lambda heated 3 min at 90°C Lanes C and H were loaded with DNA that was heated 5 min at 90°C. Lanes K and L contain previously heated DNA (90°C for 5 min) that was stored at -20°C. Distorted bands resulted in lanes A, B and C. Lanes K and L contain a single DNA band in the 21 kb range.

Figure 12. Agarose gel electrophoretogram of pBR 322 and 620-LUC plasmids extracted from E. coli with the alkaline lysis procedure, without the use of lysozyme (see Materials and Methods). Lanes A and E contain the molecular weight marker, bacteriophage lambda DNA digested with EcoR1, and heated for 5 min at 80°C. The pBR 322 lysate is in lane B. The 620-LUC lysate is in lane C. Lane D contains the lambda marker that had been heated for 5 min at 90°C, stored for 7 days at -20°C, and reheated for 5 min at 90°C. Very intense plasmid DNA bands are detectable with this method (see Results). A 1 % agarose gel was used. Wells were washed with running buffer (pH 7.95) after polymerization. Six microliters were loaded into lanes A and E, while 10 microliters were loaded into each of lanes B, C and D. Condition run: 5 min. Gel run: 120 min. Stained (EB): 67 min. Film: Polaroid 107, 3000 (speed); 93 sec exposure, IWF; 40 sec develop.

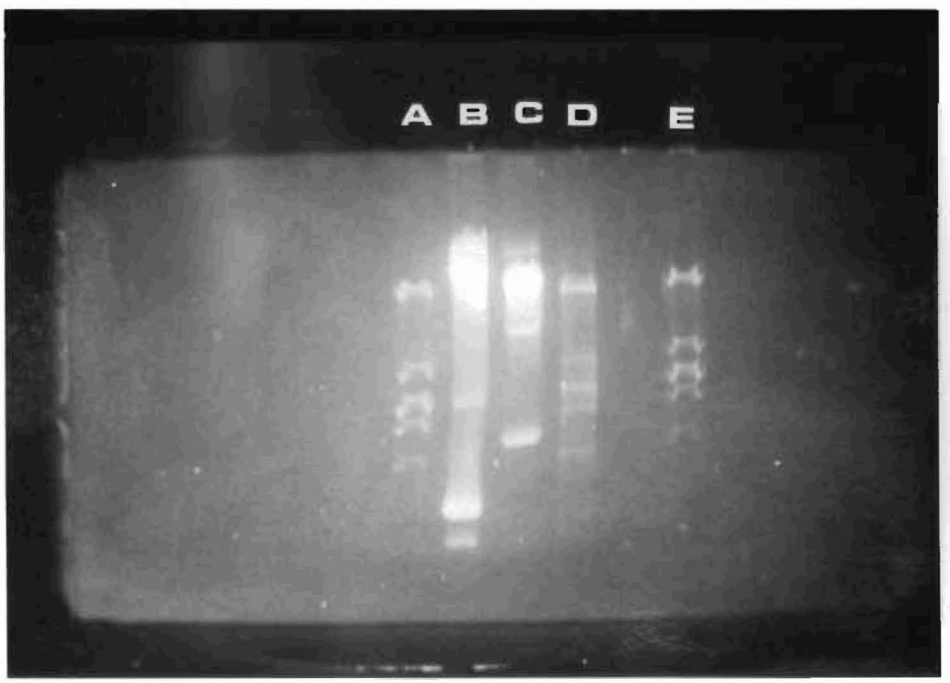
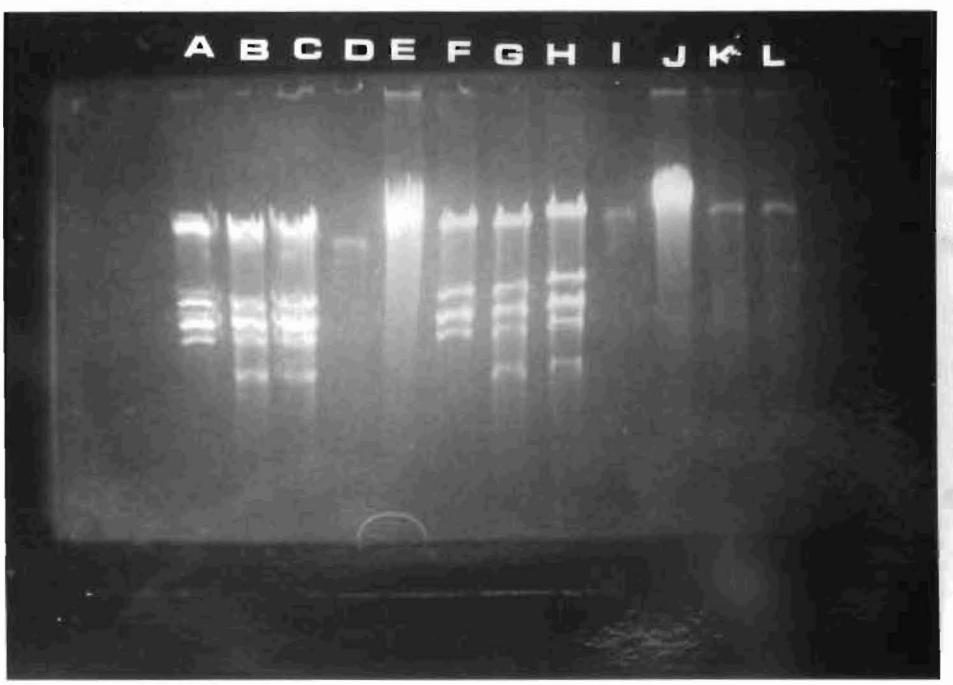

