PROVON® Gentle Lotion Soap
(continued)

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco. California.
Date: November 10, 1992
Results: Average score 0.36 (scale 0-4). Lower scores indicate lower potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Ingredients

Contents	Ingredient Class
Water	Carrier
Cocamidopropyl Betaine	Cleansing Agent, Skin Conditioning Agent Foam Booster
Coconut Acid	Cleansing Agent
Oleic Acid	Cleansing Agent
Ethanolamine	pH Adjuster
Cocamide DEA	Surfactant. Foam Booster
Sodium Sulfate	Viscosity Modifier
Allantoin	Skin Conditioning Agent
Aloe Extract	Botanical Additive Skin Conditioner

PROVON® Mild Lotion Soap

Dermatologist-tested.

Stock No. 4008, 4019, 4044

Physical Characteristics

Color: Blue Pearl
Fragrance: Fresh, Clean
Viscosity: 2,000-8,000 cps
pH: 8.8-9.6

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: February 11, 1994
Results: Average score 0.14 (scale 0-4). Lower scores indicate lower potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Ingredients

Contents	Ingredient Class
Water	Carrier
Coconut Acid	Surfactant. Cleansing Agent
Oleic Acid	Surfactant Cleansing Agent
Ethanolamine	ph Adjuster
Cocamide DEA	Surfactant. Foam Booster
Sodium Sulfate	Viscosity Increasing Agent
Chloroxylenol Agent	Preservative. Antimicrobial
EDTA	Chelating Agent
Fragrance	Fragrance
Glycol Stearate	Emollient, Pearlizing Agent
Propylene Glycol	Skin Conditioning Agent. Humectant
FD&C Blue #1	Colorant

PROVON® Enriched Lotion Cleanser

Dermatologist-tested.

Stock No. 4013, 4017, 4043

Physical Characteristics

Color: Pink Pearl
Fragrance: Floral
Viscosity: 3,000-10,000 cps
pH: 6.0-7.0

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: December 5, 1995
Results: Average score 0.27 (scale 0-4). Lower scores indicate lower potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Ingredients

Contents	Ingredient Class
Water	Carrier
Sodium Laureth Sulfate	Surfactant, Cleansing Agent
Sodium Lauryl Sulfate	Surfactant, Cleansing Agent
Cocamide DEA	Surfactant, Foam Booster
Betaine	Foam Booster
Soyamidopropyl	Cleansing Agent, Skin Conditioning Agent
Ammonium Chloride	Viscosity Increasing Agent
Citric Acid	pH Adjuster. Chelating Agent
EDTA	Chelating Agent
Fragrance	Fragrance
Glycol Distearate	Emollient, Pearlizing Agent
Preservative	Preservative Antimicrobial Agent
FD&C Red #33	Colorant

PURELL® Instant Hand Sanitizer

Dermatologist-tested, dye-free. Meets protocol for Healthcare Personnel Handwash.

Stock No. 9651, 9652. 9654. 9656. 9657. 7188

Physical Characteristics

Color: Clear
Fragrance: Fresh Lemon
Viscosity: 4,000-12,000 cps (gel)
pH: 7.5-8.5

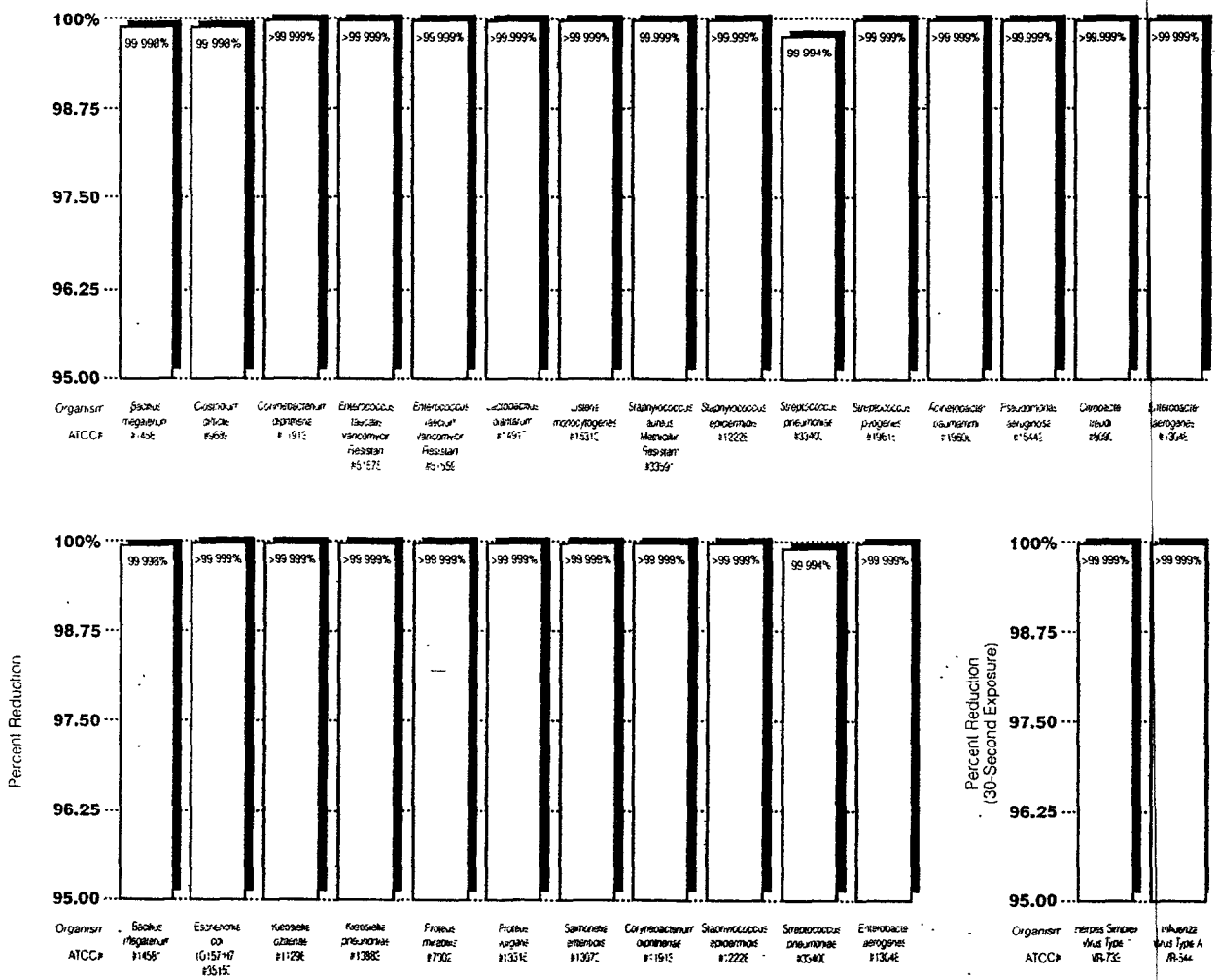
Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay.
Method: The method is that summarized by Phillips et al. (*Toxic and Applied Pharmacology* 21:369-382, 1972). The fresh materials are applied five (5) days weekly for twenty-one (21) days to the same site. Patches are not reapplied on weekends (or holidays); they remain in place for these periods. There are fifteen (15) days of reading, even when holidays intervene.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: February 20, 1996
Results: Average score 0.06 (scale 0-4). Lower scores indicate lower potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Human Repeated Insult Patch Test

Objective: Determination of the dermal irritation and sensitization potential of the product.
Description of Test: Human repeated patch test.
Method: This study was conducted utilizing a standard protocol and a total of two hundred and fifteen (215) subjects. Prior to patch application, the test area was wiped with seventy percent (70%) ethanol and allowed to dry. The test material was applied under a semi-occlusive patch (Readi-Bandage®) to the upper back (between the scapulae) and allowed to remain in direct skin contact for twenty-four (24) hours. Patches were applied to the same site on Monday, Wednesday and Friday for a three (3)-week induction period. The sites were graded for dermal irritation and sensitization twenty-four (24) hours after removal of the patches on Tuesday and Thursday, and forty-eight (48) hours after patch removal on Saturday. Standard Dermal Scores (0-4+) were used to record dermal reactions. After two (2) weeks, the subjects were rechallenged and evaluated forty-eight (48) and seventy-two (72) hours after patch removal.
Independent Laboratory: Clinical Research Laboratories, Inc., Piscataway, New Jersey.
Date: October 31, 1996
Results: No dermal reactions were observed during the induction or challenge phases of the study.
Conclusion: Test product demonstrated no potential for eliciting either dermal irritation or sensitization.

Results: Efficacy Data — *In Vitro*, 15-second exposure



Efficacy Data — *In Vitro*

Objective: These tests were designated to evaluate the antimicrobial effectiveness of product formulations when challenged with a broad spectrum of microorganisms.

Laboratories:

BioScience Laboratories,
Bozeman, Montana; March 9. 1998.

ViroMED Laboratories, Inc.,
Minneapolis, Minnesota: April 29, 1998.

Efficacy Data — *In Vivo*

Healthcare Personnel Handwash Data

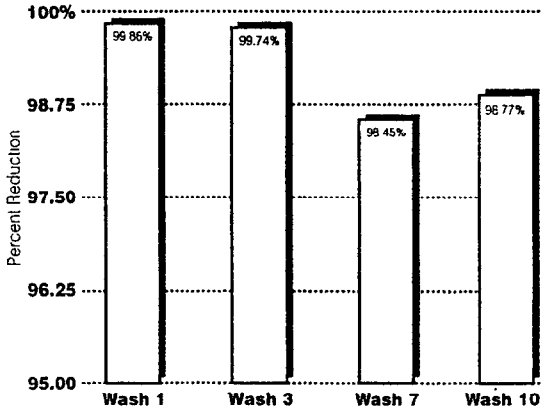
Objective: Evaluation of the antimicrobial effectiveness of the product on the hands.

Description of Test: The study evaluated both the immediate and persistent antimicrobial effects of the product over the course of ten (10) consecutive microbial contamination/product application cycles. The irritation potential of the product was measured over the course of fifteen (15) additional product cycles. This study utilized five (5) subjects for the test product. The protocol used was a modification of the ASTM Standard Method E1174-87.

Independent Laboratory: BioScience Laboratories. Inc , Bozeman, Montana.

Date: April 16. 1998

Results: Average Reduction in Transient Bacteria



Conclusion: The test data show good antimicrobial efficacy (bacterial count reduction) on the hands.

Ingredients

Active Ingredient	Ingredient Class
Ethyl Alcohol 62%	Antimicrobial Agent
Also Contains:	
Water	Diluent
Isopropyl Alcohol	Denaturant
Carbomer	Thickener
Tocopheryl Acetate (Vitamin E)	Skin Conditioning Agent
Glycerin	Skin Conditioning Agent. Humectant
Propylene Glycol	Skin Conditioning Agent. Humectant
Isopropyl Myristate	Emollient
Fragrance	Fragrance

PURELL® Instant Hand Sanitizer with Aloe

Dermatologist-tested. Meets protocol for Personnel Handwash.

Stock No. 9637, 7186, 9631, 9639

Physical Characteristics

Color: Clear, Green
Fragrance: Pleasant Floral
Viscosity: 7,000-19,000 cps (gel)
pH: 7-8.5

Irritancy Data and Allergy Test Results

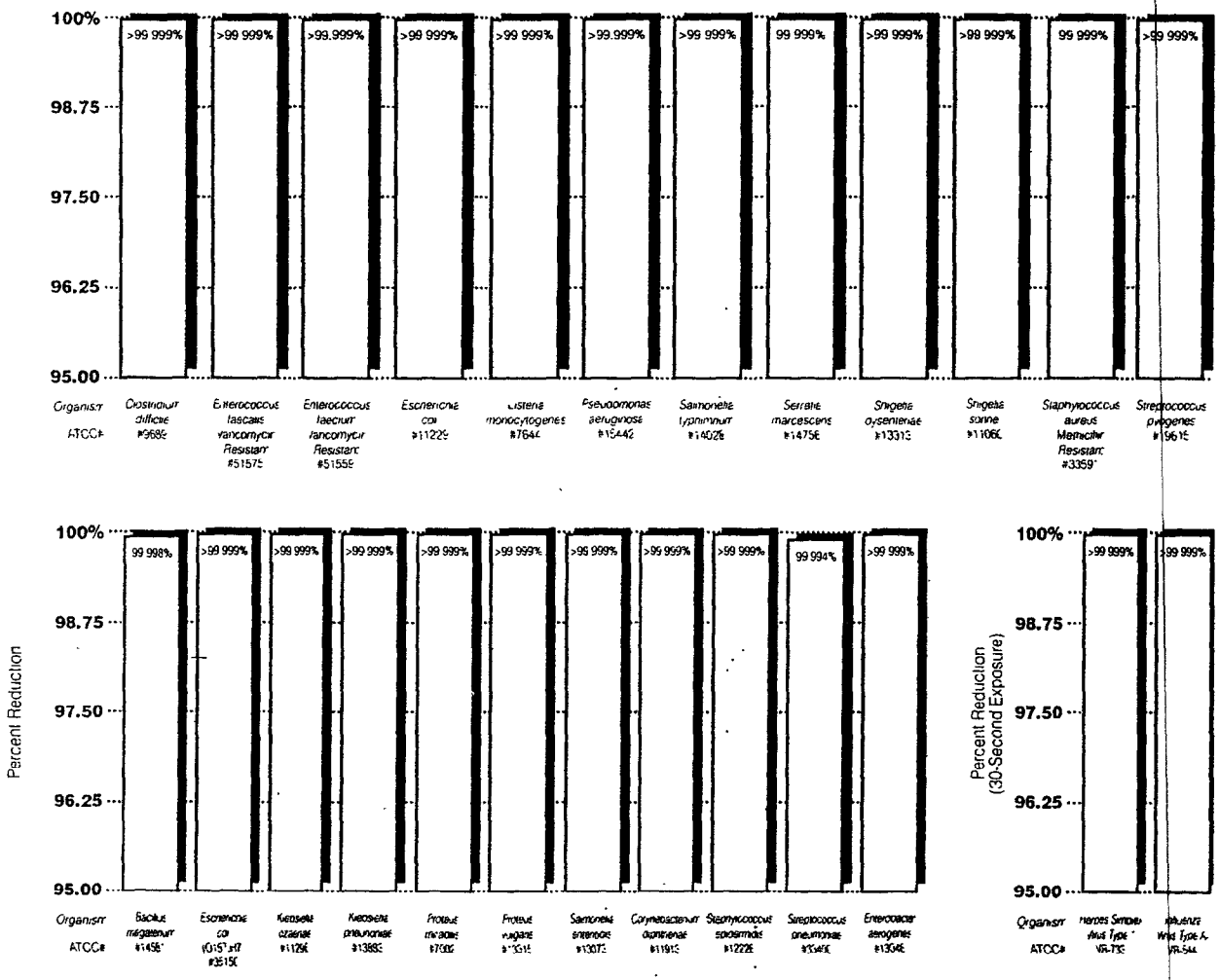
Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Cumulative Irritancy.
Method: The method is that summarized by Phillips et al (*Toxic and Applied Pharmacology* 21:369-382, 1972). The fresh materials are applied five (5) days weekly for twenty-one (21) days to the same site. Patches are not reapplied on weekends (or holidays); they remain in place for these periods. There are fifteen (15) days of reading, even when holidays intervene.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: June, 1997
Results: Average scores 0.01 (Scale 0-4)
Conclusion: Product has a very low potential for eliciting skin irritation and allergic contact dermatitis.

Human Repeated Insult Patch Test

Objective: Evaluation of skin irritation and sensitization potential in humans.
Description of Test: Human repeated insult patch test.
Method: This study was conducted utilizing a standard protocol and a total of one hundred and four (104) subjects. Prior to patch application, the test area was wiped with seventy percent (70%) ethanol and allowed to dry. The test material was applied under a semi-occlusive patch (Readi-Bandage®) to the upper back (between the scapulae) and allowed to remain in direct skin contact for twenty-four (24) hours. Patches were applied to the same site on Monday, Wednesday and Friday for a three (3)-week induction period. The sites were graded for dermal irritation and sensitization twenty-four (24) hours after removal of the patches on Tuesday and Thursday, and forty-eight (48) hours after patch removal on Saturday. Standard Dermal Scores (0-4+) were used to record dermal reactions. After two (2) weeks, the subjects were rechallenged and evaluated forty-eight (48) and seventy-two (72) hours after patch removal.
Independent Laboratory: Clinical Research Laboratories, Inc., Piscataway, New Jersey.
Date: September 10, 1997
Results: No observed dermal reactions
Conclusion: No demonstrated potential for eliciting dermal irritation or sensitization.

PURELL® Instant Hand Sanitizer with Aloe
(continued)

Results: Efficacy Data — *In Vitro*, 15-second exposure



Efficacy Data — *In Vitro*

Objective: Evaluation of the antimicrobial effectiveness of the product *in vitro.*

Description of Test: Fifteen (15) second exposure kill studies were performed utilizing twelve (12) challenge bacteria. The challenge inoculum was introduced to the test product at time zero; a portion of the samples was removed and placed in neutralizing media at the appropriate time (15 seconds). Standard plate counting techniques were used to enumerate viable challenge microorganisms.

Independent Lab:

BioScience Laboratories, Inc., Bozeman, Montana

Date: September 8, 1997.

Results: Percent reduction after a 15 second exposure to PURELL Instant Hand Sanitizer with Aloe

Efficacy Data — *In Vivo*

Healthcare Personnel Handwash Data

Objective: Evaluation of the antimicrobial effectiveness of the product on the hands.

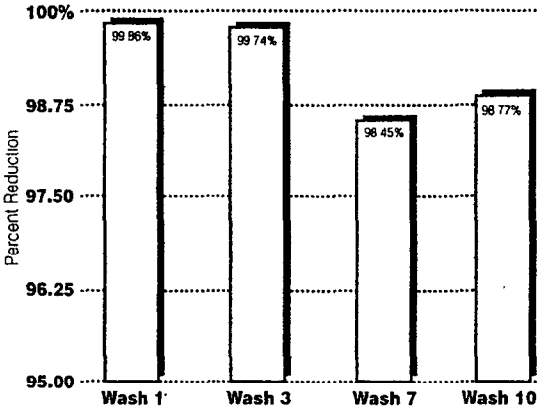
Description of Test: The study evaluated both the immediate and persistent antimicrobial effects of the product over the course of ten (10) consecutive microbial contamination/product application cycles. The irritation potential of the product was measured over the course of fifteen (15) additional product cycles. this study utilized five (5) subjects for the test product. the protocol used was a modification of the ASTM Standard Method E1174-87.

Independent Laboratory: BioScience Laboratories, Inc., Bozeman, Montana.

Date: April 16. 1998

PURELL® Instant Hand Sanitizer with Aloe
(continued)

Results: Average Reduction in Transient Bacteria



Conclusion: Very effective reductions of Gram Negative and Gram Positive bacteria were demonstrated.

Ingredients

Active Ingredient	Ingredient Class
Ethyl Alcohol 62%	Antimicrobial Agent
Also Contains:	
Water	Diluent
Isopropyl Alcohol	Denaturant
Glycerin	Skin Conditioning Agent Humectant
Carbomer	Thickener
Fragrance	Fragrance
Aloe Barbadensis Gel	Botanical Additive, Skin Conditioning Agent
Propylene Glycol	Skin Conditioning Agent, Humectant
Isopropyl Myristate	Emollient
Tocopheryl Acetate	Skin Conditioning Agent
FD&C Yellow #5	Colorant
FD&C Blue #1	Colorant

Bathing & Moisturizing

PROVON® Antibacterial Body Wash

Dermatologist-tested.

Stock No. 4026

Physical Characteristics

Color: Dye-free, Colorless
Fragrance: Fresh and Light
Viscosity: 100 cps
pH: 5.8-6.2

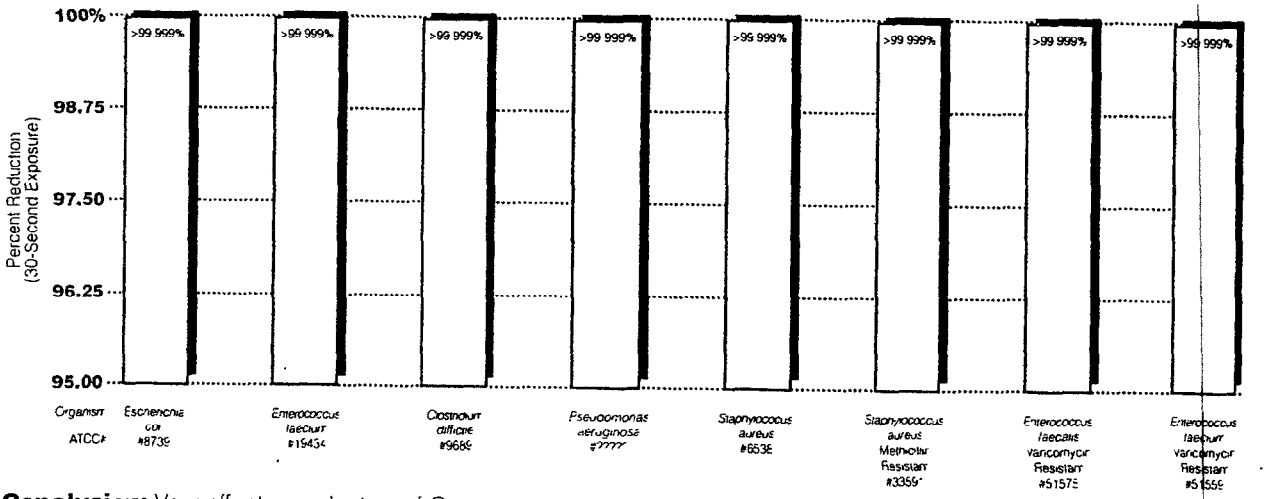
Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: June 21, 1994
Results: Average score 0.08 (scale 0-4). Lower scores indicate low potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Efficacy Data — In Vitro

Timed-Exposure Kill Tests
Objective: Evaluation of the antimicrobial effectiveness of the product in vitro.
Description of Test: Thirty (30)-second-exposure kill studies were performed utilizing nine (9) challenge bacteria. The challenge inoculum was introduced to the test product at time zero; a portion of the sample was removed and placed in neutralizing media at the appropriate time (30 seconds). Standard plate counting techniques were used to enumerate viable challenge microorganisms.
Independent Laboratory: BioScience Laboratories, Inc., Bozeman, Montana.
Date: July 19, 1994 and September 11,1995
Results: Percent reduction after 30-second exposure to PROVON Brand Antibacterial Body Wash. ***See graph top of page 19.***

Results: Efficacy Data — *In Vitro*



Conclusion: Very effective reduction of Gram-negative and Gram-positive bacteria by the product was demonstrated

Efficacy Data — MICs

Minimum Inhibitory Concentrations (MICs)

Objective: Demonstration of the broad spectrum of antimicrobial activity.

Description of Test: Bacterial tests run with agar to determine the concentration of triclosan required to inhibit growth.

Reference: Studies conducted by W. Vischer and John Regos, Pharmaceutical Division, Ciba-Geigy. Basel, Switzerland

Date: 1974

Results: The minimum inhibitory concentrations (MICs) of triclosan are listed in parts per million (ppm). Numbers in parentheses refer to the number of strains evaluated.

Microorganism	**MICs (ppm)**
Bacteria (Number of Strains)	
Bacillus subtilis	0.1
Bacillus megaterium	3
Bacillus cereus	3
Bacillus cereus var *mycoides*	3
Clostridium botulinum	3
Clostridium tetani	3
Corynebacterium acnes	3
Corynebacterium diphtheriae (3)	3
Corynebacterium minutissimum	5
Streptococcus	3
Lactobacillus fermentium	33
Mycobacterium tuberculosis	100
Mycobacterium smegmatis	1
Mycobacterium phlei	0.3
Micrococcus lutea	3
Sporosarcina ureae	0.1
Staphylococcus aureas	0.01-0.1
Staphylococcus albus (2)	0.03-0.1
Streptococcus agalactiae	3
Streptococcus haemolyticus A (2)	1-3
Streptococcus faecalis	3-10
Streptococcus pyogenes	3
Alcaligenes faecalis	>100
Brucella abortus	0.1
Brucella melitenis	0.1
Brucella suis	0.03
Escherichia coli (7)	0.03-0.1
Haemophilus influenzae	33
Klebsiella edwardsii	0.3
Klebsiella aerogenes	0.3
Klebsiella pneumoniae	0.3
Legionella pneumoniae	10
Loefflerella mallei	0.3
Loefflerella pseudomallei	1
Moraxella duplex	0.01
Moraxella glucidolytica	0.3
Acinetobacter lwoffi	0.1
Neisseria catarrhalis	33
Pseudomonas capacia	256
Pasteurella septica	0.1
Pasteurella pseudotuberculosis	10
Proteus vulgaris (2)	0.1-0.3
Proteus vulgaris (Neotype)	0.01
Proteus mirabilis (2)	0.3
Pseudomonas aeruginosa (3)	>100 - >1000
Pseudomonas fluorescens	>100
Salmonella enteritidis (2)	0.1-0.3
Salmonella typhimurium	0.3
Salmonella tyhpi (3)	0.1-0.3
Salmonella paratyhpi A	0.3
Salmonella paratyphi B (3)	0.1-0.3
Salmonella pullorum	0.3
Serratia marcescens	>100
Shigella flexneri (3)	0.1-0.3
Shigella sonnei	0.1
Shigella dysenteriae	0.1
Vibrio cholerae	10
Vibrio eltor	10
Yeasts and Fungi	**MICs (ppm)**
Aspergillus niger	30
Aspergillus fumigatus	10
Candida albicans (2)	3
Epidermophyton floccosum	1-10
Keratinomyces aielloi	10
Tichophyton mentagrophytes	1
Tichophyton rubrum	10

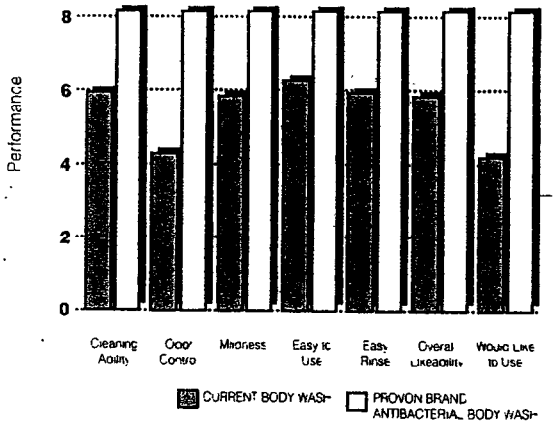
Conclusion: Triclosan is very effective against both Gram-positive and Gram-negative bacteria, yeast and fungal microorganisms.

PROVON® Antibacterial Body Wash
(continued)

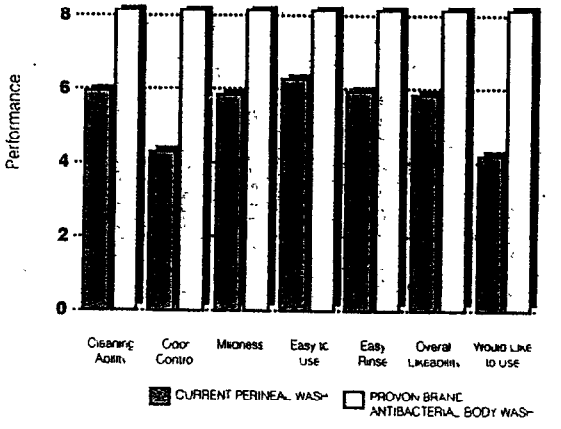
Market Research

This formula and the foaming applicator underwent rigorous market research, as well as independent lab testing, for efficacy and mildness. Tests were performed in various nursing facilities to determine overall likability of the product as a body cleanser and as a perineal wash. We also tested the cleaning ability of the product, the ability of the product to control odors, and the ease-of-use of the applicator. These facilities rated the product as both an effective body wash and an effective perineal cleanser. Overall, they preferred it to the products they were currently using for bathing and incontinent care.

Market Research
Preference Ratings: Current Body Wash



Market Research
Preference Ratings: Current Perineal Wash



Ingredients

Active Ingredient	Ingredient Class
Triclosan	Antimicrobial Agent
Also Contains:	
Water	Carrier
Lauramine Oxide	Surfactant, Cleansing Agent
Coca Betaine	Surfactant, Cleansing Agent
Sodium Lactate	Skin Conditioning Agent. Humectant
Lactic Acid	pH Adjuster
EDTA	Chelating Agent
Fragrance	Fragrance
Preservative	Preservative

PROVON® Tearless Shampoo & Body Wash

Dermatologist-tested.

Stock No. 4032, 4034, 4401, 4402, 4403, 4405, 4406, 4408

Physical Characteristics

Color: Clear Blue
Fragrance: Herbal
Viscosity: 3,000-12,000 cps
pH: 6.0-7.0

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: November 24, 1992
Results: Average score 0.23 (scale 0-4). Lower scores indicate low potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Ingredients

Contents	Ingredient Class
Water	Carrier
Cocamidopropyl Hydroxysultaine	Antistatic Agent, Cleansing Agent, Skin Conditioning Agent
Sodium Trideceth Sulfate	Cleansing Agent, Emulsifying Agent
Peg-80 Sorbitan Laurate	Surfactant
Disodium Lauroamphodiacetate	Surfactant
Peg-150 Distearate	Surfactant, Emulsifying Agent
Allantoin	Skin Conditioning Agent
Aloe Extract	Botanical Additive, Skin Conditioning Agent
Citric Acid	pH Adjuster, Chelating Agent
Fragrance	Fragrance
Inositol	Humectant
Methionine	Conditioning Agent

PROVON® Tearless Shampoo & Body Wash
(continued)

Contents *(continued)*	**Ingredient Class**
Propylene Glycol	Conditioning Agent. Humectant
Quarternium 15	Antistatic Agent, Preservative
Sodium Chloride	Viscosity Increasing Agent
Sodium Laureth-13 Carboxylate	Surfactant
Vitamin A	Skin Conditioning Agent
Vitamin B	Biological Additive
Vitamin C	Antioxidant
Vitamin E	Skin Conditioning Agent
Vitamin H (Biotin)	Biological Additive
Wheat Germ Extract	Botanical Additive
FD&C Blue #1	Colorant

PROVON® Enriched Shampoo for Body & Hair

Dermatologist-tested.

Stock No. 4012, 4014, 4015, 4016. 4018. 4033 4035

Physical Characteristics

Color: Clear Yellow
Fragrance: Herbal
Viscosity: 5,000-15,000 cps
pH: 6.0-7.0

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: November 10. 1992
Results: Average score 0.28 (scale 0-4). Lower scores indicate low potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Ingredients

Contents	**Ingredient Class**
Water	Carrier
Sodium Laureth Sulfate	Surfactant, Cleansing Agent
Sodium Lauryl Sulfate	Surfactant, Cleansing Agent
Soyamidopropyl Betaine	Cleansing Agent, Skin Conditioning Agent, Foam Booster
Cocamide DEA	Surfactant, Foam Booster
Ammonium Chloride	Viscosity Increasing Agent
Citric Acid	pH Adjuster. Chelating Agent
Fragrance	Fragrance
Preservative	Preservative, Antimicrobial Agent
Aloe Extract	Skin Conditioning Agent. Botanical Additive
Vitamin E	Skin Conditioning Agent
FD&C Blue #1	Colorant
FD&C Red #40	Colorant
FD&C Yellow #5 & 6	Colorant

PROVON® Whirlpool Skin Conditioner & Cleanser

Dermatologist-tested.

Stock No. 4412. 4416

Physical Characteristics

Color: Light Blue
Fragrance: Herbal
Viscosity: 300-1.500 cps
pH: 6.0-7.0

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: July 14, 1992
Results: Average score 0.08 (scale 0-4). Lower scores indicate low potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Ingredients

Contents	**Ingredient Class**
Water	Carrier
Mineral Oil	Emollient Skin Conditioning Agent
Coconut Acid	Surfactant Cleansing Agent
Oleic Acid	Surfactant, Cleansing Agent
Ethanolamine	ph Adjuster
Cocamidopropyl Betaine	Cleansing Agent. Skin Conditioning Agent. Foam Booster
Allantoin	Skin Conditioning Agent
Aloe Extract	Botanical Additive. Skin Conditioning Agent
Dimethicone	Skin Conditioning Agent
Fragrance	Fragrance
Hydrolyzed Protein	Skin Conditioning Agent
Inositol	Humectant
Methionine	Skin Conditioning Agent
Preservative	Preservative. Antimicrobial Agent
Propylene Glycol	Skin Conditioning Agent. Humectant
Thickener	Viscosity Increasing Agent
Vitamin A	Skin Conditioning Agent
Vitamin B	Biological Additive
Vitamin C	Antioxidant
Vitamin E	Skin Conditioning Agent
Vitamin H (Biotin)	Biological Additive
Wheat Germ Extract	Botanical Additive
FD&C Blue #1	Colorant

PROVON® Brand Moisturing Hand & Body Lotion

Dermatologist-tested.