A B C D E

Figure 13. Agarose gel electrophoretogram of plasmids extracted from clinical samples of B. pertussis and B. parapertussis using the alkaline lysis procedure without lysozyme (see Materials and Methods). Lanes A and F contain unheated, EcoRl-digested, bacteriophage lambda (marker) DNA. Lanes B and G contain marker DNA that was heated for 3 min at 90°C. Lanes C and H contain marker DNA that was heated for 5 min at 90°C. Lanes D and I contain the B. pertussis lysate. The B. parapertussis lysate is in lanes E and J. In lanes K and L is the lambda marker that was heated for 5 min at 90°C and stored for 18 days at -20°C. The B. parapertussis lysate produced the most intense 21 kb band (see Results). A 1 % agarose gel, aged 14 days, was used. The wells were washed with running buffer (pH 7.7) after polymerization. Ten microliters were loaded into each well. Condition run: 5 min. Gel run: 120 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 90 sec exposure, IWF; 40 sec develop.


A B C D E F G H I J K L

Clearly defined, intense banding occurred in lanes F, G and H. Lane H contained the most intense bands. Therefore, the best procedure is to heat the lambda 5 min at 90°C.

The same alkaline lysis method was applied to fresh Bordetella isolates grown on blood agar plates for 4 days. The lysates were then treated with one unit of Bgl I for 15 min. The DNA bands produced were extremely intense (Figure 14, lanes C-H, and L-M). The lysates were not digested by Bgl I (lanes F, H, N and O).

Acid Precipitation

The acid precipitation method was used on B. pertussis samples, and produced weak DNA bands in the 21 kb range (Figure 15, lanes D, I and N). Chromosomal DNA can be detected in the loading wells at the top of the B. pertussis lanes. Similar results were obtained when bacteria containing pBR 322 and 620-LUC plasmids were treated with this method (Figure 16, lanes E and H respectively). The acid precipitation method does produce clear, RNA-free lysates.