Stock No. 4231, 4232, 4233. 4236. 4238

Physical Characteristics

Color: Creamy Opaque
Fragrance: No Cosmetic Fragrance
Viscosity: 10,000-22,000 cps

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: November 10, 1992
Results: Average score 0.44 (scale 0-4). Lower scores indicate low potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Ingredients

Contents	Ingredient Class
Water	Carrier
Distearyldimonium Chloride	Skin Conditioning Agent
Petrolatum	Skin Conditioning Agent
Glycerin	Skin Conditioning Agent. Humectant
Isopropyl Myristate	Skin Conditioning Agent. Emollient
Cetyl Alcohol	Skin Conditioning Agent Emollient
Dimethicone	Skin Conditioning Agent
Preservative	Preservative. Antimicrobial Agent

PROVON® Skin Moisturizer with Aloe & Vitamins

Dermatologist-tested.

Stock No. 4251. 4252, 4256

Physical Characteristics

Color: Creamy Opaque
Fragrance: Herbal
Viscosity: 7,000-20,000 cps

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory. San Francisco. California
Date: November 24 1992
Results: Average score 0.08 (scale 0-4). Lower scores indicate low potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Ingredients

Contents	Ingredient Class
Water	Carrier
Glycerin	Skin Conditioning Agent Humectant
Stearic Acid	Viscosity Increasing, Skin Conditioning Agent
Cetyl Alcohol	Skin Conditioning Agent, Emollient
Dimethicone	Skin Conditioning Agent
Allantoin	Skin Conditioning Agent
Aloe Extract	Botanical Additive, Skin Conditioning Agent
Fragrance	Fragrance
Inositol	Humectant
Methionine	Skin Conditioning Agent
Mineral Oil	Emollient, Skin Conditioning Agent
Preservative	Preservative, Antimicrobial Agent
Propylene Glycol	Skin Conditioning Agent, Humectant
Triethanolamine	pH Adjuster
Vitamin A	Skin Conditioning Agent
Vitamin B	Biological Additive
Vitamin C	Antioxidant
Vitamin E	Skin Conditioning Agent
Vitamin H (Biotin)	Biological Additive
Wheat Germ Extract	Botanical Additive

Specialized Skin Care

PROVON® Perineal Wash

Dermatologist-tested.

Stock No. 4422, 4424, 4426

Physical Characteristics

Color: Green
Fragrance: Herbal
Viscosity: 100 cps
pH: 8.0-9.0

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory San Francisco, California
Date: November 10. 1992
Results: Average score 0.14 (scale 0-4). Lower scores indicate low potential for skin irritation and allergic contact dermatitis
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

PROVON® Perineal Wash
(continued)

Ingredients

Contents	Ingredient Class
Water	Carrier
Coconut Acid	Surfactant, Cleansing Agent
Oleic Acid	Surfactant, Cleansing Agent
Ethanolamine	pH Adjuster
Cocamidopropyl Betaine	Cleansing Agent Skin Conditioning Agent Foam Booster
Allantoin	Skin Conditioning Agent
Aloe Extract	Botanical Additive Skin Conditioning Agent
EDTA	Chelating Agent
Fragrance	Fragrance
Inositol	Humectant
Methionine	Skin Conditioning Agent
Propylene Glycol	Skin Conditioning Agent Humectant
Quaternium 15	Antistatic Agent, Preservative
Vitamin A	Skin Conditioning Agent
Vitamin B	Biological Additive
Vitamin C	Antioxidant
Vitamin E	Skin Conditioning Agent
Vitamin H (Biotin)	Biological Additive
Wheat Germ Extract	Botanical Additive
FD&C Blue #1	Colorant
FD&C Yellow #5	Colorant

PROVON® Antibacterial Perineal Wash

Dermatologist-tested. dye-free. U.S Pat. 5,635,462.

Stock No 4432, 4434

Physical Characteristics

Color: Colorless (dye-free)
Fragrance: Fresh and Light
Viscosity: 100 cps
pH: 5.8-6.2

Irritancy Data and Allergy Test Results

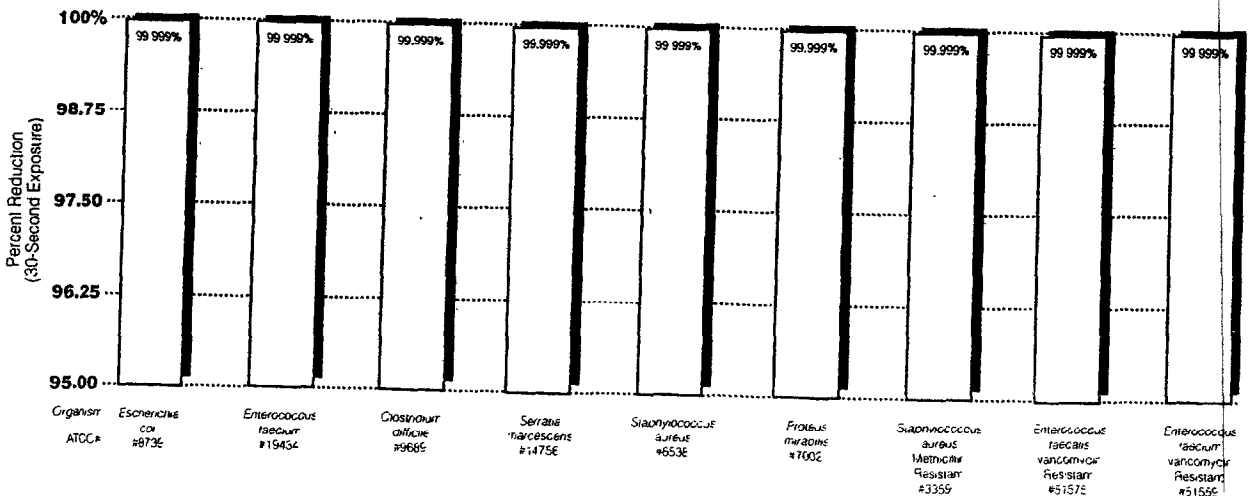
Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California
Date: June 21, 1994
Results: Average score 0.08 (scale 0-4) Lower scores indicate low potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Efficacy Data — *In Vitro*

Timed-Exposure Kill Tests

Objective: Evaluation of the antimicrobial effectiveness of the product *in vitro*.
Description of Test: Thirty (30)-second-exposure kill studies were performed utilizing nine (9) challenge bacteria. The challenge inoculum was introduced to the test product at time zero; a portion of the sample was removed and placed in neutralizing media at the appropriate time. Standard plate counting techniques were used to enumerate viable challenge microorganisms.
Independent Laboratory: BioScience Laboratories, Inc., Bozeman, Montana
Dates: July 19, 1994; October 6, 1994; September 11, 1995
Results: Percent reduction after a 30-second exposure to PROVON Brand Antibacterial Perineal Wash. ***See graph below.***
Conclusion: Very effective reduction of Gram-negative and Gram-positive bacteria by the product was demonstrated.

Results: Efficacy Data — *In Vitro*



Efficacy Data — MICs

Minimum Inhibitory Concentrations (MICs)

Objective: Demonstration of the broad spectrum of antimicrobial activity of chloroxylenol.

Description of Test: Bacterial tests run with agar to determine the concentration of chloroxylenol required to inhibit growth.

Reference: NIPA Laboratories, Inc., Wilmington Delaware.

Date: December 1991

Results: The minimum inhibitory concentrations (MICs) of chloroxylenol are listed in parts per million (ppm). Numbers in parentheses refer to the number of strains evaluated.

Microorganism	MICs (ppm)
Bacteria (Number of Strains)	
Streptococcus faecalis (7)	20-167
Staphylococcus aureus (8)	10-167
Salmonella typhi (2)	20-60
Escherichia coli (8)	40-250
Pseudomonas aeruginosa (9)	80-1,000
Providencia rettgeri	125
Bacillus subtilis (2)	50
Citrobacter sp. (2)	167-250
Corynebacterium pyogenes	60
Enterobacter aerogenes (3)	30-156
Klebsiella pneumoniae (5)	167-250
Mycobacterium avium	125
Proteus mirabilis (7)	125-167
Salmonella choleraesuis	60
Micrococcus luteus	125
Mycobacterium smegmatis	167
Proteus vulgaris (2)	100-125
Bacillus cereus	50
Providencia (2)	167
Acinetobacter calcoaceticus	83
Serratia marcescens (3)	20-167
Pseudomonas cepacia	40
Enterobacter gergoviae	250

Yeasts and Fungi	MICs (ppm)
Aspergillus niger (4)	20-200
Penicillium funiculosum	250
Trichophyton mentagrophytes (3)	20-1,000
Penicillium citrinum	250
Aspergillus flavus	60
Penicillium luteum	125
Penicillium chrysogenum	1,000
Alternaria solani	200
Trichophyton rubrum	60
Trichophyton tonsurans	250
Epidermophyton floccosum	30
Saccharomyces bayabus	50
Candida albicans (2)	50-125
Mucor racemosus	1,000
Rhizopus stolonifer	25
Torula ramosa	100

Conclusion: Chloroxylenol is very effective against both Gram-positive and Gram-negative bacteria, yeast and fungal microorganisms.

Ingredients

Active Ingredient	Ingredient Class
Triclosan	Antimicrobial Agent
Also Contains:	
Water	Carrier
Lauramine Oxide	Surfactant, Cleansing Agent
Coco Betaine	Surfactant, Cleansing Agent
Sodium Lactate	Skin Conditioning Agent Humectant
Lactic Acid	pH Adjuster
EDTA	Chelating Agent
Fragrance	Fragrance
Preservative	Preservative

PROVON® Moisturizing Perineal Barrier

Dermatologist-tested.

Stock No 4441

Physical Characteristics

Color: Dye-free, Creamy White
Fragrance: Fresh and Light
Viscosity: Nongreasy, Semisolid

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans.

Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge

Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California

Date: November 24, 1992

Results: Average score 0 (scale 0-4). Lower scores indicate low potential for skin irritation and allergic contact dermatitis.

Conclusion: Lower potential for skin irritation and allergic contact dermatitis as indicated by a score of zero (0).

Ingredients

Active Ingredient	Ingredient Class
Petrolatum	Skin Protectant, Moisturizer
Also Contains:	
Aluminum Starch Octenylsuccinate	Texture Enhancer
Preservative	Preservative, Antimicrobial Agent
Fragrance	Fragrance

PROVON® Perineal Cream

Dermatologist-tested.

Stock No. 4421, 4423

Physical Characteristics

Color: White Opaque
Fragrance: Herbal
Viscosity: 25,000 cps minimum (cream)

Irritancy Data and Allergy Test Results

Objective: Evaluation of irritation potential in humans
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California
Date: November 24, 1992
Results: Average score 0.10 (scale 0-4) Lower scores indicate low potential for skin irritation and allergic contact dermatitis.
Conclusion: Product has a low potential for skin irritation and allergic contact dermatitis.

Ingredients

Active Ingredient	Ingredient Class
Allantoin	Skin Conditioning & Protecting Agent
Also Contains:	
Water	Carrier
Lanolin	Skin Conditioning Agent, Emollient
Glycerin	Skin Conditioning Agent, Humectant
Cetyl Alcohol	Skin Conditioning Agent, Emollient
Sodium Lauryl Sulfate	Surfactant, Emulsifier
Beeswax	Skin Conditioning Agent
Aloe Extract	Botanical Additive, Skin Conditioning Agent
BHT	Antioxidant
DL Panthenol	Conditioning Agent
Fragrance	Fragrance
Methylparaben	Preservative, Antimicrobial Agent
Propylparaben	Preservative, Antimicrobial Agent
Quaternium 15	Antistatic Agent, Preservative
Thickener	Viscosity Increasing Agent
Vitamin A	Skin Conditioning Agent
Vitamin D	Biological Additive
Vitamin E	Skin Conditioning Agent

PROVON® Perineal Ointment

Dermatologist-tested.

Stock No. 4428, 4429

Physical Characteristics

Color: Blue Opaque
Fragrance: Herbal
Viscosity: Semisolid

Irritancy Data and Allergy Test Results

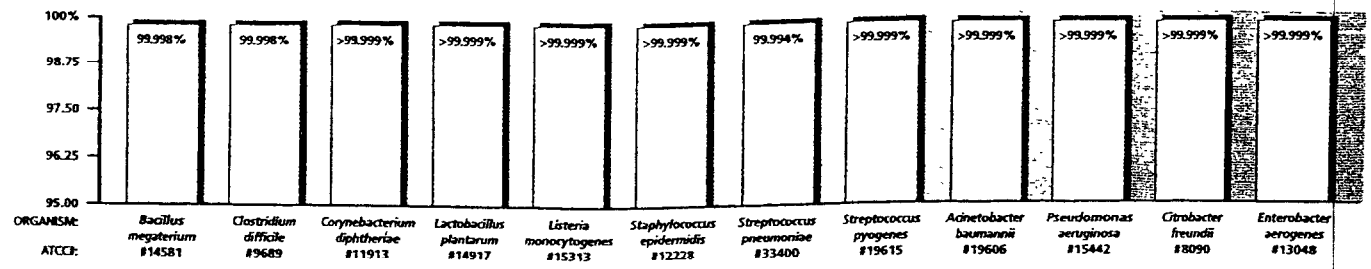
Objective: Evaluation of irritation potential in humans.
Description of Test: 21-Day Human Irritancy Assay with Delayed Challenge.
Independent Laboratory: Dermatologic Research Laboratory, San Francisco, California.
Date: November 24, 1992
Results: Average score 0 (scale 0-4). Lower scores indicate low potential for skin irritation and allergic contact dermatitis.
Conclusion: Lower potential for skin irritation and allergic contact dermatitis as indicated by a score of zero (0).

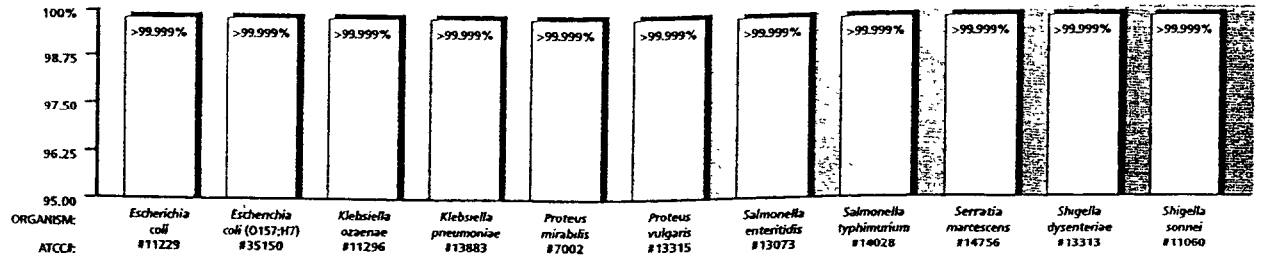
Ingredients

Active Ingredient	Ingredient Class
White Petrolatum	Skin Conditioning & Protecting Agent
Also Contains:	
Fragrance	Fragrance
Lanolin	Skin Conditioning Agent, Emollient
Preservative	Preservative, Antimicrobial Agent
Sodium Caseinate	Skin Conditioning Agent
Sorbitan Oleate	Surfactant, Emulsifying Agent
Vitamin A	Skin Conditioning Agent
Vitamin D	Biological Additive
Vitamin E	Skin Conditioning Agent
D&C Green #6	Colorant

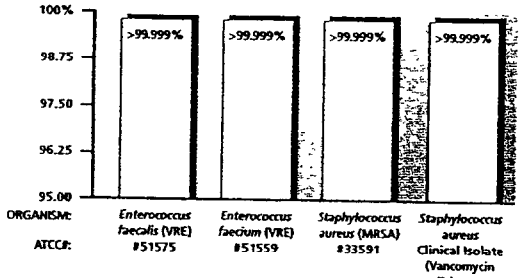
Efficacy Data: In vitro percent reduction of test organisms.[2]

Bacteria*

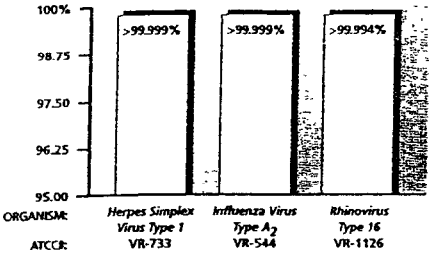




Antibiotic-resistant bacteria



Viruses**



*After a 15-second exposure
**After a 30-second exposure

PURELL® Product Recommendations

Your distributor or GOJO representative can conduct a Site Survey to help determine the most effective skin care program for your facility.

Instant Hand Sanitizers	4.25 FL OZ Bottle	8 FL OZ Pump Bottle	12 FL OZ Pump Bottle	250 mL Refill	500 mL GEMINI® Refill	800 mL Refill	1000 mL Refill	1000X* mL Refill	1250X** mL Refill	800 mL Starter Kit	1000 mL Starter Kit
Instant Hand Sanitizer Original Formula	9631-24	9672-12 9672-D2 (8 fl. oz. bottle in PLACES Holder)	9639-12 9639-D2 (12 fl. oz. bottle in PLACES Holder)	9651-12	9684-06	9656-06 9657-12	7187-06	9656-04 9657-10	7187-04	9629-D1	7187-D1
Instant Hand Sanitizer with Aloe	9637-24					9637-12	7186-06				
Instant Hand Sanitizer Soothing Gel Formula	9671-24							9676-04	7177-04		

* Fits 800 Dispenser
** Fits 1000 Dispenser
2. Data for PURELL Original Formula.

Dispensing Options	800 Dispenser	1000 Dispenser	250 mL Dispenser	GEMINI® 1000 mL Twin Dispenser	12 oz. PLACES™ Holder
PURELL	9621-12	7106-12	9601-12	9505-01	9005-12

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Inactivation of Poliovirus by Chloramine-T

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Since concern has recently been expressed about the presence of genotoxic substances due to chlorination of water and wastewater, chloramine-T (CAT) is proposed as an alternative disinfectant to chlorine. The viricidal properties of chlorine and CAT were compared. Kinetics of inactivation of poliovirus type 2 by chlorine and CAT in chlorine demand-free water were investigated by using a kinetic apparatus. Inactivation of the virus by chlorine and CAT occurred in two steps. The initial linear part of the inactivation curve followed a pseudo-first-order reaction with the virus. An obvious dose-response relationship was demonstrated with CAT. The rate of inactivation of the virus by CAT was faster in acid medium than in alkaline medium. Inactivation kinetic studies were performed at different temperatures, and the kinetic, Arrhenius, and thermodynamic parameters were evaluated. The rate of inactivation of poliovirus type 2 by chlorine was faster than that by CAT under identical conditions. A mechanism for the viral inactivation in acid conditions was proposed which led to a rate equation consistent with the experimental results. The results indicate that CAT may be an effective viricide against poliovirus type 2 in an acid medium.

Ever since the disinfection process became a standard part of drinking water and wastewater treatment in the United States and other developed nations, chlorine has been the predominant disinfectant. Recently, concern has been expressed by several investigators (2, 14, 18, 27, 28, 30, 31, 35) about the presence of potentially genotoxic substances in drinking water. Studies have shown organic compounds recovered from chlorine-treated waters in the United States and Japan to be mutagenic (14, 27). Most evidence suggests that the formation of mutagenic substances takes place during the water and wastewater disinfection processes. Chlorination, which is known to produce halogenated substances, appears to be the cause of the genotoxic activity in drinking water (27). Hence, there is need for an alternate effective disinfectant which forms a minimum of genotoxic compounds in water and wastewater. A review of the literature suggests that chemicals such as chlorine dioxide, inorganic chloramines, and ozone have been proposed or used as alternatives to chlorine for disinfection of drinking water (19, 21). Inorganic chloramine- or ozone-treated and recycled water were shown to be mutagenic in bacteria (18, 25). Chloramine-T (CAT), $p\text{-}CH_3C_6H_4SO_2NClNa\cdot 3H_2O$, may be an alternative disinfectant to standard chlorine. It behaves chemically more as an oxidant than a chlorinating agent. Thus, it would be much less likely to form genotoxic compounds. These positive qualities have led to an interest in CAT as a disinfectant of water. One of the essential features of a disinfectant is its viricidal properties.

To our knowledge, no basic research has been done on the inactivation kinetics of viruses by CAT. Inactivation kinetic studies of poliovirus have been carried out with formaldehyde (33), chlorine, hypobromite, molecular bromine, inorganic haloamines (7-9), chlorine dioxide (19, 32), and ozone (21, 22).

In this study, the viricidal properties of CAT and chlorine are compared. Specifically, we investigated the kinetic and mechanistic aspects of inactivation of poliovirus type 2 by CAT by determining (i) 99% inactivation times and rate constants at varying CAT concentrations and pH's and (ii) kinetic and thermodynamic parameters. Mechanisms of poliovirus inactivation are proposed, and rate expressions are derived which are in agreement with the experimental data and those of other investigations (21, 22). A simple method of concentration and purification was used for the first time to achieve a chlorine demand-free poliovirus type 2 suspension of high titer.

MATERIALS AND METHODS

Poliovirus stock. The same stock of poliovirus type 2 was used throughout the study. The virus was grown on human amnion WISH cells (Flow Labora-

tories, Inc., Rockville, Md) and accumulated. Cells were grown to monolayers in Eagle minimal essential medium containing Hanks salts supplemented with 10% fetal bovine serum, 100 μg of streptomycin per ml, and 100 U of penicillin per ml and infected with five 50% infective doses ($TCID_{50}$) per cell. The virus was harvested when 75 to 100% of the cells in culture showed cytopathology. Then the virus-containing fluids were frozen and thawed once and centrifuged at 1,000 × *g* for 15 min to remove cell debris.

Preparation of chlorine demand-free poliovirus. The sodium dextran sulfate(SDS)-polyethylene glycol (PEG) phase separation method of Albertsson (1) with slight modification was used to concentrate the virus (preparation of reagents is shown below). The PEG solution (210 g) containing NaCl and 20% SDS solution (10.5 g) were thoroughly mixed. The mixture was added, in equal portions (~10 ml each) to a liter separatory funnel containing 700 ml of poliovirus suspension. After each addition, the funnel was thoroughly shaken. Then the system was allowed to stand in the cold (4°C) for 24 h. The bottom phase (4 ml) harvested was treated with 3 M KCl (3.5 ml) to precipitate SDS. Since the highly purified and concentrated virus suspension harvested from the lower phase had a high chlorine demand even after removal of most of the SDS by precipitation with KCl (Table 1), we suspected that residual SDS may have been responsible. The residual SDS was removed, therefore, by treating the virus suspension with the enzyme dextranase, which degrades SDS into low-molecular-weight fragments (20) that would pass through an Amicon filter. Specifically, the purified and concentrated virus suspension obtained was diluted 10 times with phosphate buffer (0.1 M KH_2PO_4 + 0.1 M NaOH), pH 6, and treated with 4 U of the enzyme (20) per ml of virus suspension and incubated for 2 h at 37°C. Then the resultant suspension was filtered through an Amicon filter (10×M 300, 43 mm; Diaflo ultrafiltration membranes, Amicon Corp., Lexington, Mass.) and washed several times with 10-ml portions of sterile distilled water. The pure virus concentrate, shown to be virtually chlorine demand-free (Table 1) was diluted with chlorine demand-free phosphate-buffered saline (0.15 M) containing 1 M $MgCl_2$ and stored in 5-ml vials at −70°C. The titer of the purified virus concentrate was approximately 10^{10} $TCID_{50}$ per ml.

TABLE 1. *Preparation of chlorine demand-free poliovirus*

Poliovirus suspensions	Vol (ml)	Titer ($TCID_{50}$/ml)	Chlorine demand* (mg/liter)
Crude	700	3×10^7	1.65
PEG-SDS two-phase separation. Bottom phase:			
(i) Without KCl precipitation	4	10^{10}	0.80
(ii) With KCl precipitation	6	10^{10}	0.56
Enzyme (dextranase) treatment and Amicon filtration of (ii)	5	10^{10}	0.10
Addition of 45 ml of 1 M $MgCl_2$	50	10^{9}	0.08

* Chlorine used, 2 mg/liter; contact time, 60 min.

Enzyme solution. One hundred units of dextranase (α-1,6-glucan 6-glucanohydrolase) obtained from a *Penicillium* species (Sigma Chemical Co., St. Louis, Mo) was dissolved in 0.5 ml of tris(hydroxymethyl)-aminomethane-hydrochloride buffer of pH 6, and this solution was used in purifying poliovirus type 2.

Virus assays. All virus was diluted in Eagle minimal essential medium containing Earle salts supplemented with 2% fetal bovine serum and antibiotics (100 μg of streptomycin and 100 U of penicillin per ml). The virus assays (34) were performed on WISH cell monolayers grown in 96-well microtiter plates. One-tenth milliliter of each serial $\log_{10}$ dilution of each sample was inoculated into four replicate wells. The microtiter plates were then incubated for 48 h at 37°C in a 5% CO_2 atmosphere. The cultures were stained with crystal violet solution (1:100, wt/vol) in 20% methyl alcohol in water, and the cytopathic effect was read. The mean $TCID_{50}$ per milliliter was calculated by the Kärber method (29).

Chlorine. Sodium hypochlorite solution (4 to 6%; Fisher Scientific Co., Pittsburgh, Pa.) was standardized iodometrically (2), diluted to a concentration of 100 mg of chlorine per liter, and stored as a stock solution at 4°C. The concentration of the stock solution was also checked spectrophotometrically by the standard orthotolidine-arsenite method (2).

CAT. CAT (Eastman Kodak Co., Rochester, N.Y.) was freed from possible *p*-toluene sulfonamide and dichloro contaminants by washing it several times with carbon tetrachloride and dried in a vacuum desiccator over $CaCl_2$. The purity of the sample determined iodometrically was >99%, as reported by Gowda et al. (15). The stock solutions of CAT were prepared by dissolving the solid (2 g/liter) in chlorine demand-free distilled water and stored at 4°C.

Determination of chlorine demand. The chlorine demand of sterile distilled water (Abbott Laboratories, North Chicago, Ill.) used as a diluent, and of the purified poliovirus suspension was determined spectrophotometrically by the standard orthotolidine-arsenite method (2). Spectrophotometry was carried out with a Gilford model 250 dual-source spectrophotometer fitted with digital readout.

Buffer solutions. Aqueous solutions of 0.5 M monobasic sodium phosphate (A) and 0.5 M dibasic sodium phosphate (B) were prepared in chlorine demand-free distilled water, autoclaved for 30 min, and stored at 4°C. Forty-milliliter buffers having the desired pH were prepared from A and B as follows (16): for pH 6.0, 35.08 ml of A and 4.92 ml of B; for pH 7.0, 15.6 ml of A and 24.4 ml of B; for pH 7.8, 3.4 ml of A and 36.6 ml of B; for pH 10.0, 40 ml of B. After mixing, the solutions were diluted to 400 ml with water. For the pH 10 buffer, 40 ml of B was diluted to 400 ml, and the pH was adjusted with a small volume (0.32 ml) of 2 M NaOH.

SDS. An aqueous solution of 20% SDS (Sigma) was prepared and autoclaved.

PEG. A solution of PEG 6000 (Fisher Scientific Co.) was prepared by dissolving 120 g of the polymer in 360 g of distilled water containing 24.93 g of NaCl and autoclaved.

Potassium chloride. An aqueous solution of 3 M

KCl (Fisher Scientific Co.) was prepared and autoclaved. This reagent was used to precipitate the SDS.

Chlorine demand-free glassware. Preliminary experiments showed that the use of chlorine demand-free glass- and metalware is essential for maintaining a constant concentration of chlorine during the experiments. Therefore, all glassware was first treated with chromic acid and washed with detergent. Then the glassware and metalware were soaked with 1% chlorine water, scrubbed with water, rinsed with chlorine demand-free distilled water, dried, and autoclaved.

Viral inactivation kinetics experiments. Experiments for the kinetics of inactivation of poliovirus type 2 were performed by using a kinetic apparatus (32). This apparatus consisted of six stainless-steel beakers of 600-ml capacity, a water bath, a thermoregulator, and six stainless-steel stirring rods connected to an overhead stirring device. Two beakers containing control solutions were used to determine the effects of the temperature and pH of the medium on virus inactivation. The remaining beakers contained test solutions. The solutions in all beakers were stirred throughout the experiment and maintained at the desired temperature in the carefully regulated water bath. A known volume of the stock solution of chlorine or CAT and 40 ml of a mixture of the two phosphate solutions (0.5 M each) were added to all beakers except the one used as a temperature control, which contained the same volume of water. Next, the solution was diluted to 399.5 ml with sterile, chlorine demand-free water. The actual timed experiment began at the inoculation of the test virus (0.5 ml) into the rapidly stirring (100 rpm) solutions. Five-milliliter portions of the solutions containing CAT or chlorine were withdrawn at definite contact time intervals and mixed immediately with 5 ml of thiosulfate. The concentration of thiosulfate solution used was adjusted to neutralize the toxic effects of CAT or chlorine without leaving excess residual thiosulfate which was toxic to cells (1.15 ppm of $Na_2S_2O_3$ [1.15 mg of $Na_2S_2O_3$ per liter]/ppm of CAT; 4.45 ppm of $Na_2S_2O_3$/ppm of chlorine) used for virus assay. In addition, since complete neutralization of the reactants was not observed at alkaline pH, the thiosulfate solutions used under these conditions were prepared in ~0.04 M HCl. In the case of control solutions, the toxic thiosulfate solution was replaced by the same volume of water, since control studies showed that this variation had no effect on virus titers obtained.

For each experiment, the time required for 99% inactivation of the virus and the first-order rate constant, k_1, were calculated from the initial linear part of the curve.

RESULTS

Inactivation of poliovirus type 2 by CAT. The kinetics of inactivation of poliovirus type 2 by CAT was investigated at several concentrations of the reactant. Assuming that inactivation of the virus is due to a chemical reaction between CAT and some susceptible site of the virus essential for infectivity, the kinetic laws of biomolecular reactions should be followed. Since CAT was used in great excess, pseudo-first-order kinetics yielding a straight line on semilogarithmic plot might be expected. However, in practice, departures from the expected linearity have been observed for chlorine. Gard (cited in reference 32) proposed that such deviations were due to reactions between chlorine and virus that did not result in inactivation of some of the virus particles. Similar to chlorine, the first part of the CAT inactivation plots obtained by plotting log n/n_0 (n = $TCID_{50}$) versus time were linear and thus obeyed a pseudo-first-order kinetics (Fig. 1). This indicates a first-order dependence of the inactivation rate on the virus (Table 2), since the CAT concentration was in large excess and remained constant throughout the reaction.

An interesting feature observed was the two-stage shape of the inactivation curve, the initial part showing a much faster rate than the second part. Our curves are in agreement with the kinetic curves reported in the literature for the inactivation of poliovirus (9, 21, 22). A possible interpretation for the two-stage reaction might be that 0.5 to 1% of the virus consists of clumps (11, 21).

Effect of CAT concentration on the inactivation rate of poliovirus type 2. The results in Table 2 show the dose-response relationship between the rate of inactivation of poliovirus type 2 and CAT concentration. A decrease in 99% inactivation time period was observed with increase in the concentration of CAT. A plot of log CAT concentration against log 99% inactivation time was linear with negative slope (Fig. 2), showing a direct correlation between the disinfectant concentration and the duration of 99% inactivation at pH 6.0, 7.0, and 7.8. The higher the CAT concentration, the shorter was the duration of the first part of the curve. In fact, at low CAT concentrations of 10 and 20 mg/liter, the two-stage inactivation did not even appear in Fig. 1. Furthermore, a plot of log k_1 versus log [CAT] (molarity) gave a straight line

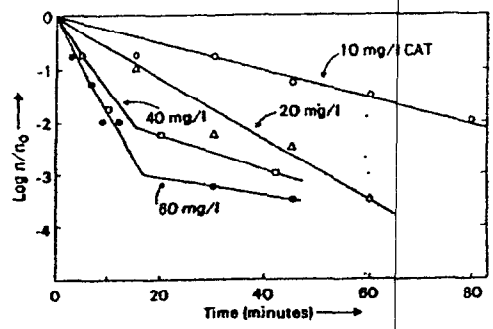

FIG. 1. *Kinetics of inactivation of poliovirus type 2 at the indicated concentrations of CAT at pH 6 and 5°C (where n/n_0 equals the surviving fraction).*

TABLE 2. *Effect of CAT concentration and pH on the rate of inactivation of poliovirus type 2 in water at 5°C*

CAT concn		pH 6		pH 7		pH 7.8		pH 10[a]	
mg/liter	M (× 10^4)	Time[b] (min)	k_1 (× 10^3) (s^{-1})	Time (h)	k_2 (× 10^3) (s^{-1})	Time (h)	k_1 (× 10^3) (s^{-1})	Time (h)	k_1 (× 10^2) (s^{-1})
10	0.355	78	0.98	4.35	2.94	8.1	1.58	33	3.88
20	0.71	34	2.26			5.5	2.33	>8	
40	1.42	14	5.29	1.35	9.48	4.8	2.67	>6	
60	2.13	11	6.98	1	12.79	2.62	4.88	27.1	4.74

[a] The curves at pH 10 showed more scattering than at other pH's, so calculated parameters are less reliable here.

[b] 99% inactivation time.

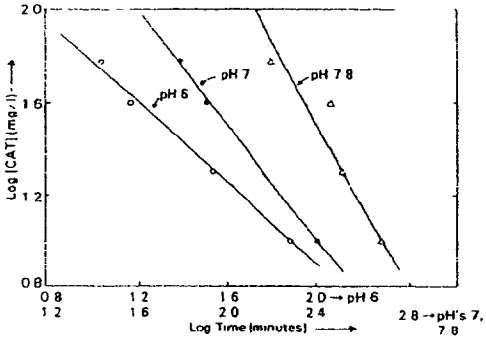

FIG. 2. *Time-concentration relationships for 99% inactivation of poliovirus type 2 at different pH's and 5°C (log-log scale).*

with a slope equal to 1.1 (Fig. 3), establishing a first-order dependence on the disinfectant concentration at pH 6 and 5°C. The same trend was observed at pH 7.0 and 7.8 (Table 3; Fig. 3). The deviation from unity may have been due to experimental error or to a change in mechanism at higher pH. Deviations of a similar magnitude have been observed previously for aqueous disinfectants (22).

Effect of pH on the inactivation rate of poliovirus type 2. The kinetics of inactivation of poliovirus type 2 by CAT at pH 6.0, 7.0, 7.8, and 10 were also two stage (Table 2). The inactivation rate decreased linearly with increase in pH, whereas the 99% viral inactivation time increased proportionately with increase in pH. Furthermore, at a CAT concentration of 10 mg/liter, a plot of log k_1 versus pH gave a straight line with a slope of −0.5, showing that the order with respect to $[OH^-]$ was a negative fraction, whereas the order with respect to $[H^+]$ was a positive fraction (Fig. 4). The same trend was noticed at higher CAT concentrations of 40 and 60 mg/liter (Table 3; Fig. 4).

Effect of temperature on the rate of inactivation of poliovirus type 2. The kinetics

TABLE 3. *Order of the inactivation reaction of poliovirus type 2 with CAT at 5°C*

CAT (mg/liter)	Order with respect to [H']	pH	Order with respect to [CAT]
10	0.47	6.0	1.10
40	0.72	7.0	0.86
60	0.64	7.8	0.60

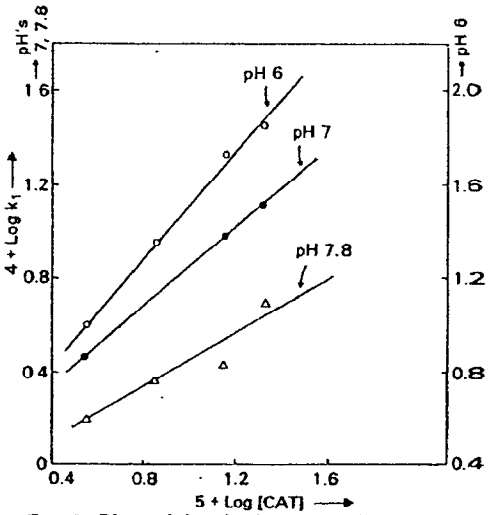

FIG. 3. *Plots of log k_1 (per second) versus log [CAT] (molar) at the indicated pH and 5°C.*

of inactivation of poliovirus type 2 by CAT (10 mg/liter) at pH 6 (0.05 M phosphate) was determined at different temperatures. The 99% viral inactivation time and k_1 were calculated, and an Arrhenius plot (Fig. 5) was obtained by plotting log k_1 against $1/T$ (reciprocal of absolute temperature). Table 4 shows the kinetic, Arrhenius, and thermodynamic parameters evaluated in the standard manner (10).

The data in Table 4 include the values of kinetic parameters (99% inactivation time and first-order rate constant), Arrhenius parameter

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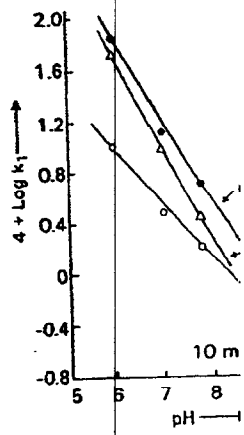

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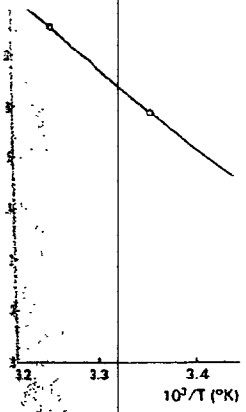

e 5. *Arrhenius plot of log*
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ter at pH 6.

Kinetic, Arrhenius

	99% inactivation time (min)
	78
	30
	13
	6

concentration, 10 mg/
opy of activation; ΔG:

(Arrhenius factor A), and thermodynamic parameters (enthalpy of activation, entropy of activation, and free energy of activation) obtained at four temperatures. However, Arrhenius and thermodynamic parameters were calculated based on the value of energy of activation, E_a (where E_a is 2.303 × the ideal gas constant, R × slope of Arrhenius plot) determined at 5 to 35°C. The low values of E_a and thermodynamic parameters, including a negative value for entropy of activation, suggest that poliovirus inactivation by CAT does not involve the rupture of many hydrogen bonds (5, 6, 12, 13).

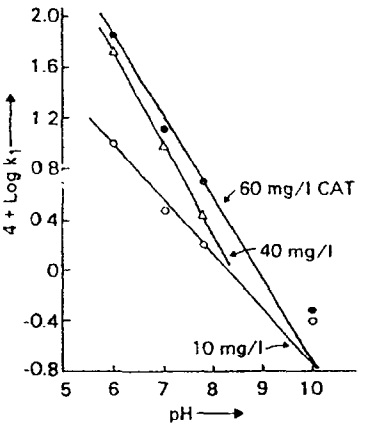

FIG. 4. *Plots of log k_1 (per second) versus pH of the medium for the indicated concentrations of CAT at 5°C.*

Inactivation of poliovirus type 2 by chlorine. For comparison with CAT, the inactivation of poliovirus type 2 by HOCl (0.5 mg of chlorine per liter) at pH 6 was observed at two different temperatures, 5 and 10°C, and two alkaline pH's, 7.8 and 10. Kinetic curves were obtained by plotting log n/n_0 against time (Fig. 6). It can be hypothesized that the kinetics of inactivation obey a pseudo-first-order reaction. Table 5 gives the kinetic, Arrhenius, and thermodynamic parameters determined from Fig. 6 and similar inactivation studies at 10°C (data not shown). It is interesting to note that the general shape of the kinetic curve of chlorine is similar to that of CAT.

DISCUSSION

The overall significance of these studies is that CAT may be a useful disinfectant for inactivat-

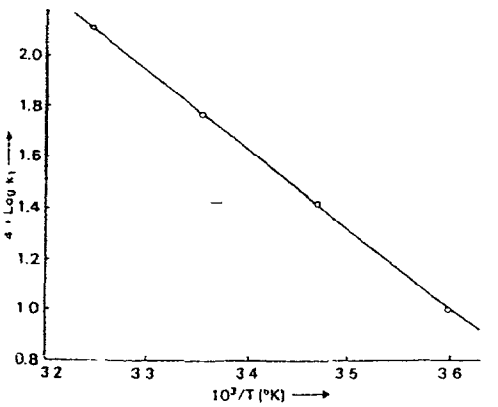

FIG. 5. *Arrhenius plot of log k_1 (per second) versus reciprocal of absolute temperature for 10 mg of CAT per liter at pH 6.*

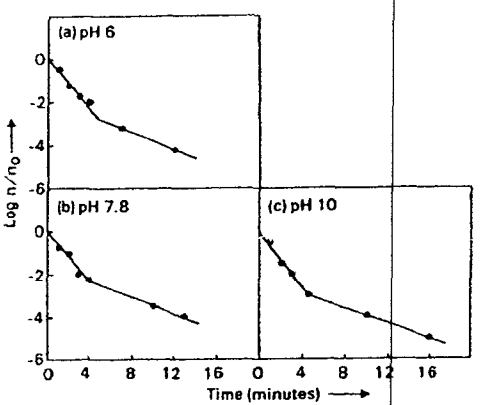

FIG. 6. *Kinetics of inactivation of poliovirus type 2 by 0.5 mg of chlorine per liter at the indicated pH and 5°C (where n/n_0 equals the surviving fraction).*

TABLE 4. *Kinetic, Arrhenius, and thermodynamic parameters for the inactivation of poliovirus type 2 by CAT in water*[a]

Temp (°C)	99% inactivation time (min)	k_1 (× 10^5) (s^{-1})	log A	$\Delta H‡$ (kcal/mol)	$\Delta S‡$ (e.u.)	$\Delta G‡$ (kcal/mol)
5	78	0.984	8.23	13.76	−20.73	19.52
15	30	2.559	8.27	13.74	−20.64	19.69
25	13	5.905	8.26	13.72	−20.75	19.91
35	6	12.791	8.26	13.70	−20.81	20.11

[a] CAT concentration, 10 mg/liter, pH 6. E_a, 14.31 kcal/mol; A, Arrhenius factor; $\Delta H‡$, enthalpy of activation; $\Delta S‡$, entropy of activation; $\Delta G‡$, free energy of activation; e.u., entropy units. 1 cal = 4.185 J.

TABLE 5. *Kinetic, Arrhenius, and thermodynamic parameters for the inactivation of poliovirus type 2 by chlorine in water*[a]

Temp (°C)	99% inactivation time (min)	k_1 ($\times 10^5$) (s^{-1})	log A	$\Delta H\ddagger$ (kcal/mol)	$\Delta S\ddagger$ (e.u.)	$\Delta G\ddagger$ (kcal/mol)
5	3.5	2.193	9.58	14.73	−13.07	18.36
10	2.15	3.571	9.60	14.72	−13.10	18.43

[a] Chlorine concentration, 0.5 mg/liter, pH 6. Ea, 15.28 kcal/mol. Abbreviations as in Table 4.

ing poliovirus in slightly acidic (around pH 6) water. The experimental results suggest that the overall reaction for the inactivation of poliovirus type 2 by CAT is complex, being first order with respect to the virus (P) and CAT, each having a fractional order with respect to [H^+] or [OH^-]. The rate laws are given by equations 1 and 2, where rates are designated by v_1 and v_2 and the rate constants are designated by k_1 and k'_1 for acid and alkaline conditions, respectively.

$$v_1 = k_1 [P] [CAT] [H^+]^{0.5} \text{ (acid conditions)} \quad (1)$$

$$v_2 = k'_1 [P] [CAT]/[OH^-]^{0.5} \text{ (alkaline conditions)} \quad (2)$$

The reaction mechanism responsible for the inactivation of poliovirus type 2 by CAT in acid medium was arrived at as follows. Bishop and Jennings (4) have shown that CAT (CH_3-C_6H_4-SO_2NClNa; RNClNa) behaves like a strong electrolyte in aqueous medium, dissociating as:

$$RNClNa \rightleftharpoons RNCl^- + Na^+ \quad (3)$$

The protonation of the anion in acid solutions gives the free acid, RNHCl, as follows:

$$RNCl^- + H^+ \rightleftharpoons RNHCl;\ K = 3.8 \times 10^4;\ 25°C \quad (4)$$

Although the free acid has not been isolated, there is ample experimental evidence for its existence in acidic solutions (17). Bishop and Jennings have shown that in a 0.05 M solution of CAT, [RNHCl] $\approx 10^{-2}$ in the range of pH 0 to 1, whereas [HOCl] $\approx 10^{-7}$. In aqueous alkaline medium, CAT undergoes hydrolysis to form RNHCl as follows (4, 26):

$$RNCl^- + H_2O \rightleftharpoons RNHCl + OH^- \quad (5)$$

It has been proposed by Mahadevappa et al. (26) that RNHCl, in fact, is the main reactive species for the alkali-retarded chloraminometric reactions. Therefore, RNHCl, the acid form of CAT, is most likely to be the main reactive species in both acid and alkaline media. The possibility of other species of CAT, such as $RNCl_2$ (dichloramine-T) and HOCl in acidic solutions and OCl^- and $RNCl^-$ (CAT) itself in alkaline solutions, being the reactive species can be discounted based on the experimental results. We propose the following mechanism to account for the observed kinetics of inactivation of poliovirus type 2 by CAT in acid conditions:

$$RNCl^- + H^+ \underset{}{\overset{K_1}{\rightleftharpoons}} RNHCl;\ \text{fast} \quad \text{(i)}$$

$$RNHCl + P \overset{K_2}{\rightleftharpoons} X;\ \text{slow} \quad \text{(ii)}$$

$$X \overset{k_3}{\longrightarrow} X';\ \text{slow and rate determining} \quad \text{(iii)}$$

$$RNHCl + X' \overset{k_4}{\longrightarrow} P_i + \text{products};\ \text{fast} \quad \text{(iv)}$$

where P_i is the inactivated poliovirus and X and X' are activated complex and reaction intermediates, respectively. Considering the first two equilibrium reactions (given above) and the expression for total concentration of CAT (equation 6), the rate expression is obtained as in equation 7:

$$[CAT]_T = [RNCl^-] + [RNHCl] + [X] \quad (6)$$

$$v = \frac{K_1 K_2 k_3 [P] [CAT]_T [H^+]}{1 + K_1 [H^+] + K_1 K_2 [H^+] [P]} \quad (7)$$

Since K_2 is small, the following equation (8) could be obtained by making the reasonable assumption $K_1 [H^+] \gg K_1 K_2 [H^+] [P]$:

$$v = \frac{K_1 K_2 k_3 [P] [CAT]_T [H^+]}{1 + K_1 [H^+]} \quad (8)$$

$$\text{or } v = \frac{k [P] [CAT]_T [H^+]}{1 + K_1 [H^+]} \quad (9)$$

where $k = K_1 K_2 k_3$ and v is the rate of inactivation. Based on similar arguments and considering a similar mechanism, a similar rate expression for alkaline conditions can be derived (details will be published elsewhere).

The observed fractional order dependence on [H^+] can be explained by equation 9. The omission of the first term makes the order with respect to [H^+] zero whereas omission of the second term produces an equation with order 1 for [H^+], showing that the reaction actually operates between the two values. The proposed machanism and the derived rate law (equation 9) are in agreement with the experimental results (equation 1). The proposed mechanism and assertion that RNHCl is the active form of CAT in both acid and alkaline media are consistent

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)	ΔG‡ (kcal/mol)
7	18.36
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The most likely interaction of CAT' with infectious virus that leads to the virus inactivation can be determined from the Arrhenius and thermodynamic parameters. The values of these parameters (Table 4) determined for the inactivation of poliovirus type 2 by CAT are compatible with those of denaturation of the ribonucleic acid (RNA) of viruses (12, 13). The low values of energy of activation (14.31 kcal/mol [59.89 kJ/mol]), enthalpy of activation (13.73 kcal/mol [57.46 kJ/mol]), and free energy of activation (19.81 kcal/mol [82.91 kJ/mol]) and the negative value of entropy of activation (−20.73 entropy units [−86.76 J/deg-mol]) are in agreement with the values of Ginoza et al. for denaturation of RNA (12, 13). This would suggest that somehow CAT' (and also chlorine) may interact with RNA of the virus rather than with protein, perhaps, as Ginoza (12) has suggested, by forming phosphotriester bonds with subsequent hydrolytic cleavage of the RNA chain.

In conclusion, these experiments indicate that (i) a clear dose-response relationship between CAT concentration and the virus inactivation rate, first order with respect to [CAT], can be demonstrated; (ii) the rate of the viral inactivation is pH dependent (faster in acid than in alkaline medium), having a fractional order with respect to both $[H^+]$ and $[OH^-]$; (iii) the kinetic reaction is first order with respect to the virus; (iv) certain anomalies in relation to virus inactivation by CAT may be associated with varying reactive species of CAT that may be formed under varying experimental conditions and relate to virus clumping and disaggregation under varying conditions; (v) the mode of attack by CAT species may be through the denaturation of the viral RNA; and (vi) CAT may be an effective viricide against poliovirus type 2 in acid rather than alkaline media. In addition, these studies suggest that CAT may be a suitable replacement for chlorine in the disinfection of slightly acidic waters.

With regard to the pH of drinking and wastewater, the waters of some rivers and lakes which receive acid rain and acid industrial and mine wastes tend to have fairly low pH's. For example, the pH of a stream of water near Johannesburg (U.K.) was found to be between 3.7 and 4.8 (23). Hence, it would be easier to adjust to pH 6 rather than a more alkaline pH before disinfection. Conventional sewage treatment has been found to be efficient in the acid range. It was shown in the United Kingdom that treatment of Bradford sewage with activated sludge gave the best results at pH 6.0 to 6.5 (24). Thus, there may be certain instances in which disinfection should be carried out in slightly acid pH. Under such circumstances, the use of CAT would be favored.

ACKNOWLEDGMENTS

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An Outbreak of Gastroenteritis in a Home for the Elderly Associated With Astrovirus Type 1 and Human Calicivirus

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We describe an outbreak of gastroenteritis, which lasted for 22 days in a residential home for the elderly. The outbreak was biphasic and affected 34/42 (80%) residents and 13/29 (44%) members of the staff. Calicivirus was associated with cases of illness during the first 9 days of the outbreak, and astrovirus type 1 with cases arising between days 16 to 22. Although the symptoms were generally mild, the resources required and the inconvenience caused were considerable.

Key words: old people's home, biphasic illness, faecal viruses

INTRODUCTION

Several viruses may cause gastroenteritis in closed communities. These include rotavirus, calicivirus, astrovirus, and the small round viruses [Kapikian et al, 1980]. Caliciviruses and astroviruses cause infantile gastroenteritis in particular [Cubitt, 1985; Ashley et al, 1978]. However, few outbreaks associated with these viruses in the elderly have been recorded [Cubitt et al, 1980; Watkins, 1984].

THE OUTBREAK

Over 22 days, 34/42 (80%) residents and 13/29 (44%) members of the staff at an old people's home were affected with an illness characterized by vomiting, diarrhoea, and abdominal cramps. The illness lasted for 48 hours, but in 4 of the residents, who appeared to have recovered, similar symptoms recurred 7 to 10 days later. The number of cases reported during the outbreak is shown in Figure 1. Between January 20 and 28, 1985, 41 cases of illness were recorded. Over the following week no new cases were reported, and it was thought that the outbreak was over. Between February 4 and 10, however, a further 13 cases arose. The pattern of the outbreak suggested person-to-person transmission.

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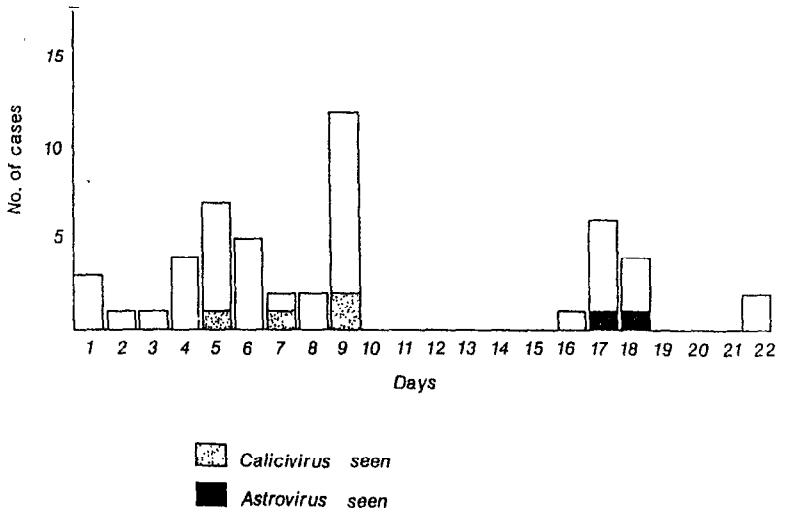

Fig. 1. Onset of illnesses in an outbreak of calicivirus and astrovirus infection in a residential home for the elderly.

MANAGEMENT

Environmental health officers visited the home on two occasions and instructed the staff about precautions they should take. General hygiene and good food handling techniques were emphasized. The home was closed to visitors and the admission of new residents halted. The community "meals-on-wheels" service, which was based in the home, was suspended and an attempt was made early on in the outbreak to confine affected residents to one part of the building. These measures had little effect and new cases continued to occur. The last case was recorded 22 days after the onset of the outbreak. The home was reopened 10 days later; no further cases were reported.

LABORATORY INVESTIGATIONS

Specimens of faeces collected from those affected and samples of food, milk, and water were examined for the presence of bacterial pathogens by means of routine bacteriological methods. Similar specimens from 9 residents and 2 staff members were examined by electron microscopy to determine whether a virus might be the causative agent. Briefly, faecal samples were suspended in phosphate-buffered saline and clarified by low-speed centrifugation (5,000 g for 10 minutes). The supernatants were collected and centrifuged at 150,000 g for 1 hour in an ultracentrifuge. The resulting pellets were resuspended in potassium phosphotungstic acid (pH 6.3), placed on a carbon-formvar coated copper grid, and examined at a magnification of 50,000× in a JOEL JEM-100 CX electron microscope.

Acute and convalescent phase serum samples were obtained from 9 residents and 1 member of the staff. Convalescent phase samples were also collected from a further 2 residents and 2 staff members. Serum samples and a pooled faecal extract were sent to the Central Middlesex Hospital for further studies. The faecal extract was purified on a

TABLE I. Detection of Antibodies to Astrovirus Type 1 by Immune Electron Microscopy

Case	Staff (S) or resident (R)	Symptoms: Days 1–10	Symptoms: Days 11–22	Electron microscopy	Antibody titer: Acute	Antibody titer: Convalescent
1	R	yes	yes	Astrovirus	20	>640
2	R	yes	yes	NA	20	320
3	R	no	yes	Astrovirus	<20	>160
4	R	no	yes	NA	<20	>160
5	R	yes	yes	Calicivirus	<20	<20
6	R	yes	no	NA	<20	<20
7	R	yes	no	Negative	20	40
8	R	yes	no	NA	<20	<20
9	R	yes	no	NA	<20	<20
10	R	yes	no	Calicivirus	NA	<20
11	R	yes	no	NA	NA	20
12	S	yes	no	NA	40	40
13	S	yes	no	NA	NA	40
14	S	yes	no	NA	NA	<20

NA = Specimen not available.

Metrizamide density gradient and fractions examined in a Philips EM 201 microscope for the presence of astrovirus and calicivirus particles.

RESULTS

Bacteriological examination of food, milk, water, and faecal samples from affected persons failed to reveal an etiological agent. Electron microscopy, however, revealed virus particles in 6/9 faecal samples collected from elderly residents. Caliciviruses were seen in 4 specimens collected between January 20 and 28 and astroviruses in 2 specimens collected between February 4 and 10.

The astrovirus was identified by immune electron microscopy [Cubitt et al, 1979] by means of hyperimmune antiserum raised in rabbits against astrovirus types 1 to 4 (kindly provided by Dr J. Kurtz, Public Health Laboratory, Oxford). Since only a few calicivirus particles were detected, it was not possible to type them. However, serological tests were performed by immune electron microscopy [Cubitt et al, 1979] with purified astrovirus type 1.

Serological tests revealed that 4 residents had a greater than fourfold rise in antibody titer to astrovirus type 1 (Table 1). Two of these residents had been shown to be excreting astrovirus in their faeces. None of the residents or staff members who were affected exclusively in the first 10 days of the outbreak had evidence of recent infection with astrovirus. This, together with the results of electron microscopy, suggest that the calicivirus was the probable cause of the initial outbreak of diarrhoea and vomiting (days 1–10) and astrovirus type 1 for the second episode of illness.

DISCUSSION

The origin of the outbreak is obscure. Food, milk, and water were tested for bacterial contamination but not for the presence of viruses. Food as the major source of infection is

unlikely if one examines the epidemic curve (Fig. 1). If a contaminated meal had been the source of infection, one would have expected to have seen a peak of infection within 48 hours rather than the steady build up of cases over several days. Furthermore, the kitchens were also used by the local authority for the community "meals-on-wheels" service. This was suspended a few days after the onset of the outbreak, but cases of gastroenteritis were not recorded among the people it served.

The pattern of infection and the presence of secondary infections among the families of affected staff members suggest person-to-person transmission. It is possible, however, that one or both viruses were introduced into the home on food prepared by an infected visitor or member of the staff [White et al, 1986; Appleton, 1979]. Among the initial three cases reported, two were members of the staff who were presumably in contact with the residents while incubating the disease. Throughout the outbreak, members of the staff continued to become ill and it was reported that because of the shortage of staff members, many returned to work while still unwell. Residents shared a communal dining room and mixed freely in several lounges and, although most had single rooms, they shared bathing and toilet facilities.

Children whose parents worked in the home had free access and often helped with the residents at meal times. Two members of the staff did report illness in their children at the time of the outbreak. Both children had diarrhoea and vomiting on January 29 and 30 (day 11 and 12) at a time when new cases were not reported at the home. Since specimens were not collected from the children, it is impossible to say whether they acquired a calicivirus infection from their parents or had an astrovirus infection that was subsequently introduced into the home or, indeed, whether their symptoms were due to infection with either of these viruses.

Although the symptoms of illness were generally mild and all those affected recovered, the resources used and the inconvenience caused were considerable. In one general practice the doctors visited patients daily, if not several times a day. A district nurse was called full-time to work in the home during the outbreak and agency nurses had to be employed to cover for those staff members who were ill. The community "meals-on-wheels" service had to be transferred to a school kitchen since early on in the outbreak a food-borne infection could not be ruled out. The services of two environmental health officers, a district community physician, and three laboratories were required in an effort to determine the cause and subsequently to control the outbreak.

Outbreaks of diarrhoea and vomiting in the elderly are common. In recent years, many have been associated with rotavirus infections [Cubitt, 1982]. As far as we are aware, there is only one report of calicivirus [Cubitt et al, 1980] and one relating to astrovirus [Watkins, 1984] causing problems in this age group. This study emphasizes the impact that these viruses can make in confined communities. There is a need to develop simpler but sensitive and specific diagnostic techniques as an alternative to electron microscopy so that the true size of this problem can be established.

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Proteins of Norwalk Virus

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The proteins of the Norwalk virus were studied by polyacrylamide gel electrophoresis. Highly purified specifically immunoprecipitated virions appeared to contain a single primary structural protein with a molecular weight of 59,000. In addition, a soluble Norwalk viral protein with a molecular weight of 30,000 was identified in fecal specimens containing Norwalk virus. The protein structure of the virion is similar to that of the Calciviridae family.

The Norwalk virus is the best-characterized member of a group of small noncultivatable viruses that cause acute epidemic gastroenteritis in humans (21). Infection with Norwalk virus is common; seroepidemiological studies have shown that antibodies to the Norwalk or antigenically related viruses are found in more than 50% of adults around the world (4, 7).

The virus is a frequent cause of gastroenteritis; it has been associated etiologically with 24 of 70 separate outbreaks of nonbacterial gastroenteritis which have occurred over the past decade (8, 9; H. B. Greenberg, R. G. Wyatt, A. R. Kalica, R. H. Yolken, R. Black, A. Z. Kapikian, and R. M. Chanock, *in* M. Pollard, ed., *Perspectives in Virology*, in press). Studies with volunteers have shown that the Norwalk virus can be serially transmitted and that in this experimental setting it causes a disease identical to the illness observed during natural outbreaks (6). By immune electron microscopy, Norwalk virus has been shown to be shed briefly, coincident with the peak of gastrointestinal illness (18). There are several other antigenically distinct small gastroenteritis viruses which resemble the Norwalk virus morphologically and epidemiologically (21), but these agents have been studied less thoroughly.

Despite the clinical importance of Norwalk virus and its antigenically distinct relatives, investigation of these agents has proven difficult. None of these small gastroenteritis viruses has been successfully cultivated in vitro, and only humans and chimpanzees have been shown to be susceptible to infection (20). Norwalk virus is shed in the feces in rather small quantities (at least 10- to 50-fold less than is hepatitis A virus when particle-rich fecal specimens are compared [unpublished data]), and the shedding period for Norwalk virus is brief (18).

Because the Norwalk virus has not been successfully cultivated in vitro and is shed only in limited amounts, it has been difficult to purify and hence classify. This is also true for the other antigenically distinct 27-nm gastroenteritis agents. In an effort to aid in the classification of the Norwalk virus, we have attempted to characterize its proteins.

MATERIALS AND METHODS

Virus. Norwalk virus and viral protein were isolated from a single diarrheal stool of a volunteer experimentally infected with the agent. This particle-positive stool was used because it contained the highest concentration of antigen, as assayed by radioimmunoassay (RIA), in a survey of sequential stools from more than 30 ill volunteers. Partially purified feline calicivirus (vaccine strain F-9) was kindly furnished by Frederick Schaffer, Naval Bioscience Laboratory, University of California, Berkeley. This calicivirus was iodinated and immunoprecipitated in a manner similar to that used for the Norwalk virus and Norwalk protein preparations.

Antisera. Preinoculation and 4-week convalescent sera from two volunteers experimentally infected with Norwalk virus, as well as acute-phase and convalescent sera from a child naturally infected with a gastroenteritis virus antigenically related to Norwalk virus (2), were used for immunoprecipitation. Paired anti-Norwalk sera were selected for having little or no measurable antibody in the preinfection or acute-phase specimen and a high titer in the convalescent specimen. Hyperimmune feline antiserum to feline calicivirus F-9 was kindly furnished by James Gillespie, Cornell University, Ithaca, N.Y.

RIA. RIA for Norwalk virus antigen or antibody was performed as described previously. The assay was shown to be both sensitive and specific (9).

Virus purification. A 10-g amount of diarrheal stool was suspended in 40 ml of TN buffer (0.01 M Tris, 0.15 M NaCl, 0.05% sodium azide, pH 8.0). An equal volume of trichlorotrifluoroethane (Genetron 113) was added to this suspension, and the mixture was homogenized for 1 min. Then the preparation was centrifuged (4,000 × *g* for 5 min) in a Sorvall GSA centrifuge rotor. The aqueous supernatant was decanted, and the Genetron layer and interface were reextracted twice more with TN buffer. The pooled

supernatants (approximately 120 ml each) were centrifuged (96,300 × *g* for 12 h) in an SW27 rotor. The high-speed supernatant was carefully decanted and saved (see purification procedure described below). The crude stool pellet (P1) was suspended in 10 ml of TN buffer and repelleted through 3 ml of 30% (wt/vol) sucrose at 150,000 × *g* for 6 h in an SW40 rotor. The supernatant was discarded, and the pellet (P2) was resuspended in 2 ml of TN buffer and layered on top of a 1.2- to 1.6-g/cm^3 discontinuous cesium chloride gradient and centrifuged at 150,000 × *g* for 18 h in an SW40 rotor. Fractions (1 ml each) of the gradient were collected and assayed for Norwalk virus antigen by RIA. The virion-associated peak fractions (fractions 4 and 5, Fig. 1) were pooled, diluted approximately sixfold, and pelleted (150,000 × *g* for 4 h in an SW40 rotor). The pellet (P3) was suspended in 0.4 ml of TN buffer and layered on a continuous 10 to 30% (wt/vol) sucrose gradient with a 1-ml, 1.6-g/cm^3 cesium chloride cushion and centrifuged at 100,000 × *g* for 90 min in an SW40 rotor. Fractions (5/10 ml each) were collected and assayed for Norwalk antigen activity by RIA (Fig. 2). The peak fraction (fraction 16, Fig. 2) was taken and layered on a continuous 30 to 60% (wt/vol) Renografin (Squibb) gradient in TN buffer and centrifuged for 15 h at 150,000 × *g* in an SW40 rotor. Fractions (0.6 ml each) were collected and assayed for antigen activity by RIA (Fig. 3). The peak fraction (fraction 7, Fig. 3) was diluted fivefold in TN buffer and pelleted at 200,000 × *g* for 5 h in an SW56 rotor. The virus pellet was suspended in 100 μl of 0.25 M phosphate buffer, pH 7.4.