Figure 14. Agarose gel electrophoretogram of plasmids extracted from clinical isolates of B. pertussis and B. parapertussis with the alkaline lysis method, followed by digestion with Bgl I (see Materials and Methods). Unheated, EcoRl-digested, bacteriophage lambda (marker) DNA is in lanes A and J. Lanes B and K contain lambda marker DNA that was heated for 3 min at 90°C. Lanes C and L contain untreated B. pertussis lysate. Lanes D and M contain unheated B. parapertussis lysate. The B. pertussis control, lysate heated for 15 min at 37°C, is in lane E. The Bgl I-treated B. pertussis control is in lanes F and N. The B. parapertussis control, lysate heated for 15 min at 37°C, is in lane G. The Bgl I-treated B. parapertussis is in lanes H and O. An unknown sample, provided by Dr. Sobieski, is in lane I. Heavy concentrations of DNA were produced with this method without RE digestion (see Results). A 1 % agarose gel was used. The wells were washed with running buffer (pH 7.9) after polymerization. Six microliters of marker were loaded into each of lanes A and J. All remaining wells were loaded with 8 microliters each. Condition run: 5 min. Gel run: 120 min. Stained (EB): 62 min. Film: Polaroid 107, 3000 (speed); 120 sec exposure, IWF; 40 sec develop.

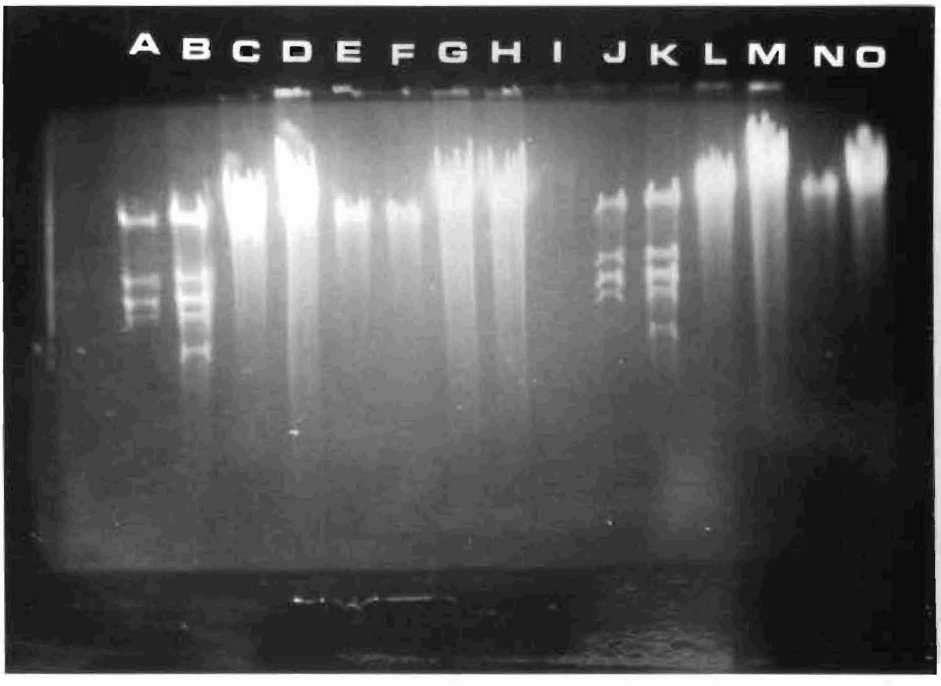

A B C D E F G H I J K L M N O

Figure 15. Agarose gel electrophoretogram of plasmids extracted from clinical samples of B. pertussis using the acid precipitation protocol (see Materials and Methods). Lanes A, F and K contain unheated, EcoR1-treated, bacteriophage lambda (marker) DNA. Lanes B, G and L contain marker DNA that was heated for 3 min at 90°C, and stored for 4 days at -20°C. In lanes C, H and M is marker DNA that was heated for 3 min at 90°C on the day of the experiment. Lanes D, I and N contain the B. pertussis lysate. Lanes E and J were not loaded with any materials. Chromosomal DNA was produced with this method along with weak 21 kb bands (see Results). A 1 % agarose gel was used. The wells were washed with running buffer (pH 7.9) after polymerization. Eight microliters were loaded into lanes D and I. Three microliters were loaded into lane N. All remaining lanes were loaded with 5 microliters each. Condition run: 6 min. Gel run: 120 min. Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 90 sec exposure, IWF; 40 sec develop.

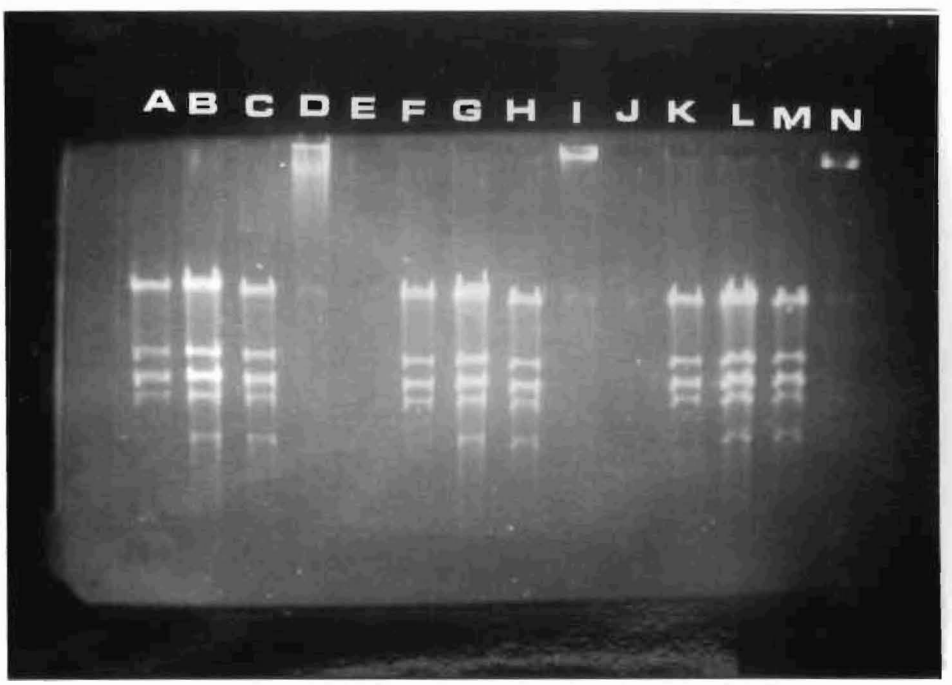
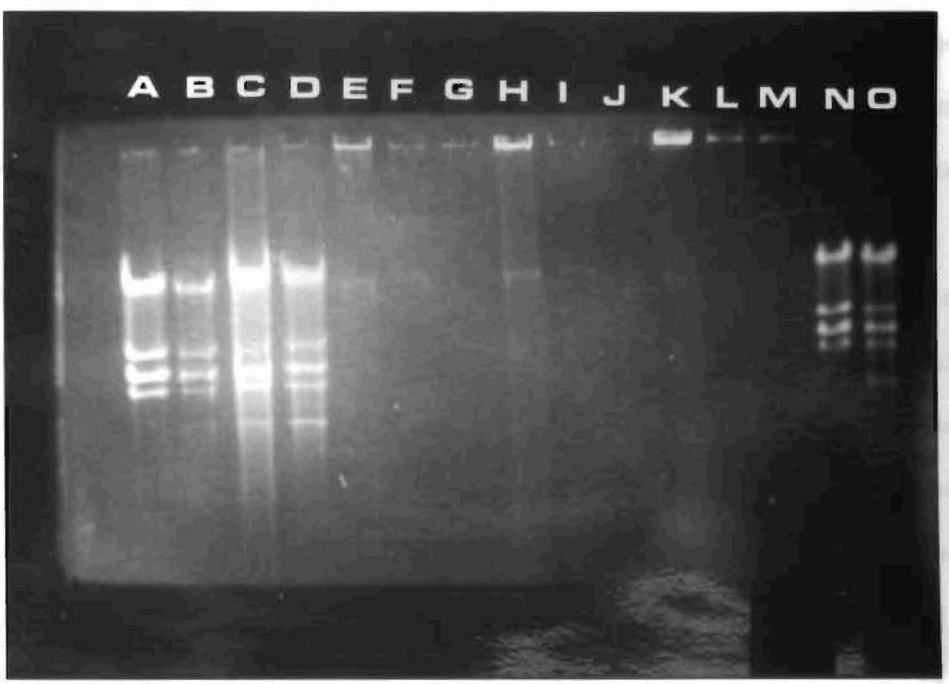