Purification of the soluble Norwalk virus protein. The pooled high-speed supernatant (120 ml; see above), which remained strongly positive for Norwalk virus antigen activity in RIA, was recentrifuged for 18 h at 96,000 × *g* in an SW27 rotor. The supernatant was again carefully collected and concentrated approximately 10-fold by pressure dialysis, using an Amicon PM10 filter. A 5-ml amount of the 10× high-speed supernatant was layered onto a gel filtration column (2.6 by 100 cm; G-200 Sephacryl superfine;

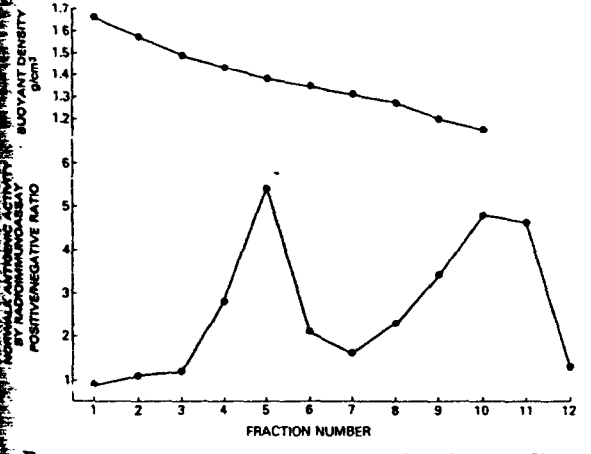

FIG. 1. *Cesium chloride buoyant density gradient of Norwalk virus. Conditions of centrifugation and preparation of Norwalk virus were as described in the text. Fractions 4 and 5 were pooled and used for further virion purification.*

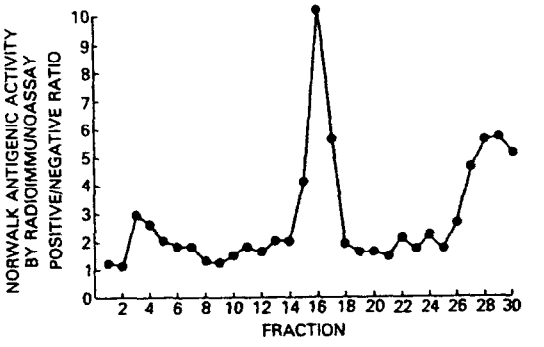

FIG. 2. *Rate zonal sedimentation of Norwalk virions in 10 to 30% sucrose gradient. Conditions of centrifugation and viral preparation were as described in the text. Fraction 16 was used for further virion purification.*

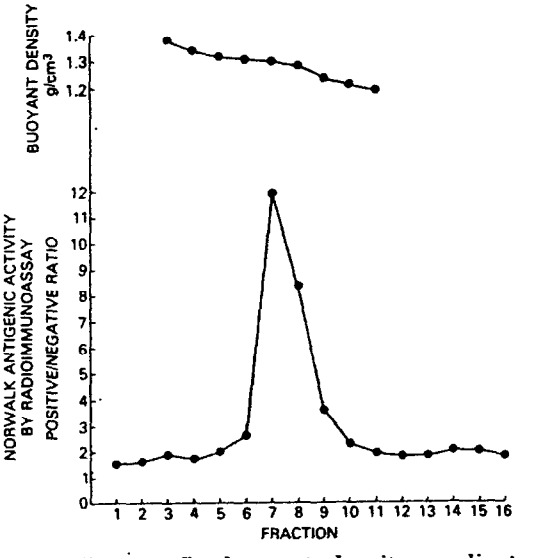

FIG. 3. *Renografin buoyant density gradient of Norwalk virion. Conditions of centrifugation and viral preparation were as described in the text. Fraction 7 was used for iodination and immunoprecipitation studies.*

Pharmacia Fine Chemicals) which was run at a flow rate of 20 ml/h with TN buffer. The column elution profile was calibrated with Pharmacia low- and high-molecular-weight standards. Fractions (4 ml each) were collected, and each was assayed for Norwalk virus antigen by RIA. The peak eight fractions were pooled (fractions 50 to 57, Fig. 4), concentrated 10-fold, and dialyzed against 0.005 M phosphate buffer (pH 8.0) by using an Amicon PM10 filter. The concentrated peak fractions from the Sephacryl gel filtration were then layered onto a DEAE-cellulose (Whatman DE 52) column (1.5 by 30 cm). A stepwise discontinuous molar phosphate buffer (pH 8.0) gradient (0.007, 0.016, 0.031, 0.06, 0.12, 0.25, and 0.5 M) with 20-ml steps was used to elute the Norwalk virus antigen. Fractions (5 ml each) were collected and assayed for Norwalk virus antigen by RIA. The peak fractions were pooled and concentrated 10-fold as before and equilibrated back to TN buffer. By RIA, greater than

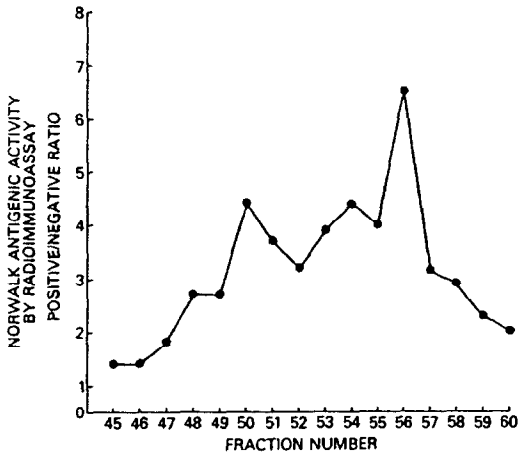

FIG. 4. *Sephacryl G-200 fine column chromatography of Norwalk virus-soluble viral protein. Conditions of elution were as described in the text. Fractions 50 to 57 were used for further purification (molecular weight range, 61,000 to 35,000).*

70% of the eluted Norwalk virus antigenic activity was contained in the 0.06 and 0.12 M fractions.

Affinity chromatography. An affinity column was prepared using immunoglobulin G from a volunteer known to possess a high level of antibody to Norwalk antigen as measured by RIA. Serum from this individual was not used in the immunoprecipitation experiments. A 5-mg amount of immunoglobulin G was coupled with 1 g of activated Sepharose 4B (Pharmacia Fine Chemicals) in $NaHCO_3$ buffer (0.1 M, pH 8.0) at 4°C overnight. The coupled Sepharose was then blocked with 1.0 M glycine and equilibrated with TN buffer in a Pasteur pipette column. The peak, pooled, concentrated fractions from the DEAE-cellulose purification of the Norwalk virus-soluble protein were bound to the affinity column in TN buffer. The column was then washed with 30 ml of TN buffer with 0.01% Nonidet P-40. The Norwalk virus protein was eluted from the affinity column with glycine hydrochloride buffer (pH 2.8), and the eluant fractions were immediately neutralized (to pH 7.0) with 1.0 M Tris buffer, pH 8. The affinity column-purified Norwalk virus antigen was again concentrated approximately 10-fold and exchange dialyzed with 0.25 M phosphate buffer (pH 7.4).

Iodination of Norwalk virion and soluble protein. A total of 4 μl of ^{125}I (Amersham; 100 μCi/μl; carrier-free NaI) and 15 μl of chloramine T (3.5 μg/μl) in 0.25 M phosphate buffer were added to 20 μl of purified virion, soluble protein, or feline calicivirus in 0.25 M phosphate buffer (pH 7.4). After 40 s, 20 μl of sodium metabisulfite (4.8 μg/μl) was added to the reaction, and the free iodine was separated from the labeled protein by gel filtration (Sephadex G-50 medium) with phosphate-buffered saline. The labeled protein was made 1% with fetal calf serum and kept at 4°C until immunoprecipitation.

Immunoprecipitation. Serum (8 μl), either preinfection, acute phase, convalescent, or, in the case of feline calicivirus F-9, hyperimmune antiserum, was added to 400 μl of labeled Norwalk virion, soluble Norwalk virus protein, or labeled feline calicivirus. After an overnight incubation at 4°C, 150 μl of *Staphylococcus aureus* A whole cells (Pansorbin, Calbiochem.-Behring Corp.) was added to the reaction, and the mixture was incubated at 37°C for 0.5 h. The mixture was then centrifuged (10,000 × *g* for 2 min), and the supernatant was discarded; the *S. aureus* A-immunoglobulin-^{125}I-labeled protein complex was washed twice with 1 ml of phosphate-buffered saline, and the final pellet was suspended in 200 μl of sample buffer (0.062 M Tris [pH 6.8], 5% mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, 0.0175% bromophenol blue) and boiled for 5 min before electrophoresis.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed at 25 to 30 mA on 12% gels by the method of Laemmli (12). Molecular-weight markers from Amersham (200,000 to 14,300 daltons) were used to estimate the size of the Norwalk virus polypeptides. Autoradiographs were made by using Kodak no-screen X-ray film.

RESULTS

Virion purification. The three-step purification procedure used for the Norwalk virion (Fig. 1 through 3) was found to be necessary to separate this virus from contaminating fecal material. Even after this purification scheme, a final specific immunoprecipitation was necessary to clearly separate the Norwalk virus protein from contaminants. As had been previously reported (10), Norwalk virus had a buoyant density of between 1.40 and 1.38 g/cm^3 in cesium chloride (Fig. 1). The buoyant density of Norwalk virus in renographin was 1.30 g/cm^3 as calculated from weighed fractions. A broad second peak of Norwalk virus antigenic activity was detected in fractions 9 to 11 (Fig. 1). When these or comparable fractions were examined by immune electron microscopy, no Norwalk virions were seen. This antigenic peak probably represents residual soluble Norwalk virus protein that had been trapped in the second fecal pellet (P2).

Purification of the soluble Norwalk virus protein. During initial purification studies of the Norwalk virion, we noticed that appreciable antigenic activity, as measured by RIA, remained in the supernatant of fecal suspensions that were centrifuged at sufficient force to pellet all Norwalk virus particles. This soluble antigenic activity was present in every stool in which we detected Norwalk virus specific antigen by RIA and was independent of suspension buffer (phosphate- or Tris-buffered saline), fecal concentration, or fluorocarbon treatment. In preliminary filtration studies of crude 10% fecal suspensions in TN buffer we used Amicon Ultra filters (PM 10, 30, and XM 100 A) with molecular weight exclusion sizes of 10,000, 30,000, and 100,000 to show that the soluble antigenic activity had an apparent molecular weight of more

than 10,000 but less than 100,000. Since in most stools studied more than 50% of the total Norwalk virus antigenic activity appeared to be associated with the soluble fraction, we attempted to further characterize this activity. The same stool was used for purification of virion and soluble protein because it contained large amounts of both materials.

Gel filtration of the concentrated high-speed supernatant (Fig. 4) showed a heterogenous elution pattern with antigen detected over a broad molecular weight range (35,000 to 61,000) with peak activity found in the 37,000-dalton range (fraction 56). The concentrated gel filtration peak of the Norwalk virus-soluble protein was eluted from the ion exchange column at phosphate buffer molarities of 0.06 and 0.125 M. Preliminary affinity chromatography studies disclosed that the soluble protein was rendered nonantigenic by 4 M guanidine, 6 M urea, 4 M KSCN, 4 M $MgCl_2$, and glycine hydrochloride buffer (pH 2.4 and 2.6). However, 0.3 M glycine hydrochloride, (pH 2.8) removed Norwalk virus antigen from the affinity column without destroying its antigenicity.

Polyacrylamide gel analysis of the virion and soluble protein. Preinfection serum from a volunteer infected with the Norwalk virus (RIA anti-Norwalk virus titer, 1:10) failed to specifically precipitate labeled protein from the virion preparation (Fig. 5). The convalescent serum (antibody titer, 1:3,200) precipitated a single major protein with a molecular weight of 59,000 from the virion preparation (Fig. 5). In another gel (not shown) the immunoprecipitated single structural protein of feline calicivirus was found to have a molecular weight of 65,000, whereas in the same gel, the Norwalk virion protein was again found to have a molecular weight of 59,000. Occasionally (not visible in Fig. 5 or 6), when gels were exposed for 3 to 4 weeks, two additionally faintly visible bands (molecular weights 40,000 and 34,000) were seen in the specifically immunoprecipitated virion preparation. The single 59,000-dalton protein was again specifically precipitated by convalescent serum from a second infected volunteer and by convalescent infection serum from a child infected during a naturally occurring epidemic (preinfection or acute-phase titer, 1:10; convalescent titer, 1:3,200 for both pairs) (Fig. 6).

A single protein band with a molecular weight of 30,000 was specifically precipitated from the soluble protein preparation with convalescent anti-Norwalk virus serum from two volunteers (Figs. 5 and 6). The two preinfection serum specimens failed to precipitate detectable iodinated protein from the soluble protein preparation (Fig. 5 and 6). The soluble viral protein was also specifically immunoprecipitated (data not shown) by convalescent serum obtained from a patient with naturally acquired epidemic gastroenteritis caused by the Norwalk virus.

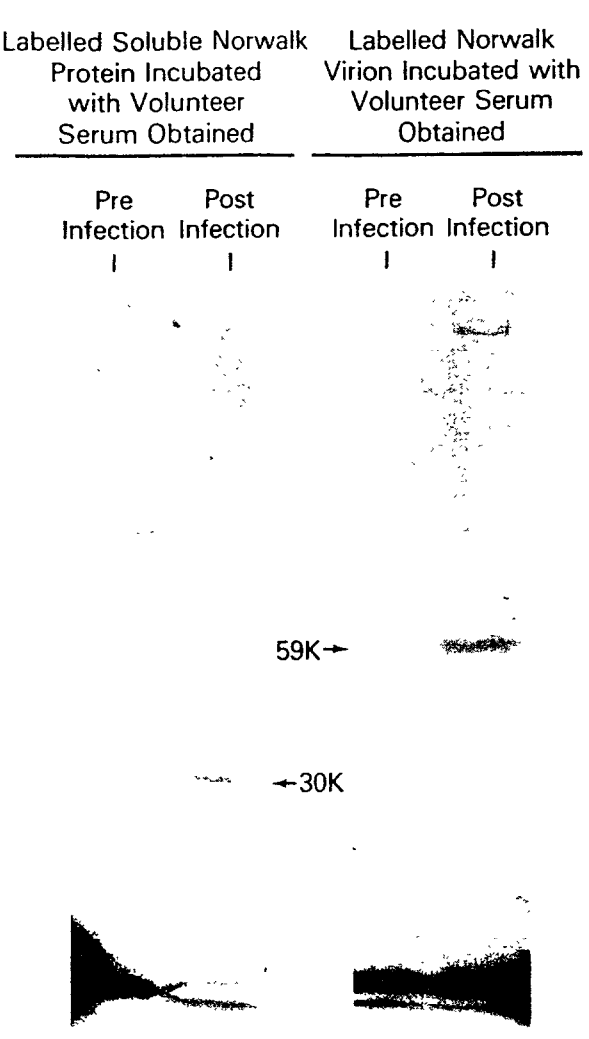

FIG. 5. *Polyacrylamide gel electrophoresis of immunoprecipitated Norwalk virus and viral protein. Conditions of gel and immunoprecipitation were as described in the text. Antisera used in immunoprecipitation were as indicated in the figure. Molecular weights of bands are indicated on the gel.*

DISCUSSION

The Norwalk virus can be considered as the prototype of a family of 27- to 30-nm gastroenteritis viruses (10, 21). These agents have been extremely difficult to study, primarily because they have a narrow host range, they are present in low titer in clinical specimens, and they have not been successfully adapted to tissue culture. Nevertheless, epidemiological studies of gastroenteritis indicate that infection with the Norwalk virus is common and is frequently a cause of epidemic gastroenteritis.

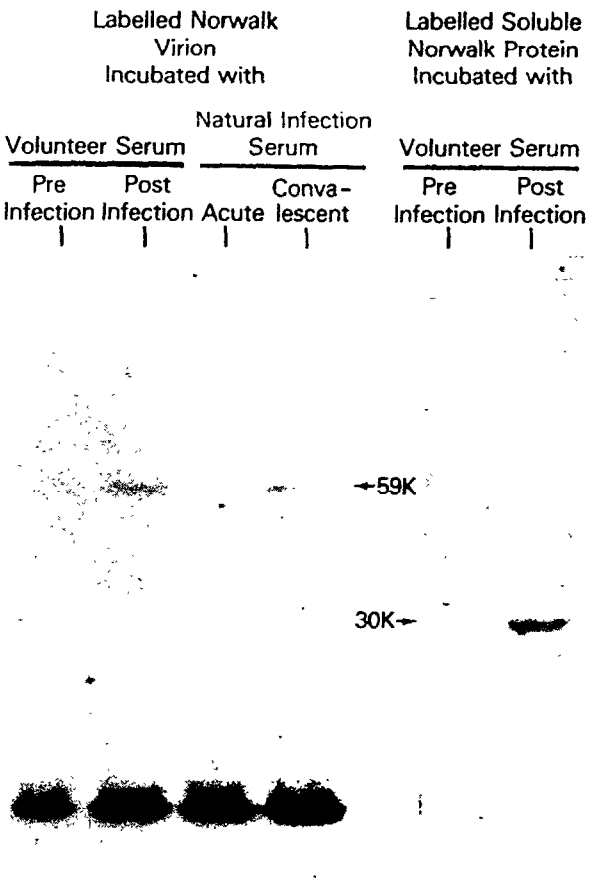

FIG. 6. *Polyacrylamide gel electrophoresis of immunoprecipitated Norwalk virus and viral protein. Conditions of gel and immunoprecipitation were as described in the text. Antisera used in immunoprecipitation were as indicated in the figure. Molecular weights of bands are indicated on the gel.*

In earlier work, the Norwalk virus was tentatively classified as "parvovirus-like" (5, 10). This preliminary classification was made because of the buoyant density of the particles, relative size, and stability in acid and ether. In this work we attempted to carry classification further by investigating the size of Norwalk viral proteins. The finding of one major structural protein with a molecular weight of 59,000 is at odds with a classification of parvovirus (16). In unpublished experiments, we used the chloramine T iodination procedure to label purified preparations of two parvoviruses, adeno-associated virus 3 and rat parvovirus (kindly supplied by David Hoggan, National Institutes of Health). As expected, three structural proteins with molecular weights of between 60,000 and 85,000 were visualized. This pattern is distinctly different from the pattern of a single major structural protein observed with Norwalk virus or feline calicivirus.

Caliciviruses are a newly proposed family of single-stranded RNA viruses that have been found to possess only one structural protein of approximately 65,000 daltons with a reported range of between 60,000 and 71,000 daltons (17). A feline calicivirus iodinated and immunoprecipitated in the same manner as the Norwalk virus was found to have one structural protein with a molecular weight of 65,000. The size and buoyant density of caliciviruses (35 to 40 nm and 1.36 to 1.39 g/cm^3) are similar although not identical to those of the Norwalk virus. In unpublished studies in which negative-stain electron microscopy was used, we found that feline calicivirus strain F-9 and Norwalk virus were comparable in size. The single Norwalk virion structural protein, when coelectrophoresed with feline calicivirus F-9, was found to be slightly smaller (59,000 versus 65,000). Caliciviruses are not known to possess minor smaller structural proteins analogous to the two faint bands occasionally seen in the Norwalk virus preparation; however, a minor protein with a molecular weight of 15,000 has been detected in some calicivirus preparations (17). The significance of these two very faintly labeled bands is unclear at present. The specific precipitation of the 59,000-dalton Norwalk virion protein by two convalescent sera from ill volunteers and one convalescent serum from a naturally infected patient, but not by the appropriate preinfection or acute-phase sera, is strong evidence for the specific relationship of this single protein to the Norwalk virion.

The finding of a single 30,000-molecular-weight soluble protein in Norwalk virus antigen containing fecal specimens was unexpected. This protein does not appear to represent an artifact of our procedure for preparing stool suspensions, since untreated fecal suspensions also appeared to contain a soluble Norwalk virus protein in large amounts. As with the virion protein, specific immunoprecipitation by several appropriate immune sera provided evidence for the specific relationship of this protein to the Norwalk virus. Whether this soluble protein represents a stable nonstructural viral protein made in great excess or whether the 30,000-molecular-weight protein is a subunit or cleavage product of the 59,000-molecular-weight virion protein is not clear at present. A nonstructural virus-associated protein with a molecular weight of 29,000 has been described in calicivirus-infected cells (17).

Caliciviruses have a unique ultrastructure characterized by cup-shaped depressions arranged symmetrically on their surfaces (17). The fine ultrastructure of the Norwalk virus has not been well visualized because of the difficulty in detecting the virion without using precipitating antibody. However, in some electron micrographs in which Norwalk virus has been seen with little or no coating antibody, a morphological similarity to calicivirus is apparent (Greenberg et al., in press). It is of interest that several

workers have recently identified morphologically characteristic calicivirus-like particles in fecal specimens from individuals involved in epidemics of nonbacterial gastroenteritis (3, 13; H. Suzuki, T. Konno, T. Kutsuzawa, A. Imai, F. Tazawa, and N. Ishida, J. Med. Virol., in press). These isolates have not been found to be serologically related to the Norwalk virus or to known animal caliciviruses.

The serologically distinct but morphologically similar small gastroenteritis viruses such as Hawaii agent (19), Ditchling agent (1), and Colorado agent (15) have not been purified or cultivated, so their taxonomic relationship to the Norwalk virus is not clear. This is also true for the morphologically distinct "astroviruses" (11, 14). Hopefully, more detailed biochemical characterization of other gastroenteritis agents will enable us to determine whether the various isolates represent several serotypes of a single viral family or several different types of viruses, all of which are relatively similar in size and biologic behavior.

The current findings are most compatible with the Norwalk virus being related to the calicivirus family. Certainly, nucleic acid studies would have been useful in clarifying this issue, but the minute amounts of Norwalk virus available and the lack of a tissue culture system have restricted us to protein analysis. In any case, from the available data we would recommend that Norwalk virus not be referred to as parvovirus-like. Hopefully, when workers identify other small gastroenteritis viruses that are present in larger quantity, nucleic acid studies can be carried out. Attempts are now under way to raise antibody to the purified 30,000-dalton protein to study its relationship with the virion.

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PAPERS AND SHORT REPORTS

Spread of rotavirus within families: a community based study

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Abstract

The spread of rotavirus infection was studied over four weeks in a sample of 28 families exposed to a child with rotavirus infection. The results showed a high incidence of intrafamilial infection, with 46% of members of these families developing rotavirus infections compared with none in another series of 18 families. Children in the families with an index case were more frequently affected than adults: 75% of the children developed rotavirus infection but only 33% of the adults. Children tended to suffer the infection in a more severe form.

Intrafamily contact is clearly important in transmitting rotavirus infection, and preventive measures should aim at reducing the likelihood of such cross infection.

Introduction

There have been several studies of the prevalence of rotavirus infection in child populations.[1] Typically between 50% and 80% of children admitted to hospital with vomiting and diarrhoea during winter are found to have rotavirus infection. The spread of infection in nurseries,[2] hospitals,[3] nursing homes,[4] and day care centres[5] and among medical personnel[6] has been described. Relatively little is known, however, about the transmission of the disease within the community, although it is commonly thought that most of the spread of infection is intrafamilial.[7,8]

We describe here a study of the spread of rotavirus infection in a series of families exposed to a child with a rotavirus infection and a series of comparison families. The aims of the study were threefold: (*a*) to estimate the likelihood that family members would develop rotavirus infection after exposure to an infected family member; (*b*) to document the frequency with which asymptomatic rotavirus infection occurs in adults and in children; and (*c*) to estimate the relative contributions of intrafamilial and interfamilial rates of infection, through a comparison of rates of infection in the series of families exposed to rotavirus and the corresponding rates in the comparison series.

Methods

The subjects for the study comprised the family members of 47 children aged under 5 years who presented to their family doctors with vomiting or diarrhoea. Only those children from families who had been without symptoms of vomiting or diarrhoea for at least four weeks before the child's attendance at the family doctor were included in the study.

DATA COLLECTION

Once the children had entered the study families were given the following series of assessments. Firstly, all the families were visited by a medically trained investigator (KG), who explained the objectives of the study and enlisted the family's cooperation. Of the 47 families who entered the study all but one agreed to participate. Secondly, at the initial interview a faecal sample was collected from the presenting child and from any other member of the family who had symptoms of gastrointestinal illness. Blood for serological examination was obtained from all members. Thirdly, all family members were given a diary record in which they were asked to record daily symptoms of rotavirus infection.[1] The symptoms examined were: vomiting, diarrhoea, respiratory symptoms, fever, and malaise. Fourthly, during the four week study the families were telephoned or visited every day, or both; any member who developed gastrointestinal symptoms was visited on the same day and faecal samples were collected. Finally, at the end of the four weeks the family was revisited and a further blood sample obtained.

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LABORATORY METHODS

Faecal samples were tested for the presence of rotavirus antigen with Enzygnost enzyme linked immunoabsorbent assays. Faecal samples from the reference child were also tested for the presence of rotavirus antigen by immunoelectrophoresis.[10] Serum rotavirus antibody titres were tested by complement fixation with a microtitre method based on that of Bradstreet and Taylor.[11]

DEFINITIONS

On the basis of the initial faecal sample from the reference child, the 46 families participating in the study were classified into two series. The index series comprised the 28 families whose child had rotavirus detected in the faeces on the basis of immunoelectrophoresis or enzyme linked assay, or both. In the remaining 18 families (the control series) the child did not have rotavirus detected in the faeces.

For this study we counted children aged over 12 as adults. The two series of families were similar in family size, age of children, and number of adults in the family (table I).

TABLE I—*Characteristics of families with (index) and without (control) in child with rotavirus infection. The reference children have been excluded*

	Index families (n = 28) (No (%))	Control families (n = 18) (No (%))
No of children:		
0	12 (43)	9 (50)
1	12 (43)	4 (22)
2	1 (4)	4 (22)
≥3	3 (11)	1 (6)
Ages of children (years):		
≤4	12 (50)	8 (53)
5-8	10 (42)	4 (27)
9-12	2 (8)	3 (20)
No of adults:		
1	3 (11)	0 (0)
2	24 (86)	17 (94)
≥3	1 (4)	1 (6)

Each family member in the two series was classified as follows: (*a*) those who showed a fourfold rise in complement fixation antibody titres with or without detectable rotavirus antigen between the initial and final assessments and who also reported symptoms of gastrointestinal illness were classified as rotavirus positive and symptomatic; (*b*) those who showed evidence of a fourfold increase in complement fixation antibody titres but reported no symptoms were classified as rotavirus positive and asymptomatic; and (*c*) those who failed to show a fourfold increase in complement fixation antibody titres or the presence of rotavirus antigen were classified as having no infection.

Results

Table II shows the rotavirus status of the subjects, excluding the referred child, at the end of the four week study period. Within the 18 control families (38 adults and 15 children) no one developed any evidence of rotavirus infection. In the index series of 28 families (54 adults and 24 children), however, there was an overall infection rate of 46%. A χ^2 test applied to these differences showed them to be highly significant ($\chi^2 = 33{\cdot}94$; $p < 0{\cdot}001$).

Within the index families there was clear evidence of differential resistance to infection depending on age. Seventy five per cent of children developed evidence of rotavirus infection, whereas 33% of adults were affected ($\chi^2 = 11{\cdot}53$; $p < 0{\cdot}001$). The mean onset of symptoms in children was 4·9 days after the onset of symptoms in the reference child, in contrast to a mean of 6·4 days for adults.

TABLE II—*Incidence of infection among family members (excluding the reference children) at the end of four weeks*

	Children (≤12 years)		Adults (>12 years)	
	Index families	Control families	Index families	Control families
No rotavirus infection	6	15	36	38
Asymptomatic infection	2	0	4	0
Symptomatic infection	16	0	14	0
Total	24	15	54	38

TABLE III—*Symptoms that occurred in 18 adults and 18 children infected with rotavirus and their mean duration*

	Mean duration of symptoms (days)	
	Children (≤12 years)	Adults (>12 years)
Vomiting	1·28	0·22
Diarrhoea	2·28	1·17
Respiratory	5·06	1·33
Fever	0·44	0·28
Malaise	3·33	1·72
Total	12·39	4·72

Finally, the children seemed to suffer the disease in a more severe form. Table II shows that four out of 18 adults with rotavirus infections were asymptomatic but only two out of 18 children. Because of the small numbers, however, this difference failed to reach statistical significance. Table III shows the number of days over which adults and children had various symptoms. Children had many more symptom days (12·4) than adults (4·7). ($t = 1{\cdot}94$; $p < 0{\cdot}05$ one tail test.)

Discussion

Our findings suggest that the primary mechanism in the spread of rotavirus infection is through intrafamilial contacts. Of 78 adults and children exposed to a child suffering from rotavirus infection 36 developed the disease in the subsequent four weeks. In contrast, within a comparison series of 53 adults and children exposed to a child who had vomiting and diarrhoea which was not due to detectable rotavirus infection none developed the condition. The model of infection which emerges is one in which there is a low probability that any family member will contract the condition but once one member becomes infected there is a relatively high probability of cross infection.

Children are more susceptible to intrafamily infection than adults. Of the 24 children in the index families 18 developed the condition, whereas only 18 of the 54 adults were infected. This difference may be due to two factors. Firstly, children are generally more susceptible to rotavirus[1]; secondly, children within a family may have more physical contact with each other, which, together with their longer rotavirus excretion rates, increases the likelihood of cross infection.[12] Not only were children more susceptible to infection; they also tended to suffer the disease in a more severe form, having more symptomatic illness, a longer duration of symptoms, and a greater number of symptoms. These findings and previous evidence[4] all suggest that rotavirus infection in children is associated with more severe symptoms than in adults.

The practical impact of this study is that it shows the important role of intrafamily contact in the transmission of rotavirus infection. The primary emphasis in the prevention of this condition should be on reducing the likelihood that other family members will be infected.

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Serum folate concentrations during pregnancy in women with epilepsy: relation to antiepileptic drug concentrations, number of seizures, and fetal outcome

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Abstract

Serum folate concentrations, blood counts, and antiepileptic drug concentrations were measured during 133 pregnancies of 125 women with epilepsy. There was an inverse correlation between serum folate concentrations and concentrations of phenytoin and phenobarbitone. The number of epileptic seizures during pregnancy showed no association with serum folate concentrations. No cases of maternal tissue folate deficiency or fetal damage attributable to low maternal serum folate were observed. Maternal serum folate concentrations for infants with structural birth defects, "fetal hydantoin syndrome," or perinatal death were similar to those for healthy babies. A low dose (100 to 1000 μg daily) of folate supplement appeared sufficient for pregnant women with epilepsy despite the antifolic action of antiepileptic medication.

Monitoring folate concentrations in pregnant women with high serum concentrations of phenytoin or phenobarbitone is recommended.

Introduction

Folic acid is required for synthesis of DNA and is essential for the normal development of the human fetus.[1] A deficiency of folate has been suspected of causing damage to the fetus, particularly if operative in the early weeks of pregnancy.[2] Treatment with antiepileptic drugs may result in lowered serum folate concentrations[2 3] and even in megaloblastic anaemia.[2] Epileptic women taking antiepileptic drugs may reasonably be expected to be at particular risk of folate deficiency during their pregnancies[1]; systematic investigations on this are, however, lacking.

We undertook a prospective study of epileptic women during pregnancy, with serial measurements of both folate and antiepileptic drug concentrations.

Patients and methods

A total of 139 epileptic women were followed up during 150 pregnancies. Eighty eight women had grand mal (tonic and clonic convulsive) seizures, 16 had psychomotor (temporal lobe) seizures, 19 both grand mal and psychomotor, 10 focal motor or sensory, and six other or unclassified. The patients recorded their seizures on a calendar, which was checked at each visit. Of the 150 mothers, 137 took combined vitamin tablets with an average daily content of 500 μg (range 100 to 1000 μg) of folic acid from the sixth to the 16th week of pregnancy until parturition; 143 received oral iron supplementation. Of the 150 pregnancies, 17 were excluded because of inadequate fast (see below) or failures in obtaining the blood samples. Subsequently, 133 singleton pregnancies in 125 women remained for analysis. Of the 133, 60 mothers had entered the study by the end of the 16th week of pregnancy.

Serum and red cell folate was radioassayed by a competitive protein binding method[5 6] at Medix Laboratories, Kauniainen, Finland. Values of serum folate above 4·0 nmol/l (1·8 ng/ml) (2·5 percentile) were considered normal. Haemoglobin concentrations and mean cell volume were measured with a Coulter S counter. Serum phenytoin and phenobarbitone concentrations were measured by gas chromatography[7] and carbamazepine concentrations by spectrophotometry.[8] Venous blood for folate and drug assessments was drawn between 1300 and 1500 hours, after a five to eight hour fast following breakfast and the last drug dose. There is evidence that antiepileptic drugs do not interfere with the radioassay of folate.[9]

Serum folate and haemoglobin concentration, mean cell volume, and serum concentrations of antiepileptic drugs were examined

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Role of Infection in Chronic Bronchitis[1-3]

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SUMMARY

Twenty-five patients with chronic bronchitis were studied intensively from 1968 to 1972. Viral, bacteriologic, mycologic, and mycoplasmal studies, both serologic and cultural, were carried out in an attempt to determine the role these agents play in exacerbations. All of the usual viral agents associated with exacerbations and 2 members of the coronavirus group, 229E and OC43, were detected. One third (33.6 per cent) of the 116 exacerbations observed could be related to viral infection or *Mycoplasma pneumoniae* (1 exacerbation). Viral infection was also noted to occur during periods of remission but was more commonly associated with periods of exacerbation ($P < 0.001$). No interrelationship between viral and bacterial infection was apparent and neither *Streptococcus pneumoniae* nor *Haemophilus influenzae* was present more frequently in the sputum of patients in exacerbation. However, the number of *S. pneumoniae* organisms present in the sputum was significantly greater ($P = 0.04$) during exacerbation than during remission and their presence was signicantly correlated with increased sputum purulence ($P < 0.01$). This was not true of *H. influenzae*. Ampicillin was effective in clearing the sputum of *S. pneumoniae* but not of *H. influenzae;* the reverse was true of tetracycline.

Introduction

Many studies have been carried out concerning the infectious etiology of exacerbations in chronic bronchitis (1). Most of these studies have considered the role of bacteria in the etiology of exacerbations, but some have also examined the role of viruses and mycoplasma (2–8). We examined the possible role of all of these agents in chronic bronchitis by intensively studying a small group of patients prospectively for several years. A variety of cultural and serologic techniques were used in an attempt to maximize the detection of infections associated with exacerbations.