A B C D E F G H I J K L M N

Figure 16. Agarose gel electrophoretogram of pBR 322 and 620-LUC plasmids extracted from E. coli, and plasmids extracted from clinical samples of B. pertussis using the acid precipitation method, digested with Hinc II or Hae II (see Materials and Methods). Lanes A and N contain unheated, EcoRl-treated, bacteriophage lambda (marker) DNA. In lane B is marker DNA, heated for 3 min at 90°C, and stored for 16 days at -20°C. Lane C contains marker DNA, heated for 11 min at 90°C on the day of the experiment. Lanes D and O contain marker DNA, heated for 3 min at 90°. The pBR 322 untreated lysate is in lane E. The pBR 322 control, non-RE-treated lysate, heated for 15 min at 37°C, is in lane F. The Hinc II-treated pBR 322 lysate is in lane G. Lane H contains the untreated 620-LUC lysate. The 620-LUC control, non-RE-treated lysate, heated for 15 min at 37°CC, is in lane I. The Hinc II-treated 620-LUC lysate is in lane J. Lane K contains the untreated B. pertussis lysate. The B. pertussis control, non-RE-treated lysate, heated for 30 min. at 37°C, is in lane L. The Hae II-treated B. pertussis lysate is in lane M. A 1 % agarose gel was used (wells washed). Five microliters were loaded into lanes A-D, N and O, while lanes E-M contain 8 microliters each. Condition run: 5 min Gel run: 120 min Stained (EB): 60 min. Film: Polaroid 107, 3000 (speed); 90 sec exposure, IWF; 40 sec develop.


A B C D E F G H I J K L M N O

DISCUSSION

Plasmid Isolation Methods

Three basic methods of plasmid isolation were utilized in this research: The boiling water bath (BWB), alkaline lysis, and acid precipitation procedures. These protocols differ generally in lysis buffer used and method of lysis, temperature, centrifugation time, and purification techniques.

After obtaining a fresh growth of the test organisms (usually 24 hours for *E. coli*, and 4 to 8 days for *Bordetella*), the pelleted bacteria were suspended in a low-ionic-strength buffer to which a strong detergent was added (SDS or LDS), or in a STET buffer containing the detergent Triton X-100. Lysozyme was used in most protocols, and heat in the form of boiling water was frequently included. This combination of reagents and heat dissolves away the cell membrane and denatures most of the chromosomal DNA to the single-stranded state (Tenover, 1984). The plasmid DNA, which is not readily denatured, remains in its circular, double-stranded molecular configuration (Vinograd & Lebowitz, 1966; Portnoy *et al.*, 1981; Tenover, 1984). The detergent buffer solutions are neutralized by high-ionic-strength reagents, causing coalescence of the large chromosomal DNA strands into bulky, insoluble networks while leaving the plasmid DNA in its circular state. Precipitation of most of the chromosomal DNA, cell wall, and cell membrane material is accomplished by adding a salt solution followed by isopropynol or phenol/chloroform treatment, and removal by centrifugation. The plasmid DNA can then be recovered from the supernatant by ethanol precipitation, centrifugation, and suspension in a low-ionic-strength buffer

(Maniatis et al., 1982; Tenover, 1984; Takahashi & Nagano, 1984).