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Materials and Methods

The patient population consisted of 25 patients who exhibited a chronic, recurrent, productive cough that was present on most days for a minimum of 3 months during each of 2 consecutive years. This criterion has been accepted by both the British Medical Research Council and the American Thoracic Society as an acceptable definition of chronic bronchitis (9). All of these patients filled out the British Medical Research Council questionnaire (10). None of our patients had bronchial asthma or was receiving corticosteroids. These patients were selected from the hospital's outpatient clinic. Any patient meeting the above definition and willing to come to the hospital every 1 to 2 weeks for a period of several years was considered acceptable. All patients lived in Burlington, Vermont, or the immediate vicinity.

Of the 25 patients, 22 had smoked cigarettes for a mean of 51 pack-years (range: 23 to 115 pack-years);

4 of these also smoked pipes and/or cigars. Seven patients were not smoking at the time of their entry into the study but none had stopped more than 5 years previously. Their mean age was 56 years, with a range of 31 to 72 years. There were 18 men and 7 women in the study population. The observations on these patients extended from the summer of 1968 until July 1972. During this time, 5 men and one woman died; all of these deaths were thought to be related to the presence of chronic pulmonary disease. One of the patients had had bronchogenic carcinoma diagnosed before death.

The patients attended an outpatient clinic in the Clinical Research Center a minimum of every 2 weeks, but during exacerbations patients were occasionally seen more frequently. The patients visited the clinic a total of 1,886 times.

Patients were considered to be experiencing an exacerbation when they demonstrated a considerable increase in their cough and sputum production. The increase in sputum production during exacerbation was marked. Thus, the mean sputum volume for 90 specimens collected during exacerbation was 28 ml compared with a mean of 19 ml for 1,311 specimens collected during remission. Generally, these findings were associated with increased purulence of sputum, shortness of breath, fatigability, and often chest pain or tightness. Fever was unusual. In most instances the physician caring for the patient decided when he or she was experiencing an exacerbation, and only those respiratory illnesses that resulted in significant increase in the patient's symptoms were considered to represent exacerbations. However, occasionally exacerbations were detected by a retrospective review of the patient's record. Microbiologic or serologic results were not used to determine when exacerbations had occurred. A total of 116 exacerbations were observed in these patients, who were studied for a total of 4,150 patient weeks. Exacerbations were treated with oral ampicillin 2 g per day for 10 days except in cases of suspected penicillin allergy, in which case oral tetracycline, 1 g per day, was used. There were 59 exacerbations treated with antimicrobial drugs; 14 occurred while the patient was receiving antimicrobial prophylaxis.

Nine patients were treated with prophylactic antimicrobial drugs during the winter of 1969-70. Eight received ampicillin, 1 g per day, and one tetracycline, 1 g per day, because of a history of allergy to penicillin. Patient 34 continued to receive ampicillin prophylaxis until September 1970, when he died.

All patients were immunized with bivalent influenza A (H_3N_2) and B vaccines in October of each year, and any serologic rises to either of these viruses that were encountered in the subsequent 4-week period were considered to be related to the vaccine rather than to infection.

In August and September 1970, 16 patients were started on an immunization program with a variety of pneumococcal vaccines, including types 2 and 5 (Cutter), 1, 7, and 8 (Squibb), and 1 to 9, 12, 14, 18, and 19 (Lilly), and a hexavalent vaccine (types 1, 3, 4, 7, 8, and 12) (Lilly). The results of this program will be the subject of a separate publication. The use of the vaccines did not decrease the incidence of pneumococci in the sputum of these patients.

In addition to a 24-hour sputum collection, the patients were requested to bring in a specimen of sputum that had been expectorated on arising on the day of their clinic visit and had been kept refrigerated. The volume of the 24-hour specimen was measured and the degree of purulence determined according to the classification of May and Delves (11). A total of 1,501 suitable sputum specimens were available for microbiologic studies. Thus, of the 1,886 clinic visits, there were 385 visits for which there were no sputums available, and of the 1,501 visits for which sputums were available 148 samples were taken from patients receiving antimicrobial drugs. Thus, 1,353 sputum samples were obtained from patients not receiving antimicrobial drugs.

Bacteriology. Sputum and throat specimens were cultured on blood, MacConkey, and chocolate agar plates and all chocolate plates were incubated in candle jars. Sputum (0.5 ml) was mixed with an equal volume of saline and injected intraperitoneally into mice. The heart blood of all mice that died was cultured on chocolate agar plates. All mice were observed for 6 days and then discarded.

Some sputum was also liquefied by the addition of an equal volume of pancreatin according to the method of Rawlins (12). The liquefied sputum was serially diluted in Ringer's solution and subsequently cultured on blood, chocolate, and MacConkey plates.

Initially, an attempt was made to type all *Haemophilus influenzae* isolates using polyvalent typing sera, but all isolates were found to be unencapsulated. All *Streptococcus pneumoniae* isolates were typed with sera obtained from Statens Serum Institute, Copenhagen (13). Potential respiratory pathogens were considered to be *H. influenzae*, *S. pneumoniae*, β-hemolytic streptococci, *Staphylococcus aureus*, *Enterobacteriaceae*, *Pseudomonas* species, and other nonfermentative bacilli.

Virus isolation. During the first 3 years of the study, viral isolation was attempted from nose and throat swabs and sputum using cell tissue cultures of human diploid fibroblasts (WI-38), human embryonic kidney, and primary monkey kidney. During the last year of the study, viral cultures were not performed.

Tube cultures were obtained from Flow Laboratories and from Microbiological Associates, Bethesda, Md. They were maintained by methods described previously (14).

Tubes were observed for 14 to 28 days for the appearance of cytopathic effect and passed at least one time if any possible cytopathic effect was seen. Pri-

mary monkey kidney cell cultures were also screened at 7 and 14 days after inoculation for the presence of hemadsorbing agents using fresh guinea pig red blood cells and also challenged with echovirus 11 to detect any interfering viruses that might be present.

Mycoplasma isolation. Standard methods were used for the isolation of *Mycoplasma pneumoniae* from sputum and throat swabs (15).

Serology. A total of 1,130 blood samples were collected from our 25 patients at least monthly and often more frequently. The complement fixation technique (16) was used to detect changes in antibody concentrations to the following viruses and mycoplasma: influenza A, influenza B, adenovirus, respiratory syncytial virus, para-influenza virus types 1, 2, and 3, *Herpesvirus hominis,* and *M. pneumoniae, M. salivarium, M. hominis, M. pulmonis,* and *M. hyorhinis.*

Coronavirus infections were detected using the complement fixation test for strain 229E virus and both the hemagglutination inhibition and complement fixation tests for strain OC43. These tests have been described previously (17). Serum antibodies to *H. influenzae* and *S. pneumoniae* were determined by the indirect fluorescent antibody technique; these findings have been described previously (18). Rhinovirus serology was carried out by screening all sera using the passive hemagglutination technique (19) with confirmation of all rises by the neutralization method.

Data processing. All of the data were coded and punched onto standard 80-column IBM cards. The data were then transferred to magnetic tape for data retrieval and analysis.

Results

Data for all 25 patients were combined because individual patients did not differ significantly in the association of exacerbations with other variables. For example, the frequency of exacerbations manifested by a patient was not related to the percentage of exacerbations that could be attributed to viral infection.

We considered our patients to be in a state of exacerbation for a total of 116 patient weeks because they experienced 116 exacerbations that were generally one week in duration. Only 12 of 116 exacerbations exceeded a week in duration and none of these was longer than 2 weeks. The patients were observed for a total of 4,034 patient weeks while in remission.

Viral infections related to exacerbations and periods of remission are shown in table 1. Evidence of viral infection was considered to be either a 4-fold rise in antibody, isolation of the virus, or both.

Also shown in table 1 is the method of diagnosis of viral infections. Regardless of whether the patient was in exacerbation or remission, the vast majority of viral infections were detect-

TABLE 1

VIRUSES (EXCLUDING *HERPESVIRUS HOMINIS*) ASSOCIATED WITH EXACERBATION OR REMISSION AND MEANS OF DIAGNOSIS

	Exacerbation (116 patient weeks)				Remission (4,034 patient weeks)			
Virus	Sero*	Iso†	Both	Total	Sero	Iso	Both	Total
Influenza A	12	2	0	14	6	1	2	9
Influenza B	1	0	0	1	2	0	0	2
Respiratory syncytial	5	0	0	5	2	0	0	2
Para-influenza 1	1	1	0	2	0	3	0	3
Para-influenza 2	0	1	0	1	0	1	0	1
Para-influenza 3	5	0	1	6	4	1	0	5
Rhinovirus	4	0	0	4	1	1	0	2
Corona 229E	2	0	0	2	7	0	0	7
Corona OC43	4	0	0	4	3	0	0	3
Adenovirus	1	1	1	3	0	0	0	0
Unidentified hemadsorbing agent	0	1	0	1	0	1	0	1
Total	35	6	2	43	25	8	2	35

Note: Four exacerbations were associated with multiple viral infections; 2 with influenza A and respiratory syncytial virus, one with para-influenza 3 and adenovirus, and one with para-influenza 2 and coronavirus 229E and OC43. During remission there was one instance of infection with 2 viruses, a diagnostic rise in antibodies to coronaviruses OC43 and 229E. Because of the serologic relationships between these 2 viruses (17) it is possible that this was a single infection.

*Infection documented by serology alone.

†Infection documented by isolation alone.

TABLE 2
ASSOCIATION OF VIRAL INFECTIONS WITH EITHER EXACERBATION OR REMISSION

Clinical Status	Patient-Weeks of Observation	Viral Infection* (Excluding *Herpesvirus hominis*)		*Herpesvirus hominis* Infection*	
		(no.)	(%)	(no.)	(%)
Exacerbation	116	38	32†	11	9.5†
Remission	4,034	35	0.9	9	0.2

*Infection defined as a 4-fold rise in antibody, isolation of the virus, or both.
†Viral infection during exacerbation was significantly more frequent, $P < 0.001$ (chi square).

ed by serology alone. It was mainly the influenza and para-influenza viruses that were isolated. The 2 methods were compared for the first 3 years of the study, because viral isolation was not carried out during the last year of the study. In those years 34 (81 per cent) of the viral infections associated with exacerbations were detected by serology alone, whereas only 6 (14.3 per cent) were detected by isolation alone. The percentage of weeks of exacerbation associated with *H. hominis* and other viral infections was significantly greater than the association of these infections with remission.

M. pneumoniae infection was documented in 2 patients; one infection, determined by isolation and serology, was associated with exacerbation, whereas the other, determined by serology alone, occurred during remission. Two *M. hominis* infections were documented by serology alone; one occurred during exacerbation and with a rise in antibody to coronavirus OC43, and the other occurred during remission. No serologic evidence of infection was detected to *M. salivarium, M. pulmonis,* or *M. hyorhinis.*

Although there was no year when there was a preponderance of infection with any particular virus, there was a definite seasonal incidence of exacerbations and viral illnesses. Seventy-five (65 per cent) of the 116 exacerbations occurred during the 6-month period from October to April and 59 (76 per cent) of the 78 viral infections occurred during the same time period. Moreover, 33 (44 per cent) of the 75 exacerbations occurring during the winter months were associated with viral infections, whereas the comparable figure for the remaining 6 months of the year was 5 (12 per cent) of 41 exacerbations.

The association of viral infections including *H. hominis,* with periods of exacerbation and remission, is shown in table 2.

The relation between viral-associated exacerbations, non–viral-associated exacerbations, and the recovery rates of *H. influenzae* and *S. pneumoniae* from sputum are shown in table 3. The recovery of *H. influenzae* and pneumococcus from the sputums of either viral-associated or non–viral-associated exacerbations was similar.

The possible relation of recovery of potential respiratory bacterial pathogens and *Candida* species from sputum and throat swabs during exacerbations and remission is examined in table 4. The isolation rate of *S. pneumoniae* from sputum during periods of exacerbation was

TABLE 3
RECOVERY OF *HAEMOPHILUS INFLUENZAE* AND *STREPTOCOCCUS PNEUMONIAE* FROM SPUTUM OF PATIENTS WITH VIRUS-RELATED AND NON–VIRUS-RELATED EXACERBATIONS

Exacerbation	Total No.	*Haemophilus influenzae* Positive		*Streptococcus pneumoniae* Positive	
		(no.)	(%)	(no.)	(%)
Viral-associated	31	21	67.7	11	35.5
Non–viral-associated	55	28	50.9	21	38.2
Total	86*	49	57.0	32	37.2

*Total number of exacerbations occurring when the patient was not receiving antimicrobial drugs and for which sputum specimens were available.

TABLE 4

RECOVERY OF BACTERIA AND CANDIDA FROM SPUTUM AND THROAT SWABS DURING EXACERBATION AND REMISSION WHEN PATIENTS WERE NOT RECEIVING ANTIMICROBIAL DRUGS

	Sputum				Throat Swabs			
	Exacerbation (86)*		Remission (1,267)		Exacerbation (94)		Remission (1,434)	
Agent	(no. positive)	(%)	(no. positive)	(%)	(no. positive)	(%)	(no. positive)	(%)
Streptococcus pneumoniae	32	37.2	419	33.1	8	8.4	100	7.0
Haemophilus influenzae	49	57.0	759	59.9	27	28.7	495	34.5
Staphylococcus aureus	12	14.0	232	18.3	9	9.6	225	15.7
Candida species	25	29.1	232	18.3	3	3.2	37	2.6
Klebsiella species	12	14.0	251	19.8	6	6.4	117	8.2
Pseudomonas species	8	9.3	118	9.3	5	5.3	88	6.1
Gram-negative bacilli (excluding hemophilus)	25	29.1	598	47.2	18	19.1	373	26.0

*Total no. of specimens examined.

slightly but not significantly higher. The recovery rate of *H. influenzae* was approximately equal for both periods. The simultaneous presence of both *S. pneumoniae* and *H. influenzae,* not shown in table 4, was also not significantly associated with the occurrence of exacerbations. The reason for the increased recovery rate of *Klebsiella* species and *Enterobacteriaceae* during periods of remission is unclear, as is the cause of the increased recovery of *Candida* species during periods of exacerbation.

Although the pneumococcus qualitatively was not significantly associated with exacerbations, such a relationship could be demonstrated quantitatively (table 5). A significantly higher concentration of pneumococci was present in sputum during exacerbations than when the patient was in remission. No such relationship could be shown for *H. influenzae.*

Sputum specimens containing pneumococci were significantly more purulent than specimens not containing pneumococci (table 6). A similar analysis did not reveal a significant correlation between the degree of sputum purulence and the presence or absence of *H. influenzae.* Sputum purulence was increased with exacerbations. Sixty-one per cent of sputum specimens collected during exacerbation were purulent, whereas this was true of only 50 per cent of those collected during remission.

The effect of antimicrobial therapy on the recovery of either pneumococci or hemophilus from sputum specimens is evaluated in table 7. From 116 sputum samples obtained from patients receiving ampicillin, only 6 (5.2 per cent) pneumococcal isolates were obtained, whereas from the 25 sputum specimens obtained from patients receiving tetracycline, 12 (48 per cent)

TABLE 5

RELATION BETWEEN NUMBERS OF *STREPTOCOCCUS PNEUMONIAE* PRESENT IN SPUTUM AND PATIENT'S CONDITION

		Sputum Culture for *Streptococcus pneumoniae*						
		Positive						
Clinical Status		$< 10^4$/ml	10^4/ml	10^5/ml	10^6/ml	$> 10^6$/ml	Negative	Total Sputums*
Exacerbation†	No.	2	0	1	2	19	59	83
	%	2.4	0.0	1.2	2.4	22.9	71.1	100
Remission	No.	20	2	18	56	172	972	1,240
	%	1.6	0.2	1.5	4.5	13.9	78.4	100

*In this analysis 30 specimens containing pneumococci were excluded because quantification was not carried out. Twenty-seven of these 30 specimens were obtained from patients during remission and 3 from patients in exacerbation. An additional 148 specimens, of which 13 contained pneumococci, were excluded because the patient was receiving antimicrobial drugs.

†During exacerbation *S. pneumoniae* present in significantly greater numbers in sputums, P = 0.04 (Wilcoxon test).

TABLE 6

STREPTOCOCCUS PNEUMONIAE AND SPUTUM PURULENCE FOR PATIENTS NOT RECEIVING ANTIMICROBIAL DRUGS

Sputum Culture for Pneumococcus		No. and Degree of Purulence of Sputum Specimens						
			Mucopurulent				Not	
		Mucoid	±	+	++	+++	Evaluated	Total
Positive*	No	124	72	87	34	53	81	451
	%	27.5	16.0	19.3	7.5	11.8	18.0	33.3
Negative	No.	284	161	153	50	75	179	902
	%	31.5	17.8	17.0	5.5	8.3	19.8	66.7
Total specimens	No.	408	233	240	84	128	260	1,353
	%	30.2	17.2	17.7	6.2	9.5	19.2	100.0

*Pneumococcal presence significantly related to purulence, $P < 0.01$ (Mann-Whitney test).

isolates were cultured. The recovery rate for *H. influenzae* was exactly the reverse: 56.9 per cent from the 116 sputum samples obtained from patients receiving ampicillin and 24.0 per cent from the 25 sputum samples obtained from patients receiving tetracycline. However, ampicillin was effective in reducing the numbers of *H. influenzae* present in sputum. *H. influenzae* was present in sputum in concentrations of $\geq 10^6$ per ml 45 per cent of the time when the patient was not receiving ampicillin, whereas the comparable figure for patients receiving ampicillin was smaller (26 per cent), but not significantly so. Antimicrobial administration was generally associated with a rapid diminution in symptoms, but because no untreated patients were included as a control group it is impossible to evaluate the efficacy of this treatment.

Mouse inoculation increased recovery rates of *S. pneumoniae*. Thus, 134 of 469 (28.6 per cent) pneumococcal isolates were obtained only from the mouse and not from the sputum culture. The routine use of mouse inoculation increased the yield of pneumococci by 40 per cent. No ready explanation is available for the failure of mouse inoculation to yield pneumococci on the 115 (24.5 per cent) occasions it was cultured from the sputum. In 220 (46.9 per cent) instances, both sputum culture and mouse inoculation yielded pneumococci. Of the 23 different pneumococcal serotypes cultured, there was only one serotype, no. 39, that was cultured exclusively from sputum and this serotype was isolated on only one occasion.

The peripheral white blood cell count (WBC) was increased 10 of the 30 times it was determined at the time of an exacerbation. These elevations were generally minimal; on only 3 occasions was the WBC > 15,000 per mm^3, and 2 of these instances occurred in one patient shortly before death. On 6 of 48 (12.5 per cent) occasions during remission the WBC was > 10,000 per mm^3 and the maximal elevation observed was WBC = 13,000 per mm^3. The mean WBC

TABLE 7

RECOVERY OF *STREPTOCOCCUS PNEUMONIAE* AND *HAEMOPHILUS INFLUENZAE* FROM SPUTUM SPECIMENS OBTAINED FROM PATIENTS RECEIVING AND NOT RECEIVING ANTIMICROBIAL DRUGS

Treatment	*Streptococcus pneumoniae*		*Haemophilus influenzae*		Total
	(no.)	*(%)*	*(no.)*	*(%)*	
Ampicillin	6	5.2*	66	56.9†	116
Tetracycline	12	48.0	6	24.0	25
Other antimicrobial drugs	2	28.6	3	42.9	7
All antimicrobial drugs	20	13.5	75	50.7	148
No antimicrobial drugs	451	33.3	808	59.7	1,353

*Significantly lower than the recovery of pneumococci from patients receiving either tetracycline or no antimicrobial drugs, $P < 0.001$ (chi square).

†Significantly higher recovery of hemophilus from patients receiving ampicillin in comparison with those receiving tetracycline, $P < 0.005$ (chi square).

during exacerbation was 10,233 per mm³ and during remission 8,250 per mm³.

The differential count was no more helpful in determining exacerbations. The mean percentage of polymorphonuclear cells present during exacerbation was 73.5 and during remission 67.7. Eosinophilia was rarely present and 7 per cent eosinophils was the maximum observed.

Discussion

Numerous studies have investigated the possible role of viruses and mycoplasmas in triggering exacerbations in chronic bronchitis (1–8). A number of these studies, as well as our study, are summarized in table 8. Except for Eadie and associates (3), we could relate fewer exacerbations to viral or mycoplasma infection than did other workers (2, 4, 5).

The definition of an exacerbation is one factor that could affect the percentage of exacerbations found to be related to viral illnesses. This is a difficult judgment to make in patients who are always experiencing symptoms of cough and sputum production. It is possible that one would be able to relate fewer exacerbations to viruses or mycoplasma if a less stringent definition of exacerbation were used and if more severe exacerbations tended to be related to viral or mycoplasma infections with a higher degree of frequency. Unfortunately, there is no precise way to define an exacerbation (20) and, therefore, it is difficult to compare the results of these studies.

The viruses that we found associated with exacerbations in our study are similar to those of other investigators. McNamara and co-workers (4), however, did not find any influenza or para-influenza viruses, but they did find a higher incidence of rhinovirus infections than most other studies. Our lower incidence of documented rhinovirus infections may have been due to the fact that 5 of 6 rhinovirus infections that we documented were serologically demonstrated and we know that the serologic (passive hemoagglutination) method used for screening was specific but not very sensitive (19). Hence, it is likely that some rhinovirus infections were missed. The one major new viral group that we found to be related to exacerbations was the coronavirus group.

Coronaviruses are a relatively new group of viruses that have been isolated from patients with acute respiratory infections (21). Both coronaviruses 229E and OC43 have been demonstrated to be the cause of colds in adults, the

TABLE 8

VIRAL AND *MYCOPLASMA PNEUMONIAE* INFECTIONS RELATED TO EXACERBATIONS

Investigators	No. of Exacerbations	Infections Related to Virus or *Mycoplasma pneumoniae* (no.)	(%)	Influenza A and B	Para-Influenza 1, 2, 3	Respiratory Syncytial Virus	Adenovirus	Rhinovirus	*Mycoplasma pneumoniae*	Coronavirus	Other
Carilli and associates (2)	46	24	52.2	4	2	8	2	0	4	*	4
Eadie and associates (3)	54	15	27.8	3	1	0	0	11	*	*	0
McNamara and co-workers (4)	42	27	64.3	0	0	5	0	18	4	*	0
Lamy and co-workers (5)	49	31	63.3	14	12	3	0	*	1	*	1
Present study	116	39	33.6	15	9	5	3	4	1	6	1†

* Not tested.

† Total of 44 agents associated with 39 exacerbations because some exacerbations were associated with more than one agent.

colds associated with coronavirus 229E being relatively mild with predominant nasal symptoms, and those due to OC43 being associated with cough and pharyngitis as well as coryza-like symptoms (22). To the best of our knowledge, the present study is the first demonstrated association of coronavirus infection and exacerbations of chronic bronchitis. Monto and co-workers (23) found that male chronic bronchitics had more infections with coronavirus OC43 than did healthy control subjects, but the design of their study did not allow them to comment on the relation of these infections to exacerbations.

Four of the 7 coronavirus OC43 infections that occurred in our patients were symptomatic, whereas this was true of only 2 of the 9 coronavirus 229E infections. This observation is in agreement with the demonstrated predisposition for OC43 infections to resemble rhinovirus colds; both viruses produce symptomatic involvement of the entire respiratory tract, whereas 229E produces mainly nasal symptoms (22). Nonetheless, both coronavirus OC43 and 229E infections were significantly ($P < 0.001$, χ^2) related to periods of exacerbation. It seems clear that future studies on the etiology of exacerbations of chronic bronchitis will have to consider these viruses.

In agreement with other studies (4, 5, 8, 20), we demonstrated that viral infection can occur without exacerbations. For example, Mufson and co-workers (8) found that fully one half of the viral and mycoplasma infections that they documented were not associated with an exacerbation. Of the 60 viral infections we demonstrated by serology alone, 25 were not associated with exacerbations. Nonetheless, because the total period of observation of our patients when they were in remission (4,034 patient weeks) was so much longer than when they were in exacerbation (116 patient weeks), there is a significant correlation of viral infection with exacerbations (table 2). This same type of relationship was observed by Lamy and associates (5), who detected viral infections during 58.3 per cent of 36 patient months of exacerbation, whereas infection was associated with only 3.7 per cent of patient months of remission. It seems clear, therefore, that asymptomatic viral and mycoplasma infection occurs in chronic bronchitics, but the occurrence of such infection is far more likely to occur during periods of exacerbation than during periods of remission. Nonetheless, the occurrence of such asymptomatic viral and mycoplasma infections raises the question of whether there is truly a causal relationship between the occurrence of infections and exacerbations. Only by challenging chronic bronchitic patients with live viruses can this question be answered.

As observed by others (5, 24) we noted that exacerbations and infections were far more common during the winter months. Furthermore, we noted that the number of exacerbations associated with viral illnesses was markedly lower during the warmer months. This observation would suggest that exacerbations in the summer are more likely to be associated with either noninfectious causes, such as increased humidity, or infectious agents that we did not detect.

Our study revealed that *M. pneumoniae* infections were uncommon in our patients. It would appear from the literature (2, 4, 6, 8) that *M. pneumoniae* infection is associated with exacerbations of chronic bronchitis but generally is not of great importance in relation to this disease. The occasional studies (4, 6) that have documented a significant role of this agent in chronic bronchitis can probably be attributed to the tendency of this agent to cause disease in a sporadic fashion in relation to both time and place (25).

Most studies of the sputum in chronic bronchitis have found that more than 50 per cent of the specimens contain *H. influenzae* (26), and our studies confirm this. However, unlike other studies (26) that found significantly higher recovery rates from purulent sputum, we were unable to show increasing rates of recovery of *H. influenzae* with increasing purulence. We have no explanation for this disparity in results.

An important question, however, is whether the presence of *H. influenzae* in sputum is correlated with exacerbations, because purulent sputum may be produced clinically by some patients regardless of whether they are experiencing an exacerbation (26). Most workers have not found any correlation between the presence of *H. influenzae* and exacerbations of chronic bronchitis (27, 28); our studies bear out these observations. Others, however, have shown an increased recovery of *H. influenzae* associated with exacerbations (20).

We did not find any increase in the numbers of *H. influenzae* present in sputum during exacerbations. This finding agrees with Davis and associates (28) but disagrees with Cooper and co-workers (29).

The rate of recovery of pneumococci from the sputum of our patients was 33 per cent when

in remission and 37 per cent when experiencing an exacerbation. These figures are similar to those observed by others (30), but some workers (20, 24) have found that rates of pneumococcal recovery increased during exacerbations.

However, even though we did not observe an increased percentage of pneumococcal recovery with exacerbations, we did note a significant increase in numbers of pneumococci present in sputum obtained at the time of exacerbations (table 5). Davis and associates (28) and Storey and co-workers (31) did not find such an increase in numbers with exacerbations.

Just as Fisher and co-workers (20) observed, we found that the presence of pneumococci was associated with increased sputum purulence (table 6). However, unlike the data of Fisher and co-workers, such a correlation could not be shown with hemophilus. Many observers (26) have been able to correlate increasing sputum purulence with an increased recovery of both pneumococci and hemophilus. However, others (31) have not been able to correlate an increased recovery of either pneumococci or hemophilus with increased sputum purulence.

As observed by others (32), inoculation of sputum into mice markedly increased the rate of recovery of this organism; in our study it increased recovery by 40 per cent and in a study of patients with pneumococcal pneumonia, by 47 per cent. In the latter study, 21.1 per cent of the positive cultures were obtained from sputum alone, which is similar to the figure of 24.5 per cent that we observed. Thus, the failure to recover pneumococci from sputum specimens that are positive on routine culture is not restricted to patients with chronic bronchitis. Furthermore, there did not appear to be any correlation between serotype and the tendency for that serotype to be cultured from sputum alone. For example, the type 14 pneumococcus, which is known to have reduced virulence for the mouse (33), was cultured from our patients on 8 occasions and never from sputum alone.

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Enumeration and characterization of bacteria in mineral water by improved direct viable count method

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S. GUYARD, P. MARY, C. DEFIVES AND J.P. HORNEZ. 1999. Fifteen strains from two emergent mineral waters were isolated and tentatively identified with API 20NE and BIOLOG GN systems. These strains were screened for their sensitivities to seven replication-inhibiting antibiotics of the (fluoro)quinolone group (nalidixic and pipemidic acid, flumequine, norfloxacin, ofloxacin, pefloxacin and ciprofloxacin). It was shown that the direct viable count (DVC) procedure could be improved by using certain antibiotic cocktails, which were active against the isolates. Geometric bacterial features were successfully determined with image analysis and adapted software (ICONIX, Perfect Image™). Elongations were significant and allowed rapid discrimination of antibiotic inhibited and non-inhibited strains. Particular isolates in a mixed culture were characterized and enumerated after only 14 h exposure with the appropriate antibiotic cocktail. This method can also be applied to other communities, such as mixed cultures in bio-fermentors or in food with known microflora.

INTRODUCTION

Mineral drinking water is characterized by a native bacterial microflora and chemical composition unique to each source. These properties are indicators of native and natural water quality (Schwaller and Schmidt-Lorenz 1981; Guillot and Leclerc 1993). Mineral drinking water is an oligotrophic environment (0·1 $\mu g\ ml^{-1}$ carbon). The viable bacterial count of emergent natural mineral water is very low, i.e. about 10 cfu ml^{-1} (Ferreira *et al.* 1994; Leclerc 1994). After bottling, this population reaches 10^3–10^5 bacteria ml^{-1} in a few days (2–7 d) with occasionally, a maximum of 10^7 bacteria ml^{-1} (Warburton *et al.* 1992). Bacteria isolated from natural mineral waters belong to about 20 genera which are mainly *Pseudomonas* and allied taxa, *Acinetobacter* and *Alcaligenes* (Leclerc 1994). Other genera, such as Gram-positive *Bacillus*, *Arthrobacter*, *Corynebacterium*, *Clavibacter* and *Micrococcus* and Gram-negative *Caulobacter*, *Sphaerotilus*, *Leptothrix*, *Flavobacterium*, *Cytophaga*, *Flexibacter*, *Chromobacterium*, *Xanthomonas*, *Vibrio* and *Aeromonas*, are also encountered (Schwaller and Schmidt-Lorenz 1981; Zheng and Kellog 1994; Gomes *et al.* 1996).

Bacterial enumeration in mineral water is usually achieved on agar media. However, medium composition and incubation conditions affect qualitative and quantitative results (Schwaller and Schmidt-Lorenz 1981; Reasoner and Geldreich 1985; Williams *et al.* 1994). Thus, it is not possible to recover all viable bacteria in water samples using specific media and experimental conditions.

Compared with standard plate count determination, the highest total population is obtained with direct epifluorescence microscopy after staining with acridine orange (AO) or 4′,6-diamidino-2 phenylindole (DAPI) fluorochrome dyes (Kepner and Pratt 1994). This can be explained by the existence of three kinds of bacteria: (i) viable bacteria non-culturable on artificial media (VBNC); (ii) viable bacteria capable of developing colony forming units (cfu); and (iii) non-viable bacteria.

Investigation of specific enzymatic activity, cell metabolism, endogenous respiration and membrane integrity can confirm the existence of viable but non-culturable bacteria. Redox dyes, such as INT (2-(p.iodophenyl)-3-(p.nitrophenyl)-5-phenyl tetrazolium chloride), CTC (5-cyano 2,3-ditolyl tetrazolium) or XTT (3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4 methoxy-6 nitro) benzene sulphonic acid hydrate) (Zimmermann *et al.* 1978; Roslev and King 1993; Coallier *et al.* 1994), reveal the respiratory activity of viable but non-culturable cells. Fluorescein diacetate (FDA) and 5-6 sulphofluorescein diacetate (SFDA) detect

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enzymatic activity (cell esterase) in viable but non-culturable bacteria (Raynel *et al.* 1994). The physiological activity of living bacteria can also be detected by studying membrane potential with rhodamine 123 (Kaprelyants and Kell 1992), carbocyanin (Mason *et al.* 1993) or calcafluor white (Mason *et al.* 1995), and investigating membrane integrity with Live/Dead® stain. The Live/Dead® stain is composed of two fluorochrome dyes, syto 9 and propidium iodide, which bind to nucleic acids and give green and red fluorescence, respectively, for viable and non-viable bacteria.

All these method present drawbacks; DAPI binds to numerous macromolecules (Kepner and Pratt 1994), Gram-negative isolates are impermeable to FDA (Tsuji *et al.* 1995) and rhodamine 123 (Kaprelyants and Kell 1992), and CTC has a toxic effect on bacterial metabolism (Ullrich *et al.* 1996).

Cell growth can also indicate active bacterial metabolism. Kogure *et al.* (1979) used nalidixic acid in liquid medium with added yeast extract to prevent cell division and observe elongation of viable micro-organisms. The method is described as the Direct Viable Count (DVC). This technique can be improved by using other antibiotics from the quinolone family. Kogure *et al.* (1984) used pipemidic and piromidic acid. The fluoroquinolone, ciprofloxacin, can discriminate between viable Gram-positive and viable Gram-negative bacteria (Barcina *et al.* 1995). Recently, Joux and LeBaron (1997) modified the DVC method using an antibiotic cocktail (four quinolones and one β-lactam) instead of nalidixic acid and obtained a more potent inhibition of the marine bacterial population under study.

The purpose of this study was to determine which antibiotics are most potent and at what concentrations for direct enumeration and characterization of viable cells in mineral water bacterial populations. The antibiotic sensitivities of 14 strains, isolated from emergent mineral water, to seven antibiotics which prevent DNA synthesis, were compared. Acridine orange dye was used for total bacterial cell counts and Live/Dead® (L/D) fluorochrome dye served to discriminate viable from dead cells on the basis of cell membrane integrity.

MATERIALS AND METHODS

Bacterial isolation from emergence mineral water and strain designation

Fifteen representative isolates from two French mineral water sources (Vauban and Source du Clos de l'Abbaye, Saint-Amand-les-Eaux, France) were examined. Strains were designated by two letters: the first designated the type of strain (A to Z) and the second, the origin of the strain (s for SDCA and v for Vauban springs).

Growth conditions and identification of isolates

The strains were initially isolated on R2A agar (Difco) incubated at 26 °C (temperature of the Vauban spring) and 18 °C (temperature of the SDCA spring). For identification purposes, sub-cultures were performed on Trypticase Soy Blood Agar (AES, AEB122870) for 24 h. The isolates were identified with the API 20 NE system (bioMérieux) and the BIOLOG GN system (AES, Combourg, France).

Determination of total bacteria and viable cell counts

Total bacterial counts were determined after staining with AO. Samples were stained with AO (0·01% w/v in acetate buffer pH 4) for 5 min and filtered onto black (0·2 μm pore size, 22 mm diameter) polycarbonate filters.

For the Direct Viable Count (DVC), Mueller Hinton (MH) broth, with an added antibiotic cocktail, was inoculated with pure or mixed cultures and then incubated in darkness at 25 °C. After 14 and 72 h of incubation, only bacteria that were elongated or wider, and fluoresced reddish-orange, were counted.

The viable bacterial cells were enumerated after Live/Dead® *Bac*Light™ (Molecular Probe, Eugene, OR USA) staining. This method is based on an evaluation of cell membrane integrity. Bacterial samples were stained with 3 μg ml^{-1} fluorochrome dye for 15 min in the dark and then filtered through black polycarbonate membranes (Haugland 1996).

For each staining method, the filters were mounted on clean microscope slides. All slides were examined at a magnification of 1000 × under immersion oil with epi fluorescence (Nikon optiphot 2) equipped with a filter block (Nikon, B2 A; Champigny-sur-Marne, France).

Determination of standard geometric features of each isolate

Strains were cultivated at 25 °C in MH broth for 20 h. After filtration through a polycarbonate black membrane, bacteria were stained with AO and then observed microscopically by epifluorescence. Some microscopic fields were photographed and treated by image analysis (ICONIX, Perfect Image™ Clara Vision, Orsay, France). A monochromatic stain was obtained with software and camera to differentiate bacteria from the background and determine morphological features. The morphological parameters retained were surface area (μm^2), height (μm) and orthogonal projections of bacteria dimensions (μm). Orthogonal projections were used for calculating the hypotenuse.

Determination of Minimum Inhibitory Concentrations (MIC)

All isolates were screened for their sensitivities to seven antibiotics. Antibiotics were dissolved in water (nalidixic acid

ciprofloxacin), ethanol (ofloxacin and norfloxacin) or alkaline solution (flumequine, pefloxacin and pipemidic acid) and sterilized through 0·2 μm pore size membrane filters (Millipore). Serial twofold dilutions of antimicrobial agents were made in MH broth (250–0·49 μg ml^{-1}).

Elongation test on bacteria cultured with added quinolones

The Ev strain was tested with five different antibiotics to follow changes in geometric bacterial characteristics according to the nature of quinolone.

Each strain was also inoculated in MH broth with the more potent antibiotic at the MIC. The media were incubated at 25 °C and the morphotypes were determined after 14 and 72 h.

Numeration and characterization of bacteria in mixed culture in high nutrient broth (MH) and in mineral water by improved DVC method

Two to five strains cultivated in MH broth were mixed. Several samples were cultivated with different antibiotic cocktails. The incubation was conducted at 25 °C for 14 h. Then, elongated bacteria were counted in each experiment after AO staining using the derived method of Kogure *et al.* (1979). The choice of antibiotics and their concentrations in the analysis depended on the nature of the bacteria under test. The initial concentration of viable bacteria for each strain in the mix was determined after Live/Dead® staining to verify the efficiency and the validity of the method.

RESULTS

Identification of isolates from mineral water

The 15 most representative phenotypes amongst the different bacterial phenotypes isolated from the natural mineral water Vauban and Source du Clos de l'Abbaye springs were selected (10 for Vauban and five for Source du Clos de l'Abbaye). All strains were non-fermentative, cytochrome oxidase-positive (or weakly positive), Gram-negative rods. Isolates from the Vauban spring were more frequently yellow or orange pigmented than isolates from the Source du Clos de l'Abbaye spring.

Two identification systems, API 20NE and BIOLOG GN, were used to tentatively identify these isolates (Table 1). API 20NE identified 60% of the isolates to levels of certainty that were acceptable or better. The other strains were scored as low discrimination (20%) or identification to the species level (20%). The BIOLOG GN system identified only 33·3% of the isolates tested to a similarity index $\geqslant$0·500 (good identification). Four isolates were identified with poor certainty as *Pseudomonas corrugata*, *Stenotrophomonas maltophilia* and *Pseudomonas putida*. Identifications with a similarity index $<$0·300 were highly improbable, notably for isolates which were assigned to the genus *Vibrio* (facultative anaerobic bacteria). Only four isolates (Fs, Ys, Vv and Wv) were identified to the same species level by the two systems. However, two of them (Fs and Wv) showed abnormal pigmentation. Two strains (As and Ev) were tentatively identified as *Ps. fluorescens* by API 20NE and *Ps. corrugata* with BIOLOG GN, although the characteristic pigments were absent. Isolates Zs, Jv, Rv and Zv were identified as *Sphingomonas paucimobilis* by the API 20NE gallery and, except for strain Zs, these strains presented a characteristic yellow, insoluble, non-fluorescent pigment. Owing to an orange pigmentation and to ecological considerations, isolate Uv was more likely to be *Brevundimonas vesicularis* (BIOLOG GN, sim 0·673) than *Agrobacterium radiobacter* (API 20NE, %id 98%). The identity of strain Jv could be *Alcaligenes latus* (BIOLOG GN, sim 0·769) rather than *Sp. paucimobilis* (API 20NE, %id 84%) because of its high BIOLOG similarity coefficient, yellow coloration and large cell width (Holt *et al.* 1994).

Susceptibility testing of isolates against (fluoro)quinolones

Isolates from the two springs were tested against three first-generation quinolones and four fluoroquinolones, and Minimum Inhibitory Concentrations (MIC) in Mueller Hinton broth were determined (Table 2). Strain Hv was not studied further because of its slow growth in Mueller Hinton broth.

Based on MIC breakpoints (Soussy 1997), strain Jv was found to be highly susceptible to all (fluoro)quinolones tested except for pefloxacin. In the same way, some isolates were highly susceptible to some antibiotics (Jv_{NOR}, $Pv_{OFX,PEF}$, Wv_{CIP}, As_{OFX}). Conversely, strain Hs showed a high level of resistance. A disparity in resistance patterns was also noted according to the antibiotic used. However, the great majority of isolates were highly resistant (MIC $>$8 μg ml^{-1} for flumequine and $>$16 μg ml^{-1} for nalidixic and pipemidic acids) against the first-generation quinolones. These antibiotics are therefore of limited interest for use in the Direct Viable Count (DVC) method. The MICs of fluoroquinolones were generally several orders of magnitude lower than those observed for the first-generation quinolones. Inhibition of 85·7% of isolates was achieved by using at least two of the four fluoroquinolones tested at a relatively low concentration ($\leqslant$7·81 μg ml^{-1}). However, in terms of basic resistance, 64·3% of isolates were found to be resistant to ciprofloxacin, 78·6% to ofloxacin and 92·8% to pefloxacin and norfloxacin.

Determination of standard geometric features of non-inhibited isolates

The standard geometric characteristics (surface area, maximal projection and hypotenuse) of non-inhibited isolates are pre-

Table 1 Characteristics and identification of bacterial isolates from mineral water springs (Source du Clos de l'Abbaye and Vauban)

Strain*	Pigment†	Cell shape	Genus‡ and species identification with API20NE (id%)§	BIOLOG GN (sim)¶
As	white	long rod	*Ps. fluorescens* (79%)	*Ps. corrugata* (0·452)
Fs	white	ovoid rod	*Ps. putida* (68%)	*Ps. putida* A (0·438)
Hs	cream	ovoid, thick rod	*C. testosteroni*/*Ps. alcaligenes* (45%) *Alc. faecalis* (39%) *Ps. putida* (12%)	nd**
Ys	cream	thin rod	*St. maltophilia* (96%)	*St. maltophilia* (0·339)
Zs	cream	small rod	*Sp. paucimobilis* (84%)	*V. tubiashii* (0·097)
Ev	white	moderate rod	*Ps. fluorescens* (79%)	*Ps. corrugata* (0·461)
Gv	pink	moderate rod	*B. vesicularis* (74%) *Aer. salmonicida* (9·1%) *Sp. paucimobilis* (8·5%)	nd
Hv	cream	long rod	*W. virosa* (48%) *E. breve* (32%) *B. diminuta* (7%)	*Ps. fluorescens* (0·504)
Jv	yellow	ovoid rod	*Sp. paucimobilis* (84%)	*Alc. latus* (0·769)
Pv	cream	small, thick rod	*C. meningosepticum* (98%)	*Si. meliloti* (0·005)
Rv	yellow	moderate rod	*Sp. paucimobilis* (84%)	*V. mimicus* (0·095)
Uv	orange	moderate rod	*Ag. radiobacter* (98%)	*B. vesicularis* (0·673)
Vv	yellow	long rod	*St. maltophilia* (99%)	*St. maltophilia* (0·512)
Wv	white	moderate rod	*B. vesicularis* (92%)	*B. vesicularis* (0·165)
Zv	yellow	moderate rod	*Sp. paucimobilis* (90%)	nd

* Isolated from springs: s, Source du Clos de l'Abbaye and v, Vauban.
† Pigmentation after 24–48 h growth on R2A agar.
‡ Abbreviations: *Ps.*, *Pseudomonas*; *C.*, *Comamonas*; *Alc.*, *Alcaligenes*; *St.*, *Stenotrophomonas*; *Sp.*, *Sphingomonas*; *V.*, *Vibrio*; *B.*, *Brevundimonas*; *Aeromonas*; *W.*, *Weeksella*; *E.*, *Empedobacter*; *C. Chryseobacterium Sinorhizobium* and *Ag.*, *Agrobacterium*.
§ Percentage identification: excellent (≥ 99·9%), very good (≥ 99%), good (≥ 90%) or acceptable (≥ 80%).
¶ Similarity index: excellent (≥ 0·750%), good (≥ 0·500), no identification (< 0·500); the 10 most closely matching species are always reported but only the first choice is presented here.
** nd, no determination.

sented in Table 3. For the isolates from Source du Clos de l'Abbaye, measured surfaces areas varied from 0·6–1·8 μm^2, maximal projection from 1·0–2·1 μm and hypotenuse from 1·3–2·7 μm; with the Vauban isolates, they fluctuated from 1·2–1·8 μm^2, 1·8–2·3 μm and 1·9–2·7 μm, respectively. A wider range of values was observed for the hypotenuse. Furthermore, the hypotenuse readily discriminated the different isolates.

Alterations in morphometry of isolates after incubation in MH with different (fluoro)quinolones

Morphometry of strain Ev after inhibition with different (fluoro)quinolones. As shown in Table 4, a 14 h inhibition period in MH with different (fluoro)quinolones used at concentrations corresponding to the MIC led to significant increases (1·8–4·8 fold) in mean geometric characteristics of strain Ev. Bacterial elongation was less pronounced after inhibition with flumequine. Increases in geometric features varied according to the antibiotic used. However, all increases were significant and the antibiotic with the lowest MIC was used in subsequent experiments.

Morphometry of different strains on incubation in MH with the most effective fluoroquinolones. Two incubation periods (14 h and 72 h) with antibiotics were studied. This method was chosen because of the need for rapid and reliable results compared with other count methods (14 h), and in order to obtain a significant elongation discrimination between

Table 2 Comparative *in vitro* activity (MIC) of different quinolones and fluoroquinolones against isolates from mineral water springs (Source du Clos de l'Abbaye and Vauban)

	MIC (μg ml^{-1}) of antimicrobial agent†						
Strain*	NA	Pi	AR	NOR	OFX	PEF	CIP
As	62·5	125	125	15·62	⩽0·49	1·95	0·98
Fs	62·5	62·5	31·25	15·63	3·91	>250	0·98
Hs	>250	>250	>250	62·5	62·5	>250	>250
Ys	62·5	250	15·63	62·5	7·81	15·63	7·81
Zs	>250	>250	>250	125	3·91	>250	7·81
Ev	125	>250	15·63	62·5	7·81	>250	0·98
Gv	>250	>250	>250	15·63	3·91	15·63	1·95
Jv	0·98	3·91	0·49	⩽0·49	0·98	7·81	0·98
Pv	15·63	31·25	62·5	15·63	⩽0·49	⩽0·49	1·95
Rv	125	>250	7·81	62·5	3·91	7·81	7·81
Uv	>250	>250	>250	62·5	15·63	31·25	15·63
Vv	62·5	250	125	31·25	7·81	15·63	7·81
Wv	125	>250	62·5	62·5	15·63	7·81	⩽0·49
Zv	250	>250	250	62·5	7·81	7·81	3·91

* Isolated from springs: s, Source du Clos de l'Abbaye and v, Vauban.
† NA, Nalidixic acid; Pi, Pipemidic acid; AR, Flumequin; NOR, Norfloxacin; OFX, Ofloxacin; PEF, Pefloxacin and CIP, Ciprofloxacin.

Table 3 Determination of mean geometric features of isolates from mineral water springs (Source du Clos de l'Abbaye and Vauban)

	Mean geometrical characteristics (± S.D.)†		
Strain*	Surface (μm^2)	Maximal projection (μm)	Hypotenuse (μm)
As	1·81 (0·32)	2·13 (0·21)	2·45 (0·17)
Fs	1·14 (0·05)	1·40 (0·05)	1·72 (0·05)
Hs	1·71 (0·30)	2·14 (0·21)	2·70 (0·19)
Ys	0·79 (0·18)	1·45 (0·10)	1·68 (0·09)
Zs	0·58 (0·091)	1·04 (0·07)	1·31 (0·05)
Ev	1·30 (0·12)	1·97 (0·11)	2·36 (0·08)
Gv	1·48 (0·19)	1·87 (0·07)	2·19 (0·06)
Jv	1·54 (0·12)	1·89 (0·11)	2·32 (0·11)
Pv	1·45 (0·08)	2·32 (0·11)	1·89 (0·12)
Rv	1·24 (0·11)	2·15 (0·13)	2·42 (0·11)
Uv	1·22 (0·18)	1·83 (0·10)	2·10 (0·09)
Vv	1·79 (0·17)	2·28 (0·09)	2·72 (0·13)
Wv	1·31 (0·18)	1·92 (0·12)	2·29 (0·12)
Zv	1·28 (0·21)	1·91 (0·09)	2·40 (0·10)

* Isolated from springs: s, Source du Clos de la'Abbaye and v, Vauban.
† Mean ± S.D. ($250 < n < 400$).

(72 h). As shown in Table 5, a 14 h incubation with antibiotics resulted in significant increases in the mean hypotenuse of isolates (⩾1·5-fold). Considerable increases were sometimes observed (2·4–3·8-fold in strains Uv, Zv, Pv, Ev, Wv and Fs). Further incubation (72 h) in MH with antibiotics led to similar hypotenuse values (strains Ys, Jv and Vv) and, generally, to lower hypotenuse values than those observed after a 14 h period. Moreover, in many instances, hypotenuse values of 72 h inhibited isolates were not significantly different from those of non-inhibited cells (strains Ev, Gv, Rv, Zv and Zs); some were even lower (strain As).

Thus, strain differentiation in mixed culture was easily accomplished in populations. The largest mean hypotenuse values of the non-inhibited isolates were smaller than those of the smallest 14 h inhibited isolate (even with the smallest dimension of non-inhibited isolates). This observation was not true for Source du Clos de l'Abbaye isolates. Therefore, a judicious choice of antibiotic cocktails had to be made to achieve enumeration and characterization of every strain in Source du Clos de l'Abbaye samples.

Enumeration and characterization of isolates in experimental mixed cultures by the improved DVC method

Tables 6 and 7 reported quantitative initial results determined by L/D staining and obtained by a modified DVC method in mixed cultures in MH broth and mineral water, respectively. Differentiation of isolates was readily achieved and with few

Table 4 Alterations of mean geometric characteristics of strain Ev after 14 h incubation time in Mueller Hinton broth with different quinolones and fluoroquinolones

Antimicrobial agent* ($\mu g\ ml^{-1}$)	Mean geometrical characteristics (± S.D.)†		
	Surface (μm^2)	Maximal projection (μm)	Hypotenuse (μm)
Without antibiotic	1·30 (0·12)	1·97 (0·11)	2·36 (0·08)
NA (125)	6·31 (0·19)	5·28 (0·21)	6·19 (0·29)
AR (15·63)	4·18 (0·29)	3·73 (0·22)	4·32 (0·11)
NOR (62·5)	5·21 (0·19)	5·88 (0·19)	6·73 (0·21)
OFX (7·81)	5·77 (0·27)	5·78 (0·31)	6·71 (0·30)
CIP (0·98)	5·91 (0·28)	5·37 (0·22)	6·13 (0·17)

* NA, Nalidixic acid; AR, Flumequin; NOR, Norfloxacin; OFX, Ofloxacin and CIP, Ciprofloxacin.
† Mean ± S.D. ($250 < n < 400$).

Table 5 Alterations of mean hypotenuse of different strains upon incubation in Mueller Hinton with the most effective fluoroquinolones

Strain*	Antimicrobial agent† ($\mu g\ ml^{-1}$)	Mean hypotenuse in μm (± S.D.)‡ after incubation time of		
		0 h	14 h	72 h
As	PEF (1·95)	2·45 (0·17)	3·78 (0·21)	1·61 (0·18)
Fs	OF (3·91)	1·72 (0·05)	6·56 (0·27)	4·70 (0·17)
Hs	NOR (6·25)	2·70 (0·19)	4·78 (0·16)	4·03 (0·17)
Ys	CIP (7·81)	1·68 (0·09)	2·46 (0·18)	2·47 (0·22)
Zs	OF (3·91)	1·31 (0·05)	2·02 (0·09)	1·51 (0·12)
Ev	CIP (0·98)	2·36 (0·08)	6·12 (0·26)	2·67 (0·28)
Gv	CIP (1·95)	2·19 (0·06)	3·42 (0·21)	2·39 (0·31)
Jv	CIP (0·98)	2·32 (0·11)	3·63 (0·21)	3·67 (0·19)
Pv	CIP (1·95)	1·89 (0·12)	4·91 (0·29)	3·02 (0·28)
Rv	OF (3·91)	2·42 (0·11)	4·63 (0·19)	2·59 (0·30)
Uv	CIP (15·63)	2·10 (0·09)	5·05 (0·27)	3·32 (0·33)
Vv	OF (7·81)	2·72 (0·13)	3·50 (0·17)	3·31 (0·26)
Wv	PEF (7·81)	2·29 (0·12)	7·02 (0·41)	3·27 (0·38)
Zv	CIP (3·91)	2·40 (0·10)	6·05 (0·33)	2·64 (0·31)

* Isolated from springs: s, Source du Clos de l'Abbaye and v, Vauban.
† NOR, Norfloxacin; PEF, Pefloxacin; OFX, Ofloxacin and CIP, Ciprofloxacin.
‡ Mean ± S.D. ($250 < n < 400$).

exceptions, DVC results were within the same order of magnitude and were not statistically ($P = 0·05$) different from initial inocula counts.

DISCUSSION

The number of bacterial species recovered from the two springs was within the range (1–20 species per brand) previously reported for different brands of mineral water (Manaia *et al.* 1990; Ferreira *et al.* 1996). The species *Sp. paucimobilis*, *Ps. putida* and *B. vesicularis* have been reported in other studies of aquatic bacteria and mineral water (Mora and Da Costa 1990; Amy *et al.* 1992; Leclerc 1994; Brow and Leff 1996; Ferreira *et al.* 1996). These species, togeth with *Ps. fluorescens* and *Ps. stutzeri*, are common to seve French mineral waters (Guillot and Leclerc 1993). The spe ies *Chryseobacterium meningosepticum* (formerly *Flav bacterium*) has occasionally been isolated from aquat environments (Manaia *et al.* 1990; Leclerc 1994; Zheng a Kellogg 1994). The presence of *Ps. corrugata* in ground wat has been reported only once previously (Jain *et al.* 1997).

Although widely used, the API 20 NE has been repo

Table 6 *Enumeration and recognition of a particular strain from bacterial mix in Mueller Hinton broth by the improved DVC method*

Strain*	Log initial viable cell ml^{-1} (±S.D.)† in experimental mix	Log viable cell ml^{-1} (±S.D.)‡ as determined by the improved DVC method
Zs	6·72 (0·10)	6·72 (0·08)
Ys	6·53 (0·15)	6·54 (0·11)
Fs	6·14 (0·09)	6·62 (0·09)
As	5·84 (0·11)	6·32 (0·10)
Fs	5·00 (0·07)	6·36 (0·14)
Ys	6·00 (0·12)	6·51 (0·13)
Hs	7·58 (0·09)	6·69 (0·10)
As	7·75 (0·07)	7·04 (0·08)
Ys	7·92 (0·07)	7·74 (0·10)
Fs	7·89 (0·11)	7·80 (0·05)
Zs	8·32 (0·08)	8·04 (0·11)
Pv	6·47 (0·07)	6·89 (0·09)
Gv	6·84 (0·05)	6·50 (0·10)
Vv	6·30 (0·08)	6·99 (0·06)
Rv	6·47 (0·11)	6·88 (0·12)
Ev	6·69 (0·09)	6·96 (0·14)
Ev	6·30 (0·12)	6·25 (0·09)
Uv	6·14 (0·09)	6·67 (0·11)
Wv	6·04 (0·10)	6·25 (0·07)

* Isolated from springs: s, Source du Clos de l'Abbaye and v, Vauban.
† Log initial viable cell ml^{-1} were determined after staining with Live/Dead.
‡ Mean ± S.D. ($n = 3$).

Table 7 *Enumeration and recognition of a particular strain from bacterial mix in natural mineral water by the improved DVC method*

Strain*	Log initial viable cell ml^{-1} (±S.D.)† in experimental mix	Log viable cell ml^{-1} (±S.D.)‡ as determined by the improved DVC method
Zs	7·39 (0·13)	7·23 (0·09)
Ys	7·27 (0·11)	7·25 (0·07)
Fs	7·27 (0·06)	7·14 (0·10)
As	6·80 (0·12)	6·92 (0·11)
Fs	7·27 (0·09)	7·14 (0·07)
Ys	7·27 (0·11)	7·20 (0·12)
Hs	5·96 (0·09)	5·73 (0·11)
As	6·80 (0·09)	6·92 (0·08)
Ys	7·27 (0·11)	7·70 (0·11)
Fs	7·27 (0·12)	7·14 (0·08)
Zs	7·39 (0·10)	6·98 (0·11)
Pv	7·71 (0·09)	7·23 (0·07)
Gv	6·96 (0·05)	7·12 (0·05)
Vv	7·70 (0·08)	6·38 (0·09)
Rv	7·53 (0·11)	7·14 (0·07)
Ev	6·77 (0·12)	7·30 (0·06)
Ev	6·77 (0·09)	7·11 (0·13)
Uv	7·14 (0·06)	7·30 (0·08)
Wv	7·49 (0·07)	7·17 (0·08)

* Isolated from springs: s, Source du Clos de l'Abbaye and v, Vauban.
† Log initial viable cell ml^{-1} were determined after staining with Live/Dead.
‡ Mean ± S.D. ($n = 3$).

to be unable to identify a large proportion (around 70%) of aquatic bacterial isolates (Morais and Da Costa 1990; Amy *et al.* 1992; Brown and Leff 1996; Ferreira *et al.* 1996). The 60% identification rate reported here is thus higher than those of previously published studies. However, some identifications (Zs, Uv and Wv) remained questionable owing to abnormal, or lack of, pigmentation at least on R2A agar. Tentative identification of isolates with BIOLOG GN was found to be largely unsuccessful, in accordance with other studies (Amy *et al.* 1992; Zheng and Kellogg 1994; Balkwill and Boone 1997). Nevertheless, a 74% identification rate has been achieved with this system for isolates from deep groundwaters (Jain *et al.* 1997). Owing to the difficulties frequently encountered in identification of mineral water isolates, it is not surprising that new species are described, notably among the fluorescent *Pseudomonas* (Coroler *et al.* 1996; Elomari *et al.* 1996) and the yellow-pigmented pseudomonad group (Gomes *et al.* 1996).

Difficulties in obtaining a reliable assessment of antibiotic resistance in aquatic bacteria has been reported previously (Jones *et al.* 1986). Jones *et al.* (1986) recommended the use of the disc method and careful temperature standardization. The incubation temperature in our MIC determination was different from that normally employed (Anon. 1996). The temperature used to determine MIC is usually in the 35–37 °C range, much higher than the *in situ* conditions for the species studied here. Indeed, the temperature of the sources were 26 °C and 18 °C for Vauban and Source du Clos de l'Abbaye springs, respectively. Therefore, 25 °C was chosen as a compromise between experimental and environmental temperature, and bacterial growth rate. The purpose of our study was to determine the more potent quinolones for use

in the Direct Viable Count of mineral water bacteria growing at 18–26 °C and not to detect basic resistance to these antibiotics. Thus, our determinations were done in Mueller Hinton broth incubated at 25 °C. However, based on MIC breakpoints and in terms of basic resistance, intermediate susceptibility or resistance patterns against (fluoro) quinolones were observed for the great majority of the isolates. Resistance to first-generation quinolones and particularly, against nalidixic acid, is frequently reported for a sizeable number of aquatic and mineral water bacterial isolates (Amy *et al.* 1992; Papapetropoulou *et al.* 1994; Massa *et al.* 1995; Joux and LeBaron 1997). However, there is no clear cut evidence that the antibiotic-resistant bacteria naturally found in mineral water are a public health risk (Rusin *et al.* 1997). Among (fluoro)quinolones, ciprofloxacin was found to be the most effective compound according to Papapetropoulou *et al.* (1994) and Joux and Lebaron (1997). At least two of the four fluoroquinolones tested were able to achieve growth inhibition of the different isolates at concentrations within the range of the first-generation quinolone concentrations used in the antibiotics cocktail described by Kogure *et al.* (1984). However, more potent, new fluoroquinolones, which have been reported to be highly active against the genus *Pseudomonas* and allied taxa (Ford *et al.* 1993; Cunha *et al.* 1997), should be tested in order to ensure growth inhibition at lower antibiotic concentrations.

In order to achieve efficient distinction between inhibited and resistant strains (able or unable to divide), surface area, maximal projections and hypotenuse were determined. Surface area and projections were measured using computerized image analysis. Calculating the hypotenuse can be useful as it gives a real measurement of cell length. In addition, the hypotenuse can attenuate some of the errors generated by image focusing with Perfect Image™ software. Focusing generates multidirectional defaults on surface estimation, whereas the hypotenuse only has a bidirectional effect. We found the hypotenuse to be more discriminating than surface area and projections.

Peele and Colwell (1981) used area measurements for cultures of sea water which generally show swollen cells after incubation with various nutrients and under different growth conditions. In spite of the presence of ovoid cells, length measuremen as more discriminating in our study. Barcina *et al.* (1995) emphasized that biovolume measurements offer a better estimation of cell increases due to nutrient incorporation. However, in the case of bacterial rods, growth mainly implies variations in length, so biovolume measurements would be more appropriate for cocci, as determined by Barcina *et al.* (1995).

Singh *et al.* (1989) found that a 1·5-fold elongation is a suitable measurement for determining the viability of bacteria in the DVC test. In our study, this elongation coefficient fluctuated from 1·8 to 2·8 for strain Ev and was always $\geqslant 1{\cdot}5$ for other isolates. Therefore, elongation was significant w ever the antibiotic tested.

Dye breakdown inside the cells explains the decrease mean hypotenuse after 14 h. Indeed, bacterial leng increased even after a 14 h period of incubation, but im analysis and fluorochrome stain revealed heterogeneous sta ing in the bacterial body. Elongations after a 14 h exposur antibiotics were significant and allowed a rapid differentiat between inhibited and non-inhibited morphotypes between viable and dead cells.

In morphometric determination of inhibited antibi strains, the elongation coefficient varied from 1·5–3·8. Val tended to be higher in the Vauban strains. Source du Clos l'Abbaye isolates appeared to be less substrate responsi This phenomenon has already been observed in a previ study concerning the culture of these different isolates various media (PCA, PCA/10 and R2A).

The sensitivity of a strain to a given antibiotic should considered in terms of the antibiotic concentration. In method, and as recommended by Buchrieser and Kasp (1993), the concentration that inhibits replication witho affecting other synthetic pathways was required. High an biotic concentrations could lead to metabolic inhibition sometimes, to cell death or disruption of some sensitive ce as emphasized by Joux and LeBaron (1997). Neverthel the rather high antibiotic concentrations used in our st (quinolone: $\geqslant 16\ \mu g\ ml^{-1}$ and fluoroquinolone: $\geqslant 4\ \mu g\ m$ did not produce such an effect as isolates are not lyse subject to non-specific secondary effects.

The absence of atypical cellular shapes after incubat with various nutrients and growth conditions (MH, and m eral natural water, with or without antibiotic), which observed in cultures from sea water for example (Peele a Colwell 1981), confirms the value of measuring lengths rat than surface areas.

One of our objectives was to avoid adding nutrients for DVC method applied to mineral natural water in order conserve the water's native oligotrophic state and thus avo nutritive inhibitor effects or eutrophic contaminants. Th exogenous substrates added came only from the pre-cultu media (less than $10\ \mu g\ ml^{-1}$ organic matter).

In both experiments, the method appeared to be effectiv and efficient under the defined conditions. Some new fluor quinolones, which appear to be very active on many types micro-organisms (Ford *et al.* 1993; Cunha *et al.* 1997), migl also be useful.

The aim of this method was to count and characterize bacteria in relatively well known and simple bacterial miner water communities, both rapidly and directly. It can also b applied to estimate some mixed cultures in biofermentors in food with known a microflora. The DVC method may b a practical tool in view of the fact that specific morphologic physiological and metabolic features, and some comme

nucleic probes, are not always available for identifying and demonstrating the viability of target micro-organisms.

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CHAPTER 26

Rhinoviruses

Jack M. Gwaltney, Jr.

1. Introduction

Rhinoviruses are the most important common-cold viruses to be discovered. The name *rhinovirus* reflects the prominent nasal involvement seen in infections with these viruses. The large rhinovirus genus, which is a member of the *Picornavirus* family, contains over 100 different immunotypes. The discovery of the rhinoviruses led to the realization that the common cold is an enormously complex syndrome. The number of antigenically distinct rhinoviruses is so large that one can be infected with a different rhinovirus each year and still not experience all the known types in a lifetime. The antigenic diversity of the rhinovirus group has proved an insurmountable obstacle to rhinovirus vaccine development. It is now known that the cellular receptor site for rhinovirus is shielded from the immune system, eliminating it as a target for vaccines and further discouraging prospects for control of rhinovirus colds by this approach. Recent work on rhinovirus has focused on understanding pathogenesis and on developing control measures such as chemoprophylaxis, chemotherapy, and interruption of transmission.

2. Historical Background

Rhinovirus colds may have affected humans and higher primates for many thousands of years, although natural rhinovirus colds have not been documented in nonhuman primates.[83] A closely related member of the *Picornavirus* family, poliovirus, is known to have caused human disease in ancient times, so it is probable that rhinoviruses were in existence then also. Colds were a nuisance in early civilization; then, as now, many useless remedies were proposed for their treatment. In 400 BC, Hippocrates noted that bleeding was a frequently used, although worthless, treatment for colds. In the first century, Pliny the Younger prescribed "kissing the hairy muzzle of a mouse" for colds. The first sound epidemiologic knowledge about acute respiratory disease came with the observations that sea voyagers and the inhabitants of isolated communities were free of colds while not in contact with the outside world but developed colds when such contact was reestablished. This led to the important conclusion that colds are contagious.