Boiling Water Bath

The experiment employing ethanol/dry ice-cooled isopropylnol (see BWB section of Results) may have failed for two reasons. One cause may have been due to a lack of incubation time after the addition of the lysozyme. Most protocols (Manaitis et al., 1982; and Takahashi & Nagano, 1984, for example) recommend 3-5 min incubation at RT after adding the lysozyme. The sample was immediately placed in boiling water before the lysozyme had sufficient time to hydrolyze the cell walls. The other reason for the failure may have been because the final sample was not dried before sterile deionized water was added. Researchers recommend drying the final plasmid pellet in a vacuum desiccator for 5-20 min, or inverting the tube containing the pellet on a paper towel for 20-30 min to remove precipitating reagents. The desire is to cause solubilization of the pelleted plasmid, which apparently does not occur if precipitating materials are present (Maniatis et al., 1982).

Plasmid DNA was detected by the BWB method (Figures 1-5). The nuclease experiment (Figure 4b) provided evidence that the 21 kb band is DNA. The reextraction experiment (Figure 3) substantiated the fact that the 21 kb band present in the Bordetella lysates is plasmid and not chromosomal DNA. When the insoluble network of chromosomal DNA and cellular inclusions was subjected to the BWB method (Figure 3), no bands were detected. While the 21 kb band is apparently plasmid DNA, it should be pointed out that contaminating chromosomal DNA may also be present in plasmid-containing lysates and, therefore, detectable in the gel.

Chromosomal DNA is so large (millimeters or centimeters) that their size cannot be determined by agarose gels. However, most preparations of large DNAs are susceptible to extensive shearing by pipets, particularly when subjected to high pH (alkaline lysis) or high temperature (BWB) methods. This shearing causes fragments of contaminating, linear, chromosomal DNA that migrate faster in agarose gels than the open circular, plasmid DNA. Under the artificial conditions of analytical extractions, plasmid DNA often develops internal stresses where the two ends are twisted in opposite directions (superhelical). These molecules are more compact than linear or open circular molecules of the same length; therefore, they would migrate faster in agarose gels (Davis et al., 1980).

Sonication (Figures 4a and b) produced a large amount of undesirable nucleic acid. The lower band, very evident in Figure 4a and less intense in Figure 4b, is either contaminating linear chromosomal DNA or a second plasmid DNA band. Both bands (the upper, 21 kb band and the lower band) were eliminated by DNase treatment. The DNase had no effect on the nucleic acid smear, but 4 microliters of RNase did appear to reduce it somewhat. For these reasons, it is believed that the nucleic acid smear is RNA. The loss of the 21 kb band after the second treatment of the sonicated lysate with RNase (Figure 4b, lane G) should not have happened since the treated lysate was only heated 18 total min. (9 min each treatment) at 37^{o}C. However, heat-treated lysates did tend to lose some of the DNA intensity. This was demonstrated in Figure 9. The uncut, unheated, pooled lysate (lane F) contained 3 distinct DNA bands including a very intense 21 kb band. The control lysate (lane B) was heated 60 min at

37°C. The 21 kb band is much less intense than that in lane F. The 2 lower bands are not detectable. Therefore, the fact that the RNase treatment was 4-times the normal concentration used in published procedures (Maniatis et al., 1982), coupled with the heat, apparently caused the 21 kb band to be lost.