Direct evidence of the infectious nature of colds came in 1914 from the volunteer studies of Kurse,[140] who produced experimental colds in volunteers by intranasal inoculation of cell-free filtrates of nasal secretions from persons with colds. Similar experiments by Dochez *et al.*[50] in 1930 confirmed that colds could be transmitted by bacteria-free filtrates, suggesting that the responsible agents might be viruses. At the same time, epidemiologic studies of acute respiratory disease in populations had been started. Van Loghem[227] measured the incidence of colds and observed their relationship to the seasons. Frost and Gover[72] made the perceptive observation that common respiratory disease appearing during the months of high prevalence, September to March, was composed of a series of short epidemics of irregular sequences and magnitude. This suggested that colds were caused by a variety of different agents occurring in succession. In the 1940s and 1950s, long-term studies of colds in the home by Dingle *et al.*[48] yielded precise information on attack rates by age and the importance of the home as a site for transmission of respiratory infections. During the same period, a group at the Common Cold Research Unit at Salisbury, England, headed by Andrewes and later Tyrrell, was vigorously pursuing questions related to the etiology and epidemiology of colds.[2] Colds were successfully transmitted in volunteers using nasal secretions that were later shown

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to contain rhinoviruses. Attempts at the time to establish growth of the virus in artificial culture were unsuccessful.

Specific work on rhinoviruses began in 1956 when Pelon *et al.*[175] and Price,[185] working separately, reported the isolation of a new virus that was subsequently given the designation *rhinovirus IA*. Within a few years, Ketler *et al.*,[137] using the highly sensitive human embryonic lung cells developed by Hayflick and Moorhead[113] and employing growth methods developed at the Salisbury Common Cold Unit,[226] isolated a number of different serological types, indicating that the rhinovirus group would not be small. Epidemiologic studies conducted by Hamre and Procknow[102] during the same period established that rhinoviruses were responsible for a significant amount of acute respiratory disease. Specific rhinovirus infection rates and the finding of recurrent fall peaks of rhinovirus colds were reported from a longitudinal study by Gwaltney *et al.*[87] In further studies of rhinovirus epidemiology by Monto,[158] Dick *et al.*,[46] and Hendley *et al.*,[116] the importance of the family setting and of schoolchildren in particular in favoring rhinovirus transmission was demonstrated. Couch *et al.*[38] noted the surprisingly small amount of virus necessary to initiate experimental infections in volunteers. This group also provided important information on the pathogenesis[54] and immunology of rhinovirus infections.[193] In 1967, a collaborative program directed by Kapikian *et al.*[132] assigned numbers 1A-55 to the rhinovirus types then known. In 1971, a second phase of this program added types 56–89.[133] Results of a third phase of the numbering program completed in 1987 has extended the numbering system to include 100 rhinovirus types.[97] More recently, work has focused on understanding routes of viral transmission[85,92,117] and mechanisms of pathogenesis.[214,222,223] Also, the structure of the viral shell[194,195] and the composition of the viral genome[22] have been determined and a new therapeutic approach consisting of the simultaneous administration of an antiviral agent and of compounds that block the action of selected inflammatory mediators has shown promise.[82]

3. Methodology Involved in Epidemiologic Analysis

3.1. Surveillance and Sampling

Longitudinal studies of rhinovirus epidemiology have provided data on rhinovirus attack rates. Surveillance of a population of young adults at an insurance company in Charlottesville, Virginia, was conducted by collecting illness data on symptom-record cards in conjunction with weekly personal contact by a nurse-epidemiologist.[87] This nurse also collected samples at the time of illness. In addition, samples were obtained weekly from asymptomatic persons in a randomly selected sample of the study population. In another study, families from representative segments of the population in Tecumseh, Michigan, were surveyed by weekly telephone contact with a single household respondent who provided illness information for the family.[160,165–167] In the third investigation, mothers of families with newborn infants in a group health cooperative in Seattle, Washington, recorded illness information on their families and were visited twice weekly for routine sampling.[70] In the latter two studies, specimens were collected during home visits by a nurse-epidemiologist when illness was reported to the study team by telephone. Specimens for viral culture are usually collected from adults by nasal swabs or nasal washes. In young children, nasopharyngeal aspirates have been reported to be superior to nasal swabs for rhinovirus isolation.[41]

3.2. Methods of Virus Isolation, Propagation, and Identification

Cell culture is the standard method for rhinovirus isolation and propagation. Rhinoviruses grow best at temperatures of 33–34°C under conditions of motion[130] and will not grow in embryonated eggs or suckling mice. Most epidemiologic studies have employed human embryonic lung cells, strains W138 and MRC5, or strains of human embryonic lung cells originated by the laboratory conducting the study. Rhinovirus cytopathic effect in W138 and MRC5 cell cultures is readily discernible, making these easy systems with which to work. The sensitivity of these cells to rhinoviruses appears to be similar to that of the nasal mucosa of volunteers. Volunteer challenge experiments comparing rhinovirus median human and tissue culture infectious dose (HID_{50} and $TCID_{50}$) have shown 1 HID_{50} to be equivalent to 0.03–0.75 $TCID_{50}$.[51]

There are problems, however, with the use of human embryonic lung-cell cultures. The sensitivity to rhinovirus of cell strains of different origin may vary 100-fold or more, for poorly understood reasons.[13] Also, different lots of the same strain, such as W138, may have unpredictable variations in rhinovirus sensitivity that are unexplained.[84] Interpretations of rhinovirus morbidity data must take these variations into account, since rates of rhinovirus-associated illness are directly related to the sensitivity of the cell cultures used.

Rhinoviruses will grow in other cell lines and strains derived from human and primate tissues, including rhesus monkey kidney, human embryonic kidney, and KB. The sensitivity of these cells for rhinoviruses tends to be less consistent than that of W138 cells. A strain of HeLa cells with enhanced sensitivity to rhinoviruses has been devel-

oped and proven useful for propagation of antigen and for serological procedures.[31] These M-HeLa cells have been used to grow rhinovirus harvests with exceptionally high titers (10^9 PFU/ml)[33] and to prepare large quantities of antigen in suspension cultures.[220] Certain rhinovirus serotypes were recovered from original specimens with M-HeLa cells but not with human diploid-cell cultures.[34,143] All the first 55 numbered rhinovirus types have been plaqued using a method that employs HeLa cells and an agarose overlay containing medium with added magnesium and DEAE-dextran.[66]

The earlier division of rhinoviruses into H and M strains on the basis of growth in cells of human or monkey origin has been of limited epidemiologic importance. M strains tend to grow better in cell culture and thus were more easily recovered with the less sensitive systems used in earlier studies.[90] Consideration should be given to the greater ease of recovery of M strains when epidemiologic data are being evaluated, since this variable could result in overestimation of the importance of M rhinoviruses. Recent work has shown that H strains can be adapted to grow in monkey-kidney cells, suggesting that the division into H and M strains is not based on major differences in the biological properties of rhinoviruses.[52]

Organ cultures of fetal human trachea and other ciliated epithelium have been used to isolate rhinoviruses that did not grow initially in cell culture.[122,225] Comparison of the sensitivity for rhinovirus isolation of standard cell culture and of organ culture has failed to show clear superiority of the organ-culture system[118]; both systems are necessary for optimal recovery of these viruses. Once isolated in organ culture, rhinoviruses can usually be adapted to cell culture. The organ-culture strains have been found to be types that have also been recovered in cell culture. Because of the limited supply of fetal material, it has not been possible to use organ-culture systems in large epidemiologic studies.

The use of the polymerase chain reaction with nucleic acid probes has also been adapted to detection of rhinovirus in clinical specimens.[6,129] The sensitivity of this method compared to sensitive human embryonic lung cell cultures and its practicability for epidemiologic studies have not been well defined. Also, an enzyme-linked immunosorbent assay (ELISA) has been developed for detection of rhinovirus.[44]

Experimental infections with human rhinoviruses have been produced in chimpanzees[45] and gibbons,[183] and a variant of human rhinovirus type 2 has been adapted to replicate in the lungs of Balb/c mice.[232] Rhinoviruses have been isolated from cattle,[157] and respiratory viruses with characteristics similar to those of human rhinoviruses have been recovered from cats[40] and horses.[49]

3.3. Methods Used for Serological Surveys and Antibody Measurements

The multiplicity of rhinovirus types and their relative immunologic specificity have prevented the general use of serological techniques for measuring infection rates. Serological study of infection rates is possible, however, when the types of rhinoviruses circulating in small populations, such as families, are known from viral cultures. Testing for the presence of rhinovirus antibody has been done with the neutralization (N) test. The N test has been used to identify specific antigenic types of viruses and to measure antibody in human serum and nasal secretions. An ELISA has recently been developed for measuring rhinovirus antibody in serum and nasal secretions that was reported to correlate well with the N test.[8]

In experimental rhinovirus infection, virus shedding was found to be more sensitive than antibody response as a means of detecting infection,[114] whereas in studies of natural infections, either procedure alone identified only about two thirds of the diagnosed infections.[9] In family studies, 20–40% of total infections were detected only by serology in persons who had both tests performed.[46,116]

For typing rhinoviruses, hyperimmune rhinovirus antisera have been produced in a number of animal species, including rabbits, guinea pigs, calves, goats, and baboons. Some goat and calf antisera have contained cytotoxic substances that have caused difficulties in the interpretation of N test results.[31] The large number of rhinovirus serotypes has led to the use of antisera pools for serotype identification. An efficient method of antisera pooling is the combinatorial method.[135] Serological identifications of rhinoviruses in large epidemiologic studies can be done with pooled antisera used in microneutralization systems.[78,139]

The accepted standard for serological identity of an unknown rhinovirus is neutralization of virus concentrations ranging from 10 to 300 $TCID_{50}$ by 20 units of antibody.[130] For measuring N antibody in human serum and nasal washings, it is necessary to use small doses of virus (3–30 $TCID_{50}$) for the test to have satisfactory sensitivity.[57]

4. Characteristics of the Virus that Affect the Epidemiologic Pattern

4.1. Physical and Biochemical Characteristics

Rhinoviruses have physical and biochemical properties that put them in the picornavirus family (Table 1).[172,173,206,228] The human rhinovirus virion is a 30-nm-diameter, nonenveloped particle with a shell composed of

Table 1. Characteristics of Rhinovirus

Physical and biochemical	*Biological*
Size: 30 nm	Optimal temperature of growth 33–35°C and restriction of growth at 37°C
Shape: capsid with icosahedral symmetry with proposed structure of 60 copies each of four polypeptides (VP1–VP4)	Inability to survive and replicate in the intestinal tract
Nucleic acid: single-stranded RNA of $2.6 \pm 0.1 \times 10^6$ daltons (30% of total particle mass)	Survival on skin and environmental surfaces
Ether: resistant	Two receptor families for host cells
Acid: labile (pH 3–5)	
Virus: synthesis and maturation in cytoplasm	

Antigenic

Native antigenicity: type-specific (D antigenicity)
One hundred or more numbered native antigenic types
Direct and indirect antigenic relationships between some native antigenic types demonstrable with hyperimmune sera
Altered antigenicity (by heat or urea): cross-reactive between types (C antigenicity)

three proteins (VP1, VP2, VP3).[195] The rhinovirus shell is more loosely packed than that of enterovirus, accounting for rhinovirus' greater buoyant density and its susceptibility to inactivation on acid exposure. X-ray defraction studies of the rhinovirus shell have disclosed the presence of a depression on the surface at the junction between the plateau of VP1 and those of VP2 plus VP3.[194] This depression contains the recognition site for the host cell receptor.

The genome of several rhinovirus types has been sequenced, that of rhinovirus type 14 being 7209 nucleotides long.[22] Rhinovirus genomes have been found to share 45 to 62% homology with poliovirus genomes. Similarity in physical nature of the two groups may help explain similarities in epidemiologic behavior, i.e., increased prevalence in late summer and fall and possible spread by direct contact with infectious secretions.

4.2. Biological Characteristics

The biochemical basis for the optimal temperature range for rhinovirus growth is unknown, but this property may be of major epidemiologic importance (Table 1). The mean temperature of nasal mucosa, 33–35°C, corresponds to the optimal temperature for rhinovirus replication. At 37°C, virus yields fall to 10–50% of optimum.[215] In natural infection in man, rhinovirus concentrations are higher in nasal secretions than in pharyngeal secretions, saliva, or secretions obtained by simulated coughs and sneezes.[117] Attempts to isolate rhinovirus from blood have not been successful,[54,59] nor does rhinovirus survive and replicate in the intestinal tract. Studies of rhinovirus survival in the gut suggest that the temperature of 37°C may be a decisive factor in inhibiting growth, although gastrointestinal secretions and transmit time may also have adverse effects on virus survival.[25] On the basis of these observations, it may be possible that one reason for the different pathogenic and epidemiologic behavior of enteroviruses and rhinoviruses is the difference in the optimal temperature for growth of the two groups of viruses.

Rhinoviruses have been divided into three groups on the basis of their cellular receptors.[28] Ninety-one of the viral immunotypes, the "major group," use the intercellular adhesion molecule-1 (ICAM-1) cellular receptor.[77,212] Another 10 immunotypes (1A, 1B, 2, 29–31, 44, 47, 49, 62) use another unknown receptor, while type 87 requires sialic acid for attachment. ICAM-1 binds into the depression on the viral surface, a site that is inaccessible to antibody.[172] Viral attachment to cellular receptor can be blocked when an immunoglobulin G (IgG) molecule binds to the surface of the virus in a position that spans the canyon.[208] ICAM-1 expression in fibroblasts is induced by some cytokines and inhibited by others.[182]

4.3. Antigenic Characteristics

Rhinoviruses in their native state contain type-specific surface antigens (Table 1). By means of atomic resolution,[194] four previously recognized neutralizing immunogenic regions[202] have been identified as external protrusions on the viral shell. On the basis of collaborative programs, rhinoviruses have been classified as serotypes 1–100 and subtype 1A.[97,131,132] Using antisera for types 1–89, it was possible to identify over 90% of wild rhinovirus strains recovered in three epidemiologic studies.[97,159] This suggests that most rhinovirus immunotypes, at least those currently circulating in the United States, have now been identified and that new types are not continuously emerging.

The criterion for the selection of numbered prototype viruses was the absence of cross-neutralization with other prototype candidates using animal hyperimmune antiserum at dilutions of 1 : 2–20 in a standard N test. There was a virtual absence of cross-reactions with the antisera that were used in the numbering program. Recent work with high-titered hyperimmune antisera, discussed below, has disclosed antigenic relationships among some of the numbered types that were not discovered in the collabora-

tive program. Despite these findings, which are discussed in the next paragraph, the large number of antigenically different types of rhinoviruses is undoubtedly an important characteristic of the group, influencing epidemiologic behavior and accounting for the frequency of rhinovirus colds.

In an early study, antigenic relationships among different rhinovirus types were reported, using hyperimmune bovine antisera in N tests.[65] The bovine antisera were later recognized to contain anticellular antibody. When this antibody was removed, the antigenic cross-reactions largely disappeared.[30] More recently, potent monotypic animal antisera were used to demonstrate both reciprocal and one-way cross-reactions among numbered rhinovirus types studied.[35,198] The cross-reactions were usually minor. A number of these relationships were indirect and were demonstrable only by primary immunization with one rhinovirus type followed by immunization with a different but related type.

The importance of cross-reactions in immunity in humans is currently unknown, and the results of work in this area are contradictory. Neutralization tests carried out with paired sear from patients have usually not shown significant cross-reactions following natural rhinovirus infections.[102] On the other hand, in a study of experimental infections in volunteers, heterotypic antibody responses were relatively common after infection with some types.[67]

The native antigenicity of rhinoviruses can be altered by experimental means. Treatment at pH 5 at 56°C or in 2 M urea produces virus particles that react in immunodiffusion and CF tests with heterologous types.[146] When the virus is in this C-antigenic state, which results from a configurational change that exposes normally hidden determinants, it is unable to attach to cell receptors. This alteration in antigenicity, which also occurs after virus attachment to host cells, may be an important step in the initiation of infection[145] but probably plays no role in immunity to infection.

5. Descriptive Epidemiology

5.1. Incidence and Prevalence of Infection

5.1.1. Age- and Sex-Specific Infection and Illness Rates. Rhinovirus infections are the most common of the acute respiratory infections[32,90] and probably the most common of all acute infections of humans. Infection rates based on virus isolations from routine specimens from family members in Seattle with and without symptoms were 0.59 per person-year (Table 2). Rates in this population ranged from 1.21 in the 0 to 1-year age group to 0.20 in mothers; values were intermediate in children 2–9 years of age. Similar data collected from medical students in Chicago[101] and insurance company employees in Charlottesville[87,90] gave rhinovirus infection rates of 0.74 and 0.77 per person-year, respectively.

Table 2. Rhinovirus Infection Rates: Calculated from Surveillance and Sampling of All Persons—Well and Ill

Location	Age (yr)	Person-year of observation	Infections per person-year
Seattle, Washington[32]	0–1	144	1.21
	2–5	135	0.54
	6–9	22	0.55
	Mothers	208	0.20
	All ages	510	0.59
Chicago, Illinois[101]	19–32	466	0.74[a]
Charlottesville, Virginia[87]	16–45	500	0.77[b]

[a]Rhinovirus isolation percentages for well and ill persons 1.5 and 25.4%, respectively; sampling interval of well persons 6 weeks; data collected over four periods of 9 months and adjusted to annual rates.
[b]Rhinovirus isolation percentages for well and ill persons 2.1 and 23.3%, respectively; sampling interval of well persons arbitrarily adjusted to 6 weeks; data collected over 1 year.

True rhinovirus infection rates are probably higher than reported, since currently available rhinovirus culture methods lack optimal sensitivity (see Section 3). The overall rhinovirus infection rates of 0.74 and 0.77 per person-year in Chicago and Charlottesville, respectively, are probably minimum values for the true incidence of rhinovirus infections in young adults. Adjustment of the Seattle rates for children to those measured for young adults in Chicago and Charlottesville gives projected rhinovirus infection rates in young children of up to 1.5 per person-year. Of particular interest was the increase in incidence of rhinovirus infections in females 20–39 years in the Michigan population[166] and 16–24 in the Charlottesville population. These findings may relate to the importance of young children in disseminating rhinovirus in the home, particularly to mothers. This is discussed in Section 5.2.1.

Rhinovirus illness rates have been measured in long-term studies of families and insurance company workers. The estimated incidence of rhinovirus respiratory illness in the Tecumseh, Michigan, study for all ages was 0.83 per person per year.[166] The annual incidence in different age groups based on actual viral isolation results ranged from 0.59 in 0- to 4-year-olds to 0.09 in persons over 40 years of age (Table 3). Data collected from the insurance company population of young adults yielded a rhinovirus illness

Table 3. Rhinovirus Illness Rates: Calculated from Surveillance and Sampling of Persons with Colds

Location	Age (yr)	Person-years of observation	Number of respiratory illnesses per person-year	Number of rhinovirus illnesses per person-year
Tecumseh, Michigan[166]	0–4	539	4.9	0.59
	5–19	1541	2.8	0.13
	20–39	1523	2.2	0.21
	40+	1757	1.6	0.09
Charlottesville, Virginia[87,90]	Males			
	16–24	240	2.2	0.51[a]
	25–34	204	2.1	0.50
	35–44	111	2.3	0.54
	45+	24	2.2	0.51
	All males	579	2.2	0.51
	Females			
	16–24	477	2.6	0.60
	25–34	237	2.1	0.49
	34–44	84	2.1	0.49
	45+	24	1.3	0.31
	All females	822	2.4	0.55
	All persons	1401	2.3	0.53

[a]All rates calculated using rhinovirus isolation percentage of 23.3% (observed: 22.9% in males, 23.6% in females).

rate of 0.53.[87] Rates for males and females derived from this study were 0.51 and 0.55, respectively. The higher rate in females reflected a greater incidence of total colds in females and not an increased incidence of rhinovirus recovery from females, since the rhinovirus isolation percentages from males and females were not different. The reason for the differences in rhinovirus illness rates in these studies is not clear but may relate to variables such as the methods of surveillance, criteria used in counting colds, and varying sensitivities of the cell cultures used for virus recovery.

5.1.2. Prevalence of Antibody and Geographic Distribution. Studies of the prevalence of rhinovirus antibody support the conclusion that rhinovirus infections begin in early childhood and continue into adult life (Fig. 1). Antibody to the various rhinovirus types begins to appear at a early age and increases in prevalence throughout childhood and adolescence.[99,163,218,224] The prevalence of antibody reaches a peak in young adults (mean percentage positive: 50%), probably reflecting the effect of exposure to young children in the home.[98] Antibody prevalence then declines to a slightly lower level that persists throughout adulthood. Studies of antibody in sera collected serially from the same person show persistence of antibody at relatively stable levels for years.[218] The mechanisms by which rhinovirus serum antibody levels persist are unknown and could include inherent stability of antibody formed initially, recurrent antigenic stimulation from infection with the same or related types, or both. The slight decrease in prevalence of antibody after the early adult peak (Fig. 1) suggests that a decline in antibody occurs when viral exposure is lessened. Limited work has also shown that artificially induced N antibody in nasal secretions may persist for at least 330 days following intranasal vaccination.[17]

Information is also available on the prevalence in adults of serum N antibody to each of the different serotypes, 1A–55. In the groups studied, antibody was present in all the types tested (Fig. 2).[98] The prevalence of antibody ranged from a low of approximately 10% to a high of approximately 80%, and there was no sharp dividing point between types associated with high and low antibody prevalence.

Studies of rhinovirus-antibody prevalence in specimens from many different parts of the world have shown that rhinoviruses have a worldwide distribution.[219] Broadly speaking, there were differences in prevalence of antibody among countries for any particular virus tested. Rhinovirus-antibody prevalence in tropical areas is equal to or greater than that in the temperate zone.

5.1.3. Seasonal Distribution of Infections. In an early epidemiologic study of acute respiratory disease in which virological methods were not available. Frost and Gover[72] noted that "during the season of high preva-

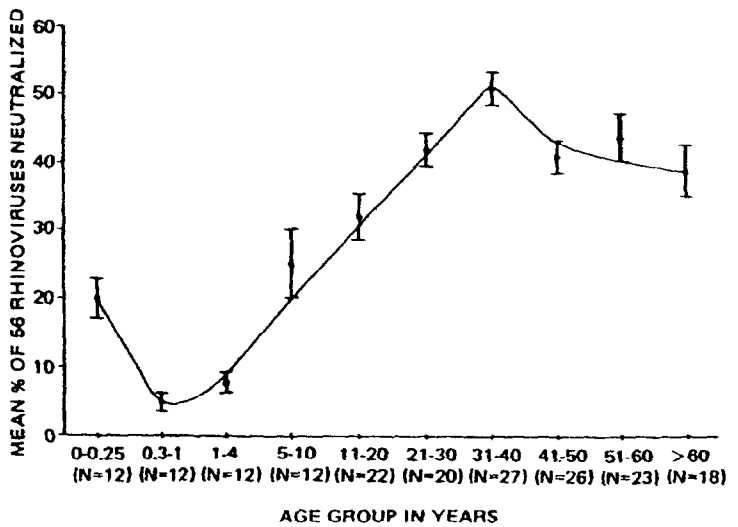

Figure 1. Distribution of N antibody in human sera according to age. A total of 184 sera were tested at 1:4 dilutions versus rhinovirus types 1A–55. The vertical brackets represent the S.E.M. With permission of Hamparian *et al.*[98]

lence, from September to March, inclusive, the incidence curve [for colds] in each locality exhibited a series of oscillations, constituting a succession of epidemics, each of several weeks' duration, rather irregular in sequence and magnitude, but clearly not attributable to mere chance fluctuation." The data from this study showed that one of the recurrent epidemic peaks of colds occurred in the early fall, usually in September. Later, in the Cleveland family study of minor illness, a September peak of colds was a prominent feature of the seasonal pattern of illness, although no respiratory viruses could be associated with this period.[48] Studies using virus cultures have now shown that rhinoviruses account for a major part of this early fall outbreak of colds that annually initiates the respiratory disease season (Fig. 3),[87] although this has not been observed in all locations.[166] In adults with colds in the eastern United States, rhinovirus infection rates reached their highest annual point (3.5 illnesses/1000 per day, 1.28 per person-year) in September. At this time, rhinoviruses accounted for approximately 40% of all colds and greater than 90% of diagnosed colds. Rhinovirus infection rates fell and remained low (1–1.5/1000 per day) in the winter and early spring. A second peak of rhinovirus illness occurred in April and May. Although total respiratory

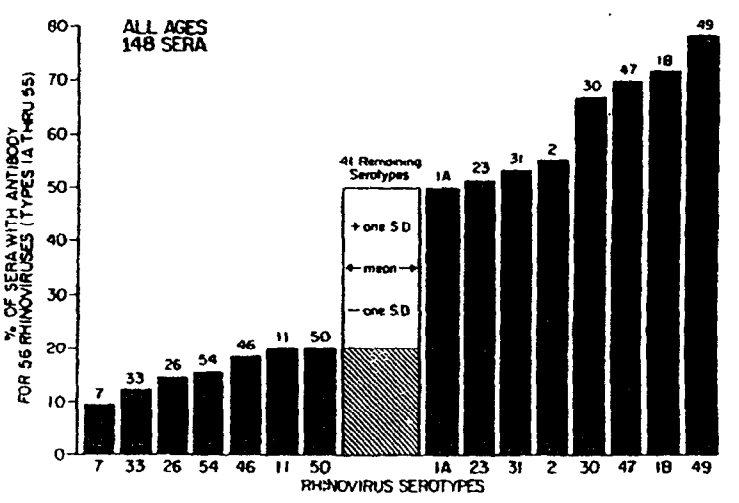

Figure 2. Percentages of human sera with N antibody to rhinovirus types 1A–55. A total of 148 sera were tested at a 1:4 dilution. With permission of Hamparian *et al.*[98]

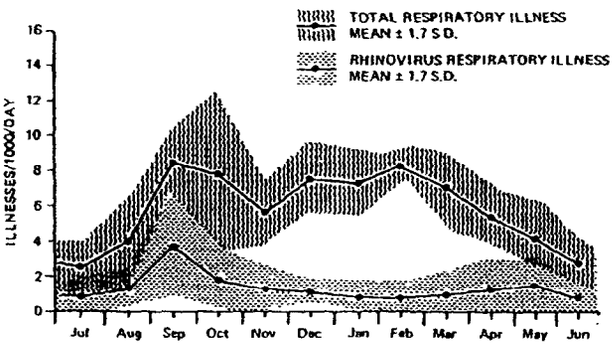

Figure 3. Total and rhinovirus respiratory illness rates (± 1.7 S.D.) in young adults. Data collected over a 7-year period (1963–1969). Adapted from Gwaltney *et al.*[87]

rates were falling in the spring, rhinoviruses were associated with a substantial fraction of all colds during that time. Throughout the summer, rhinovirus infections continued to account for an important part of all colds, although respiratory illness rates reach their lowest point at this time.

In the tropics, the respiratory disease season coincides with the rainy season, beginning in May and June and ending in November and December.[164] Rhinovirus infections were most prevalent during the rainy season in Panama.[163] In the continuously humid climate of Fortaleza, Brazil, rhinoviruses were recovered from 17% of young children with colds, and over a 2-year period, the prevalence of infection appeared to show an inexact correlation with the amount of rainfall.[7] In arctic locations; where the respiratory disease season coincides with cold weather as in temperate climates, rhinovirus outbreaks have been observed, but precise patterns have not been studied.[231]

Although there is a well-established correlation between the lowered temperatures during the fall, winter, and spring months and the increased occurrence of acute respiratory disease during that period,[125] there is no evidence to support a direct causal relationship between thermal cold and increased rates of infection.[81] As to meteorological effects that specifically influence rhinovirus infections, a thorough study of weather and colds showed that none of nine weather variables including temperature had a distribution remotely resembling the autumn (presumed rhinovirus) peak of colds.[144] This is in keeping with the observations from two long-term studies of rhinovirus infections in which prominent September peaks of rhinovirus colds were associated with mild seasonal fall weather and not with the more severe cold of winter that requires heating of homes.[81,162] More direct evidence on this question comes from a volunteer study with rhinovirus type 15 in which exposure to thermal cold showed no adverse effect on susceptibility to experimental infection or severity of illness.[58]

The reason for seasonal variation in the incidence of colds remains a mystery.[81] Speculations include the idea that cold weather, like rain in the tropics, leads to crowding indoors, thus providing better conditions for virus spread.[126] Also, school openings in the fall bring together into large groups a segment of the population susceptible to rhinoviruses and other respiratory viruses. There has also been speculation on the effect of weather changes on virus survival and infectivity. Changes in humidity have been shown to influence the survival of respiratory viruses.[14] Rhinovirus survives best at relative humidities of over 55%. In temperate areas of the United States, such as Charlottesville, Virginia, indoor relative humidity remains in the favorable range for rhinovirus survival from April through October, which is the period of highest rhinovirus prevalence.[81]

5.1.4. Distribution of Immunotypes. A tally of rhinovirus immunotypes in the United States based on published studies revealed wide dispersal of most types throughout the country.[100] Of the first 55 numbered types, only type 5, a virus first isolated in England, had not been recovered in the United States. The serological survey cited earlier[98] showed antibody to type 5 virus in sera from United States populations. Thus, the conclusion that rhinovirus types are widely distributed throughout the United States and the world is supported by both virus-isolation and serological data.

The current impression, based on longitudinal studies, is that multiple types circulate in a geographic area at any given time with no discernible pattern to their appearance or reappearance.[89,100] Over several years, some types were endemic, whereas others appeared only once or twice. It has been proposed that certain rhinovirus types

might possess a higher degree of infectivity than others, increasing their importance as a cause of colds and making them prime candidates for inclusion in vaccines.[161] Analysis of the frequency of isolation of the various rhinovirus types, however, does not show a sharp division between "common" and "uncommon" types. Also, types most commonly encountered in one study have not necessarily been the same as those in other studies. From the analysis of combined data from several studies, it was not possible to designate a particular year as a nationwide epidemic year for a particular type, nor was it possible to detect pathways of rhinovirus transmission by type across the country.[100]

Long-term studies have shown a gradual change with time in the overall distribution of immunotypes in a given geographic location.[21,69] Immunotypes with lower numbers, which in general were discovered earlier, have been replaced by higher-numbered, "newer" types and by strains that could not be typed with available antisera. The reason for the shift in types in a given area over time appears to be the large number of stable immunotypes in existence and not the rapid emergence of new types of rhinovirus.[97]

5.2. Occurrence in Different Settings

5.2.1. Family. A major site for rhinovirus spread in civilian populations is the home.[46,116,158] The characteristic epidemiologic pattern in this setting is for a schoolchild or child in day care to introduce virus into the home, after which transmission occurs to other members of the family (Fig. 4). Secondary infections are most common in young children and mothers, but all members of the household including fathers, other adults working outside the home, and adolescents are affected. Intervals of 2–5 days are commonly seen between onsets of cases.

In one study, total respiratory illness rates were highest in preschool children.[116] Rates in housewives were similar to those in schoolchildren and were consistently higher than rates in adults working outside the home. During the height of the epidemic, the frequency of rhinovirus infection as determined by culture and serology was similar in all age groups. Later, in October, total illness rates were seen to decline in adults and older children, while young children continued to have frequent colds for which no etiology could be established. The presence of children in the home was associated with total respiratory illness infection rates for adults that were higher than for adults who did not have this exposure. At the height of a September peak of illness, rhinovirus respiratory illness rates for all family members, adults and children, were approximately 8/1000 per day (2.92 per person-year), calculated on the basis of rhinovirus causing 40% of fall colds.

In one family study, the rhinovirus attack rates for two epidemic types were 25 and 50%,[46] and the attack rate for type 16 in an outbreak in families in a small Alaskan community was nearly 70%.[231] In another study, the secondary attack rates for members of families into which a rhinovirus had been introduced were inversely proportional to preexposure serum antibody levels: 71, 50, and 21% of persons with titers of ≤2, 4, and 8–32, respectively, were infected.[114,116] Based on the results of a study of colds in the tropics,[158] the secondary attack rate with type 39 was calculated to be 56% in antibody-free persons.[116]

5.2.2. Schools. A key study has shown that rhinoviruses spread efficiently among children in nursery

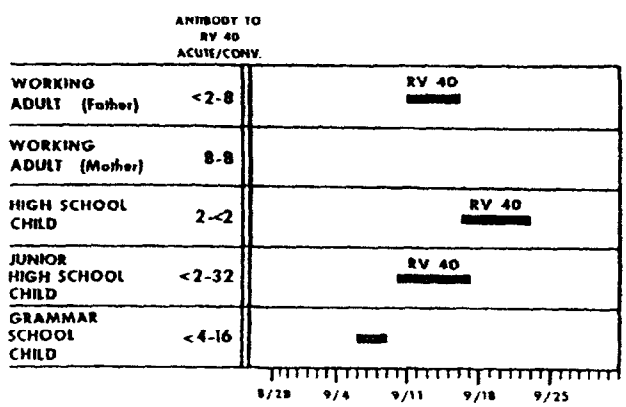

Figure 4. A family outbreak of colds caused by rhinovirus type 40. (■) Periods of symptomatic illness; (RV 40) positive virus culture. The diagnosis of rhinovirus infection in the index case (grammar-school child) was made by serology. Adapted from Hendley *et al.*[116]

school,[9] thus establishing transmission in school as an important step in rhinovirus dissemination in civilian populations. Spread of some types in the schoolroom was extensive, involving up to 77% of children. However, half the serotypes introduced into the groups showed no evidence of spread. The reason for the lack of spread of some types is unknown, but the authors concluded that it was not related to characteristics of the associated illness, patterns of virus shedding, or levels of immunity. Spread was most pronounced during March and April, a recognized time of increased prevalence of rhinovirus colds. The study unfortunately did not extend through the September peak of rhinovirus infections.