Geneclean

Geneclean is a rapid plasmid purification procedure that is reputed (by Bio 101, Inc.) to be an inexpensive, easy method for desalting or purifying DNA solutions, or removing DNA from agarose. The procedure should remove and purify DNA from agarose gels within 20 min. It should remove unreacted tracer-nucleotides from labeling reactions and/or desalt and concentrate DNA solutions within 15 min without columns, alcohol precipitations or organic extractions. It should eliminate protein, small RNA and other enzyme-inhibiting impurities from DNA solutions without protease, RNase, organic extractions or alcohol precipitations. Traces of residual enzyme-inhibiting phenol, chloroform or ether from DNA extractions should be removed in 15 min. Finally, the protocol should be able to "quickclone" from high melting-point agarose gels. The Geneclean method is fast and did produce clean lanes (Figures 6a, b, 7), but some of the claims of the Geneclean manufacturer were not substantiated during this research. The DNA bands that resulted with this method were not very intense. In some cases, they were not detectable at all (Figure 7, lanes B-G). As noted above, this procedure is supposed to work without organic (solvent) extractions. When the Geneclean method was used in a protocol employing organic (phenol/chloroform) extraction, however, a much more intense band was

produced (Figure 10, lane C).

Alkaline Lysis

Probably one of the most powerful plasmid isolation methods used in this research was the alkaline lysis technique. Two variations were employed: alkaline lysis with lysozyme (Figures 10, 11), and alkaline lysis without lysozyme (Figures 12, 14). These protocols use 0.2 N sodium hydroxide to facilitate a highly alkaline solution (pH of about 12.4) for the SDS treatment of the sample. As pointed out above, the chromosomal DNA is denatured leaving the intact plasmid DNA. Lysozyme is not a vital agent for the dissolution of the cell wall with this procedure. The results point out clearly that the lysate obtained without the lysozyme (Figures 13 and 14) yielded more intense DNA bands than that that resulted with lysozyme (Figure 11). In fact, the intensity was so great that nucleic acid smears occurred (Figure 14).

Phenol/chloroform extraction

All lysates treated with phenol/chloroform (Figures 10-14) produced intense DNA bands. Both reagents were maintained in bottles covered with aluminum foil and stored at room temperature. The phenol solution became brownish in color after 45 days. This same solution was used in an experiment and produced very good results (Figure 12). Kado and Liu (1981) pointed out that brown oxidation pigments usually occur with phenol solutions alone. Maniatis et al. (1982) recommend storage of the phenol solution at $4^{o}C$ for periods of up to 1 month.

Acid Precipitation

The acid precipitation method is a rapid plasmid isolation

procedure that uses dilute acetic acid (0.2 % v/v in distilled water) for the precipitation of bacterial proteins; the detergent action of lithium dodecyl sulfate; and the catalytic effect of heat produced by boiling water. Neither chloroform, phenol, lysozyme, nor ether is required for this technique. It yielded less plasmid DNA than the alkaline lysis protocol and most of the BWB procedures, but produced much cleaner, RNA-free preparations. There frequently is, however, strong evidence of contaminating chromosomal DNA in the wells of agarose gels containing plasmid lysates derived with the acid precipitation method (Figure 15, lanes D, I and N, for example). While this chromosomal DNA is undesirable, it does provide further evidence that its bulky molecular configuration prevents its migration into the gel. This lends further support to the belief that the 21 kb bands detectable in lanes D, I and N of Figure 15 are plasmid DNA. Chromosomal DNA contamination was detected in the BWB method (lane B, Figure 4a) and the alkaline lysis procedure (lane B, Figure 5c, and lanes D and E, Figure 14). This argument, therefore, can be extended to all 21 kb bands detected with plasmid isolation protocols in this investigation.

Restriction Endonuclease Treatment

Table 1 lists the RE's used in this investigation for the digestion of the Bordetella lysates produced by the various plasmid isolation techniques. These enzymes cut DNA within or near their particular recognition sequences, which typically are four to six nucleotides (base pairs) in length with a twofold axis of symmetry (Maniatis et al., 1982). Neither of the RE's produced clearly defined, distinct plasmid profiles. Figure 5(a-c) provides evidence for the

Table 1. Restriction edonucleases used to treat Bordetella samples.

RE NAME	CUT SEQUENCE *
Bgl I	5'GCCNNNN^NGGC 3' 3'CGGN^NNNNCCG 5'
EcoRl	5'G^AATTC 3' 3'CTTAA^G 5'
Hind III	5'A^AGCTT 3' 3'TTCGA^A 5"
Hinc II	5'GTPy^PuAC 3' 3'CAPu^PyTG 5'
Hae II	5'PuGCGC^Py 3' 3'Py^CGCGPu 5'

* The cleavage sites are indicated by the mark (^).

failure of Hind III and EcoR1 digestion of B. pertussis. The EcoR1 does appear to have caused a large reduction in the intensity of the 21 kb band (Figure 5b, lane A), but there does not appear to be any migration of fragmented plasmid DNA into the gel. The Hinc II appears to have over-digested the B. pertussis (Figure 7, lane I). This would indicate that it has promise as a possible tool for the fingerprinting of the plasmid. Bgl I appears to have effected neither the B. pertussis nor the B. parapertussis. However, it should be noted that the lysates treated with one unit of Bgl I were those produced by the alkaline lysis without lysozyme method. These lysates contained the most intense concentration of DNA and may be susceptible to digestion by 3 or 4 units of Bgl I. The one unit of Hae II appears to have over-digested the B. pertussis, and could be a possible candidate for plasmid profiling.

Cold Storage and Plasmid Stabilization

Lysates that were stored for 24 hrs or more at -20°C produced more intense DNA bands on agarose gels. This finding was observed throughout this investigation (see, for example, Figures 4b, 10 and 11). The increase in DNA intensity of chilled lysates is due to the fact that closed circular molecules that are subjected to artificial conditions of analysis are prone to great stress. These stresses are partly relieved by local denaturation of AT (adenine and thymine) rich segments, where the interstrand bonds are weakest. Heat-denatured DNA tends to re-form duplexes (regain their native configuration) when cooled. Particularly when it is made up of relatively small, homogeneous molecules, as are plasmids (Davis et al., 1980).

CONCLUSIONS

All three plasmid isolation procedures employed in this research were useful in producing plasmid DNA detectable by agarose gel electrophoresis. Acid precipitation was the least effective method because the DNA bands produced were extremely weak in intensity, and contaminating chromosomal DNA was prevalent. The boiling water bath method produced good results, including well defined, fairly intense banding. However, treatment of the Bordetella BWB lysates with RE's failed to produce desirable profiles. The most effective procedure used was the alkaline lysis method without lysozyme. This technique yielded extremely intense bands that were not effected by RE treatment. The implication is that a small amount of the lysate obtained with this protocol should produce an intense plasmid DNA band that could be used for further RE investigations.

SUMMARY

The purpose of this study was to evaluate variations on three protocols for plasmid isolation: the boiling water bath method, the alkaline lysis procedure, and the acid precipitation technique. Clinical samples of Bordetella pertussis, obtained from the Kansas State Health Laboratory, were the test organisms. All protocols required less than two hours for actual plasmid isolation and purification. No laboratory equipment more complicated than a microcentrifuge was required. The isolated plasmids were visualizes by staining agarose gels with ethidium bromide and observing the resultant fluorescence under UV illumination. The results showed that all three techniques produced a distinctive 21 kb DNA band, ant that the band was similar for all B. pertussis samples tested. The intensity of the DNA bands was greatest in the gels resulting from alkaline lysis derived lysates. The acid precipitation technique produced the least intense bands. The boiling water bath method provided bands that were more intense than the acid precipitation procedure, but less intense than the alkaline lysis protocol. The use of phenol/chloroform for plasmid purification proved extremely effective, while Geneclean treatment of the lysates produced barely detectable DNA bands. No distinctive profiles were obtained when the Bordetella lysates were treated with restriction enzymes, but Bgl I, Hae II and Hinc II did display some promise as possible tools for the plasmids fingerprinting of this organism.

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