Rhinovirus activity has been observed in dayschool groups at various grade levels[176] and in boarding school, university, and medical school populations.[91,101,134,156,181] Rhinoviruses are a prominent cause of morbidity in these groups, although information on their specific epidemiologic behavior is not available. Presumably, spread in older children, adolescents, and young adults who are part of closed populations such as boarding schools occurs among roommates, friends, members of athletic teams, and the like.

5.2.3. Military. Rhinoviruses account for a large amount of the morbidity associated with upper respiratory tract infections in military populations.[68,128,156] In a prospective study of Navy and Marine recruits, 90% of the men developed rhinovirus infection during a 28-day period in basic training, giving an attack rate for this period of 11.7 per person-year![191] Of these infections, 75% occurred within the first 2 weeks of training, and simultaneous or closely spaced infections with two different serotypes in the same man were common. The epidemiologic behavior of the numbered rhinoviruses in military populations is generally similar to that in civilians, showing a constantly changing mosaic of different types.[168]

6. Mechanisms and Routes of Transmission

Although considerable work has been done on the question, the exact mechanism by which rhinoviruses are passed from person to person is unknown.[115] As discussed above, children are the most important reservoir of the virus, and home and school are the places where spread most often occurs. In volunteer experiments, close personal contact appears to be necessary for virus to spread efficiently from an infected to a susceptible subject.[42,121] These facts alone suggest that spread is most often by some type of short-range exposure to infectious secretions. Information on the various steps in the sequence of transmission is best evaluated in relation to the question of spread by direct manual contact with infectious secretions versus spread by contact with virus in contaminated aerosols of large or small particle sizes.[85]

Virus shedding, the first step in the sequence, occurs primarily from the nose. Under experimental conditions, the amount of rhinovirus in the nasopharyngeal washes of volunteers peaked (832 $TCID_{50}$/ml) on the third day after inoculation and then fell to low levels that persisted for up to 2 weeks.[54] Some volunteers showed a different pattern of nasal shedding characterized by delayed onset and slow buildup over 7 days to relatively low maximum virus concentrations (41 $TCID_{50}$/ml). Comparisons of rhinovirus concentrations in respiratory secretions from subjects with natural colds have shown that the quantity of virus in nasal mucus tends to be 10- to 100-fold greater than in pharyngeal secretions.[117] Also, virus was present only 50% of the time in saliva, where it was found in low concentrations. In keeping with the relative scarcity of rhinovirus in saliva was the finding that virus was infrequently recovered from simulated coughs and sneezes.

The relatively poor yield of virus in saliva can be interpreted as evidence against spread through the air, since aerosols produced by coughing and sneezing are mainly of oral origin, coming primarily from the pool of saliva in the anterior part of the mouth.[12,127] On the other hand, the idea of nasal mucus as a direct source of transmissible virus is appealing because of the relatively high titers of virus in mucus and the great potential for people with colds to contaminate the environment, including fingers, with this substance. Rhinovirus has been recovered from the hands of 40–90% of adults with natural[117] and experimental colds[43,92,187] and from 6 and 15% of selected objects in the environment of persons with experimental and natural colds, respectively.[92,187] Information obtained on the second step in transmission, virus survival in the environment, indicates that rhinovirus in concentrations found in nasal mucus survives regularly for up to 3 hr on skin and a variety of surfaces such as wood, plastic, steel, Formica, and hard fabrics.[117]

Evidence in favor of spread through the air comes from experiments in which biological tracers, the spores of *Bacillus mycoides*, were placed in the nose. These experiments showed that blowing the nose and especially sneezing could produce droplets containing the tracer that were small enough to remain airborne and yet in the size range (3–16 μm) that is likely to be trapped in the nose.[15] Rhinovirus survival in aerosol is enhanced by low temperature and high humidity.[120]

Whatever the method of transfer, virus must reach an appropriate portal of entry to complete the sequence of

events leading to successful spread. Under experimental conditions, small quantities of rhinovirus (the HID_{50} equivalent to 0.032–0.75 $TCID_{50}$) placed in the nose in coarse drops will efficiently initiate infection.[51] There is indirect evidence that similar small amounts of virus may initiate infection under natural conditions.[114] Experimental infections have been produced by the inhalation of rhinovirus aerosols with particle sizes in the true droplet nuclei range (0.3–2.5 μm) but require approximately 20-fold greater concentrations of virus than intranasal challenge. Thus, it appears that the nasal mucosa is more susceptible to rhinovirus than is the lower respiratory tract.[38] In this experiment, it was not possible to exclude the possibility that infection resulted from the fraction of the viral aerosol that was deposited in the nose rather than that reaching the lower respiratory tract. Experimental rhinovirus colds have also been produced by dropping small amounts of virus on the conjunctiva,[20,117] indicating that the eye may be another portal of entry for rhinovirus. In contrast, rhinovirus placed in the mouth does not readily initiate infection.[117] In related experiments in which infected and susceptible volunteers kissed under controlled conditions, oral contact was an inefficient method of causing spread.[180]

From the results of the work cited above, it appears that rhinovirus must reach the nasal mucosa for efficient initiation of infection. Observations carried out on adults at medical conferences and in Sunday school show that normal behavior includes placing fingers into the nose or onto the conjunctiva with regularity.[117] Episodes in which finger contact with nasal and conjunctival mucosa occurred were measured on the average of two per 3 person-hours of observation. This type of behavior provides sufficient opportunity for accidental self-inoculation if the fingers are contaminated with virus. The alternative method of spread, transmission via airborne particles with deposition in the respiratory tract, is also feasible. The average adult is effectively exposed by inhalation to large amounts (approximately 10 liters/min) or air; thus, small concentrations of virus in the air may be sufficient to transmit infection.

Indirect evidence on the relative importance of these different methods of spread under natural conditions has been obtained in studies of experimental infections. In one study, airborne transmission of rhinovirus did not occur across a wire mesh barrier from infected to susceptible volunteers in closed barracks.[51] In another, infected volunteers who engaged in singing and other activity designed to create infectious aerosols failed to spread rhinovirus to susceptible subjects confined in the same closed room.[42] More recently, transmission models have been developed for the hand contact/self-inoculation and aerosol routes of rhinovirus transmission. The hand contact model was shown to be quite efficient in one study, with 11 of 15 volunteers infected after brief hand-to-hand contact compared to 1 of 12 infected after exposure by large-particle and none of 10 after small-particle aerosol.[92] The hand contact model has been used to determine the usefulness of viricidal hand treatment,[93] an environmental disinfectant,[86] and viricidal nasal tissues.[112]

Another transmission model, based on an antarctic hut setting has accomplished experimental rhinovirus transmission by aerosol.[151] In this model in which elbow restraints were used to prevent finger-to-nose contact,[47] a linear relation was observed between transmission rates and the number of hours of exposure between donors and recipients. A large pool of coughing donors and a long period of exposure is required for transmission to occur with this model.

While the transmission models allow speculation about what might occur under natural conditions, they cannot provide definitive answers to that question. To discover the natural routes of rhinovirus transmission, the performance of selected intervention methods must be tested in the natural setting (Table 4).[85] Two such intervention studies in a natural setting have addressed the hand route of rhinovirus transmission. In one, contact prophylaxis with a viricidal hand treatment was associate with a 60% reduction in total colds and the elimination of rhinovirus colds in the treated group.[115] In the other study, a programmed reduction in the self-inoculatory behavior of young children was associated with a 45% reduction in the incidence of asthmatic attacks and a 47% reduction in the laboratory-confirmed respiratory virus infection rate.[37] No attempts to interrupt rhinovirus transmission in a natural setting have been reported using methods that would block aerosol spread.

Two studies of contact prophylaxis with natural in-

Table 4. Postulates to Test a Hypothesis of Microbial Transmission

1. Infectious microorganism must be produced in infected host at proposed anatomic source.
2. It must be present in secretions or tissues that are shed from host by proposed route.
3. It must be present and survive in or on the appropriate environmental substance or object.
4. The contaminated environmental substance or object must reach the proposed portal of entry.
5. Interruption of transmission by the hypothesized route must prevent spread of infection under natural conditions.

terferon, while not designed to address transmission routes, nevertheless provide useful insight into the question.[60,104] In these studies, interferon was applied topically into the nose and was associated with marked reduction in the natural rhinovirus infection rate, implicating either finger-to-nose and/or large-particle aerosol as the natural routes of spread. Small-particle aerosols reach the lower airway and lungs, and thus the intranasal instillation of interferon would not be expected to prevent infection at these sites. Thus, in summary, a limited amount of direct evidence suggests that rhinovirus is transmitted by direct hand contact or by a combination of this route and large particle aerosol.

7. Pathogenesis

The incubation period of experimental rhinovirus colds is 16 to 24 hr,[171] but in some cases may extend for up to several days.[51,54] Virus may be recovered from nasal pharyngeal washes in small amounts by 24 hr after inoculation. Virus concentrations then rise rapidly to peak values on days 2 and 3. Maximal virus shedding is followed within 24 hr by the release of large quantities of protein from the mucous membrane.

The virus' ability to evade mucociliary clearance and other nonimmunologic defenses of the nasal passages appears to be important in the initiation of infection. Thus, small inocula of virus placed into the nasal passages of nonimmune persons routinely lead to infection.[51,81,216] In a study employing serial brush biopsies of selected sites in the upper airway, point inoculation of the nasal passage with rhinovirus by way of one tear duct was followed by transport of virus to the posterior nasopharynx and initiation of infection at that site.[230] Infection remained localized in the nasopharynx in some patients but usually spread forward to one or both nasal cavities over several days. Viral shedding persisted for up to 3 weeks. After an experimental challenge, maximum clinical illness usually occurs during the first 4 days of infection.

Infection of the nasal cavity with rhinovirus produces little or no detectable damage to the nasal epithelium as determined by histological examination of nasal biopsies (Fig. 5),[52,229] although occasional ciliated epi-

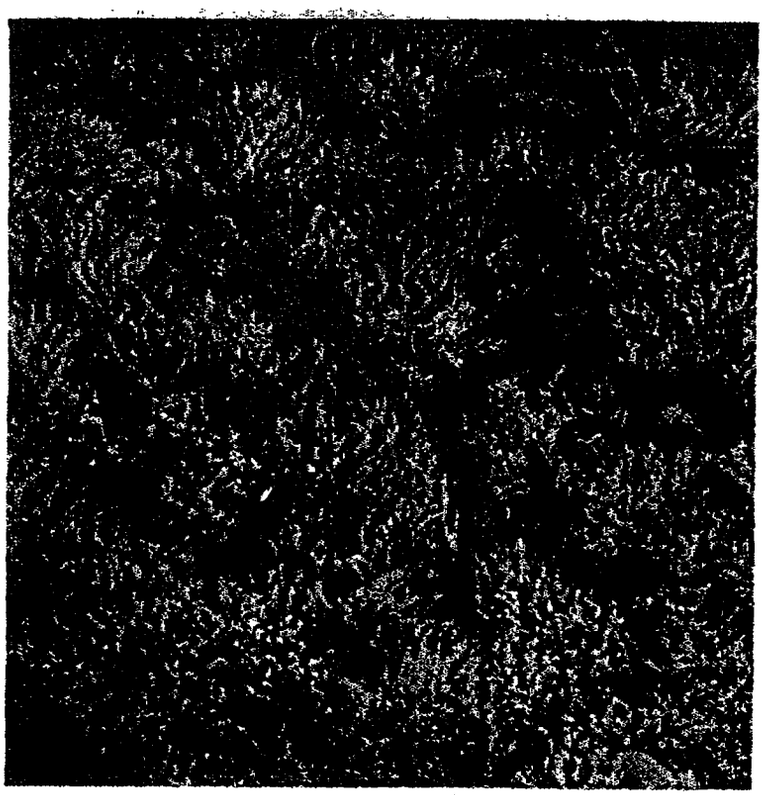

Figure 5. Scanning electron photomicrograph of a nasal biopsy from a volunteer with an experimental rhinovirus cold, showing no evidence of cellular damage.

thelial cells containing rhinovirus antigen have been found in nasal secretions of infected volunteers,[223] and nasal mucociliary flow rates are decreased.[197] This led to the suggestion that the viral infection acts primarily to trigger inflammatory responses by the host, which, in turn, lead to the symptomatic illness.[216,223]

The role of various mediators and neurogenic reflexes in the pathogenesis of rhinovirus colds is now being studied. This is being approached by measuring mediator concentrations in respiratory secretions of persons with colds, by blocking mediator activity with specific compounds, and by challenging volunteers with mediators instilled into the upper airway. Using these approaches, several mediators have been associated with symptom occurrence in rhinovirus colds, including bradykinin and lysyl-bradykinin,[61,171] prostaglandin,[61,210] histamine,[61,62,73] and interleukin-1 (J. M. Gwaltney and D. Proud, personal communication). Also, parasympathetic[74] and alpha adrenergic[211] pathways have been implicated in rhinovirus pathogenesis.

In addition to the nasal cavity, rhinovirus colds also affect the lower airway, the middle ear, and the paranasal sinuses. In reports on children with wheezing[124] and adults with chronic bronchitis or asthma,[141] rhinovirus was recovered more often from sputum than from the nose or throat, suggesting that viral replication was occurring in the lower respiratory tract. A study using a sampling device designed to minimize upper airway contamination of specimens suggested that rhinovirus replication was occurring in the large airways of volunteers with experimental rhinovirus colds,[95] although it was not entirely possible to exclude the possibility that the specimens had been contaminated by upper airway secretions. There still remains no direct evidence on the question as might be obtained by transtracheal aspiration or lung puncture.

Rhinovirus infections have been implicated as an important precipitant of asthmatic attacks in children.[119,123,124,148,153,154] The mechanism for this is unknown, but a decrease in granulocytic β-adrenergic and H_2 histamine receptor responses has been observed in volunteers with peripheral airway obstruction associated with experimental rhinovirus infection.[18] In another study, 4 of 19 young adults with mild to moderate asthma had decreases in FEV_1 and increases in histamine sensitivity during experimental rhinovirus infection.[94]

Rhinovirus infections have also been associated with periods of acute exacerbation in patients with chronic bronchitis.[63,149,213] A decline in pulmonary function has been observed in patients with chronic obstructive pulmonary disease in association with rhinovirus infection.[207] However, the abnormalities have been mild and transient. Similar changes in pulmonary function have been seen in cigarette smokers[71] and healthy adults[10,26] with rhinovirus infection. The mechanisms by which rhinovirus infection might alter pulmonary function are unknown. Direct invasion of the lower respiratory tract by the virus is a possibility, but reflex mechanisms or secondary bacterial infection might also play a role.

In addition to asthma and chronic bronchitis, there have been multiple reports of rhinovirus infection in patients, especially children, with other diseases of the lower respiratory tract.[29,39,75,179] The possibility cannot be excluded that concurrent infection with other viral or bacterial pathogens may have been present and caused the illness seen in some cases. The opinion of several workers in the field has been that rhinoviruses are not an important cause of viral pneumonia, croup, and bronchiolitis in children.[11,76,169,184,231]

Rhinovirus colds have been associated with the frequent development of abnormalities in eustachian tube function and middle ear pressures in young adults with experimental rhinovirus colds.[147] Also, rhinovirus has been recovered from middle ear aspirates of patients with acute otitis media.[5,80] These findings support the clinical and epidemiologic impression that colds have a major role in the pathogenesis of otitis media. Rhinovirus infections also have recently been shown to cause abnormalities in the paranasal sinuses. In one study, a third of young adults with experimental rhinovirus colds had acute abnormalities of the sinus cavities detected by magnetic resonance imaging.[222]

8. Immunity

Work on the immunology of rhinovirus infections has focused on humoral immunity, particularly the role of antibody in respiratory secretions. Serum N antibody titers rise in up to 75–80% of persons with natural or experimental rhinovirus colds[23,88,90,116]; once present, antibody in serum is well maintained.[218] The level of naturally acquired serum N antibody prior to natural or experimental challenge is inversely proportional to the subsequent infection rate. Under conditions of exposure to rhinovirus in the home, naturally acquired serum antibody at a level of 8 was associated with a sharp reduction in the infection rate, and serum antibody levels of ≥ 16 were associated with solid immunity.[116] With artificial challenge, it is possible to infect, although at a reduced rate, volunteers who have higher titers of naturally acquired serum antibody. In one study using relatively small challenge doses of virus (0.05–50 $TCID_{50}$), no infections

occurred in volunteers with prechallenge titers of 64 or higher.[114] In other studies in which the infecting inocula contained more virus (17–10,000 $TCID_{50}$), infections were observed in volunteers with prechallenge titers of up to 512, presumably as a result of the overwhelming of normal immunity by an artificially large virus challenge.[27,170]

The findings cited above do not necessarily indicate that serum N antibody is the primary immune mechanism responsible for resistance to rhinovirus, since naturally acquired serum antibody is found in close association with antibody in nasal secretions.[171,178] The ratio of nasal secretion to serum N antibody after recent infection (approximately 1:2) appears to be higher than that after remote infection (approximately 1:16), suggesting a decline in nasal secretion antibody with time.[27] Actual measurements of nasal immunoglobulin concentrations have confirmed that significant falls in titers did occur over a 5-month period after infection.[121]

Attempts have been made to determine the relative importance and specific roles of serum and nasal secretion antibody in protection against rhinovirus infection. Naturally acquired antibody in nasal wash specimens and serum was associated with resistance to "infection" if present in sufficient titer before artificial challenge, but it did not appear to modify "illness" or virus shedding.[27] Because of the close association of naturally acquired antibody in serum and nasal secretions, the findings of this study did not answer the questions posed. Another approach to the problem was to administer inactivated rhinovirus vaccine by either the parenteral or the intranasal route to elicit selectively nasal secretion or serum antibody or both. Vaccine given intranasally in large amounts led to the production of antibody in both serum and nasal secretions, whereas parenteral vaccination resulted primarily in serum-antibody production.[177,178] Intranasal challenge with rhinovirus at a later date resulted in the reduction of *illness* and virus shedding only in volunteers who received the intranasal vaccine.[17,177,178] In these studies, intranasal vaccination was not associated with a clear-cut reduction in *infection* rate determined by antibody response. Therefore, this work suggested that the primary effect of nasal antibody was to modify illness and reduce virus shedding. This conclusion is in conflict with that of the investigation cited above[27] and of other reports that have found that the major effect of humoral (serum) immunity was prevention of infection and not modification of illness.[54,114,116,170] Other studies have reported on finding an association between naturally acquired[87] and vaccine-induced[56] serum antibody and reduction of illness and, in the latter study, diminished virus shedding. Thus, currently available data from studies of the relative importance of nasal and serum antibody associated with immunity are not in complete agreement; further work is necessary to provide a clear understanding in this area.

Naturally acquired neutralizing activity against rhinovirus in serum has been found to sediment primarily in the 5–7 S region and to be associated with fractions containing immunoglobulin A (IgA) and IgG.[27,192] After recent experimentally induced rhinovirus infection or intranasal vaccination with inactivated rhinovirus vaccine, neutralizing activity has also been associated with 19 S IgM.[27,138,192]

Under normal conditions, nasal secretions contain 12 different identifiable proteins found in serum as well as six antigenic components not present in serum.[193] Secretory IgA, the most abundant protein in nasal secretions, is synthesized locally at sites adjacent to the mucosa and accounts for 30% or more of total protein in nasal secretions. Rhinovirus-neutralizing activity in nasal secretions is associated primarily with IgA in 9–11S fractions, although secretory IgA is not entirely homogenous in its sedimentation characteristics, being found also in 7 and 19 S regions.[138] The symptomatic period of rhinovirus illness is associated with considerable transudation of serum proteins, including IgG, into nasal secretions.[19,193] After cessation of illness, the concentration of serum proteins in nasal secretions falls rapidly; at this time, the IgA concentration begins a progressive sustained increase. The IgA that appears during this period is not associated with an increase in specific neutralizing activity for the infecting virus. Specific N antibody first appears in nasal secretions and serum at approximately 2 weeks in volunteers lacking detectable antibody. Antibody concentrations increase most rapidly between the third and fourth weeks, by which time virus shedding is completed. Volunteers with preexisting serum antibody may show rises in nasal antibody titers by as early as day 7. Neutralizing antibody to rhinovirus has also been found in tears and parotid saliva, where it is associated with the IgA fraction.[59]

Because of the sequence of events described above, it is felt that recovery from rhinovirus infection and illness is not dependent on humoral immunity.[27] It has been shown that interferon is released into respiratory secretions during the course of experimental rhinovirus infection. This has led to the suggestion that in rhinovirus colds, as in other viral infections, interferon may have an important role in recovery.[24]

Limited work has been done on the role of cellular immunity in rhinovirus infection. Natural-killer-like cytotoxic cells were induced in peripheral blood mononuclear leukocytes incubated with rhinovirus.[142] Also, cross-

immunotype reactivity was elicited in murine lymphocytes from mice immunized with either of two rhinovirus types.[103]

9. Patterns of Host Response

9.1. Clinical Features

Rhinoviruses produce a typical common cold characterized by rhinorrhea, nasal obstruction, sneezing, pharyngeal discomfort, and cough. The medial length of natural illness in young adults is 7 days, with peak symptomatology occurring on the second and third days of illness.[88] Symptoms last up to 2 weeks in one fourth of cases and may be prolonged to 1 month, although secondary bacterial infection may play a role when this occurs. Volunteers with experimental rhinovirus colds have had an average (± SD) of 23 g (± 22) of nasal secretions over the first 5 days of illness.[174] The profile of rhinovirus illness can be distinguished from that of influenza by the relative severity of systemic complaints and cough that occur with influenza (Fig. 6). Rhinovirus colds differ from group A β-hemolytic streptococcal pharyngitis in having more nasal involvement and cough and less severe and prolonged pharyngeal discomfort. This information is unfortunately of limited value to the clinician. In the individual patient, it is impossible to distinguish, on clinical grounds, rhinovirus colds from those caused by other common respiratory viruses.

In children, rhinoviruses also produce the common cold syndrome.[11,190] Whether rhinoviruses cause more serious disease in children, such as viral pneumonia, croup, and bronchiolitis, is still not clear. As discussed in Section 7, the prevailing opinion is that rhinoviruses, unlike parainfluenza viruses and respiratory syncytial virus, do not commonly cause these diseases.

Cough is a prominent feature of rhinovirus colds in patients of all ages, indicating that involvement of the lower respiratory tract of some type does occur. The frequency and duration of cough are markedly increased in cigarette smokers, particularly females, with rhinovirus colds.[88] Also, it has been reported that up to 40% of exacerbations in patients with chronic bronchitis may be associated with rhinovirus infections.[63,141,149,213]

Rhinoviruses are among the respiratory viruses that precipitate asthmatic attacks in children.[119,148,154] They appear to play an especially important role in causing wheezing in older children.[123,124,154] Multiple serotypes have been implicated.[154] Also, asthmatic children have been found to experience a significantly greater number of viral respiratory infections, primarily caused by rhinoviruses, than do nonasthmatic controls.[152] These findings are of interest in view of an earlier report that volunteers with a history of allergy have enhanced susceptibility of experimental colds.[126]

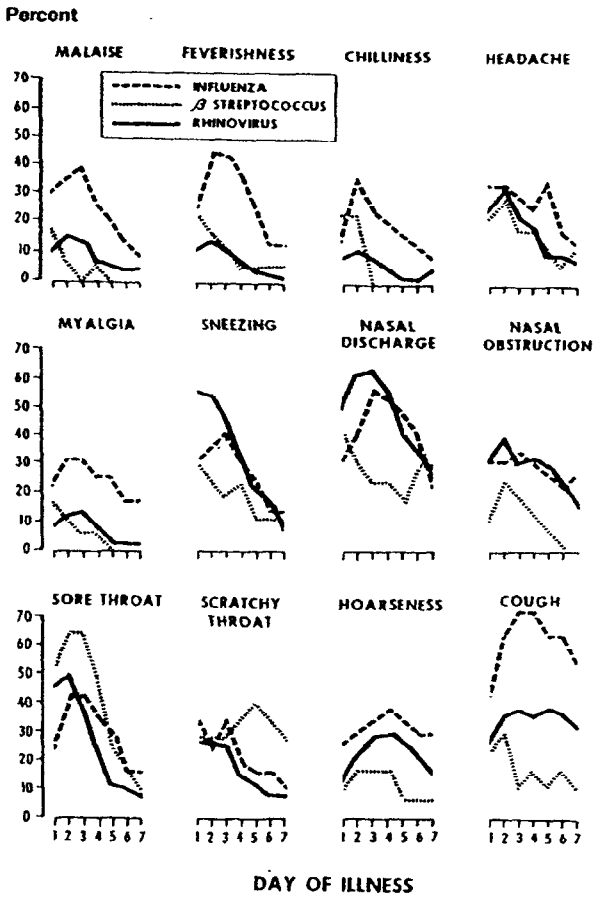

Figure 6. Comparison of symptom profiles of rhinovirus colds (139 cases), type A_2 influenza (33 cases), and group A β-hemolytic streptococcal pharyngitis (17 cases). Adapted from Gwaltney *et al.*[88]

During acute rhinovirus illness in volunteers, there is a modest increase in circulating neutrophils.[23] Later in the infection, moderate elevations in the erythrocyte sedimentation rate may occur. The diagnosis of rhinovirus infection is best accomplished by isolation of the virus from nose-and-throat-swab or nasal-wash specimens. There is currently limited availability of facilities for the laboratory diagnosis of rhinovirus infections in routine medical practice.[179]

9.2. Apparent–Inapparent Infection Ratios

Data based on virus isolations are available from several studies for calculating apparent–inapparent infection ratios for rhinoviruses. The results, which are in good agreement, indicate that the majority of rhinovirus infections are associated with symptomatic respiratory illness. The percentages of rhinovirus infections associated with illness were 63% in families,[136] 88% in medical students,[101,102] 69% in insurance company employees,[87] and 70–74% in miliary trainees.[128,168] Thus, the ratio of apparent to inapparent infections is approximately 3 : 1.

10. Control and Prevention

A vaccine approach to prevention of rhinovirus colds, a long sought after goal,[3,36,55,56,69,96,155,170,186,198,214] has not been successful. The antigenic diversity of the group has proved too much of an obstacle for currently available vaccine technology (Table 5). Other approaches that have been investigated include antiviral agents for prophylaxis and treatment, combined antiviral antimediator treatment, receptor blockade, and interruption of transmission.

Interferon was the first antiviral substance shown to provide effective prophylaxis against rhinovirus in humans.[150] Recombinant interferon-α_2 applied topically in the nasal cavity is a highly efficacious way of preventing both experimental and natural rhinovirus colds. The use of interferon in this way has reduced infection rates by up to 80% and clinical illness rates by 60–100% following experimental virus challenge.[107,196,200] In the natural setting, a strategy of contact prophylaxis for persons in the home exposed to family members with rhinovirus colds reduced rhinovirus illness rates by 80%.[60,104] This approach may prove to have practical value in clinical practice.

On the other hand, long-term administration of topical intranasal interferon does not appear feasible because of side effects including nasal dryness and stuffiness, blood-tinged nasal mucus, pinpoint bleeding sites, and occasional small ulcerations.[64,110,111] These side effects, which occur after 5 to 7 days of interferon administration, are reversible after discontinuation of the drug. Also, studies of the therapeutic effect of topical intranasal interferon given alone have not been promising. When administered as early as 28 hr after virus challenge, there has been an inconsistent effect on clinical illness and nasal mucus weights, although viral titers in nasal secretions were reduced.[108]

Control of rhinovirus infection by chemoprophylaxis and chemotherapy with compounds other than interferon has also been under investigation.[209] In early work, rhinoviruses were found to be susceptible to 2-(α-hydroxybenzyl)benzimidazole and related compounds that have specific actions on virus replication. Since then, a number of other compounds with activity against rhinoviruses *in vitro* have been discovered.[4,16,79,188,199,201,204,205,217] Most have not been effective in volunteers given experimental virus challenge.[106,188,203,221] Two members of a group of compounds called capsid binders, which bind to the hydrophobic pocket within the rhinovirus shell, have shown prophylactic but not therapeutic activity in volunteers with experimental infection.[1,105]

A new approach to treating rhinovirus colds is based on the idea that effective treatment requires simultaneously suppression of viral replication and blocking of the associated inflammatory events triggered by the infection. In volunteers with experimental rhinovirus colds, early treatment with topical intranasal interferon-α_{2b} and ipratropium combined with oral naproxen reduced the number of full-blown colds that developed in the treated subjects and significantly lowered symptom scores.[82] Another approach that has attracted interest is to block viral attachment with either monoclonal antibody to the cellular (ICAM-1) receptor or to treat with artificial soluble ICAM-1 to bind virus before it reaches natural receptor. One study in which monoclonal antibody was used prophylactically in experimental rhinovirus colds showed promising results.[109] No reports of human trials with soluble receptor have been published.

Interrupting viral spread also remains an area of interest. In one controlled, blinded study conducted under natural conditions in the home, the treatment of the fingers with a virucidal solution containing iodine reduced the

Table 5. Approaches to Control of Rhinovirus Colds

Vaccines
Not feasible with current technology
Chemoprophylaxis
Interferon-α_{2b} effective in experimental and natural colds
Capsid binders (R61837, R77975) effective in experimental colds
Chemotherapy
Combined antiviral antimediator treatment effective in experimental colds
Receptor blockade
Monoclonal antibody effective as prophylaxis in experimental cold
Soluble receptor, no reports of human testing
Interruption of transmission
Antiviral hand treatment and training to reduce self-inoculatory behavior effective as prophylaxis in natural colds

incidence of all colds by 60% and eliminated laboratory-proven rhinovirus colds.[115] However, the iodine solution is not practical for routine use, and other virucidal treatments for use on the hands that are effective, safe, and cosmetically acceptable have not been found for testing. In another study, training children to avoid self-inoculatory behavior resulted in a reduction in cold-associated asthmatic attacks and the laboratory-proven respiratory virus infection rate.[37]

11. Unresolved Problems

Although the atomic structure of rhinovirus is now known, much remains to be learned about how the virus interacts with its human host. Information is being obtained on the sites of infection, types of disease, and roles of mediators and neurogenic reflexes in pathogenesis but knowledge in these areas is limited. Also, the question of how rhinovirus colds are naturally transmitted has not been fully answered. Since the prospects for developing successful rhinovirus vaccines do not appear good, prevention of viral transmission may offer the best hope for an epidemiologic approach to control. Also, better therapy for rhinovirus colds may be possible through the development of combined antiviral antimediator treatment.

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Epidemiology and Control

FOURTH EDITION

completely revised and expanded

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RHINOVIRUS INFECTIONS IN AN INDUSTRIAL POPULATION*

I. The Occurrence of Illness

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CHARLOTTESVILLE, VIRGINIA

RHINOVIRUSES have been associated with 10 to 30 per cent of acute upper respiratory illnesses in several groups of adults.[1-6] In March, 1963, a study was undertaken to define the etiology and epidemiology of acute respiratory disease in a population of working adults, and the role of a number of viruses was studied initially. Coincident with the beginning of the investigation, improvements in technology occurred that facilitated the isolation of rhinoviruses and shifted emphasis to these agents. During the first three years of the study, the period considered in this report, approximately a quarter of the acute respiratory illnesses were caused by members of this large group of viruses. There was marked seasonal variation in the incidence of rhinovirus infection, with annual epidemics in the early fall being most striking.

METHODS

Population

The population consisted of all employees of the Eastern Regional Office of State Farm Mutual Insurance Companies located in Charlottesville, Virginia. As time progressed 2 small divisions of employees, such as maintenance men, were dropped because it was difficult to maintain satisfactory surveillance. The number under study ranged from 520 to 570 during the first twenty-four months, and from 320 to 350 thereafter. The major decrease in population was due to the assignment of an entire division to a new branch office in another city. Annual employee turnover for the three-year period varied from 20 to 30 per cent.

Eighty-three per cent of the employees were younger than thirty-five years of age; 59 per cent were females; 50 per cent had children, and 30 per cent had schoolchildren. Two thirds lived in Charlottesville; the remainder were scattered among 49 different neighboring towns or rural areas.

Environment

Approximately 70 per cent of the employees worked in one large, incompletely partitioned area. The rest were located in smaller groups in separate areas. Opportunities for employees to leave their immediate working area were so frequent that constant mingling occurred. During the year temperature in the building varied from 70 to 85°F., and the relative humidity from 30 to 70 per cent.

Surveillance

Basic. Employees were asked to record, for the appropriate day or days, the occurrence of a number of respiratory, gastrointestinal and general symptoms, as well as vacation periods and other times away from work. From the start of the study until April 19, 1964, recording was done on check sheets distributed at the beginning of each month. On April 20, 1964, these were replaced by prepunched IBM cards distributed every fourteen days with the employee's time card. Employees were directed not to record symptoms unless they were unusual for them. In this way it was hoped that entities such as allergic rhinitis and chronic bronchitis would not be reported as acute illness. Diagnostic classification

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was based on the recorded symptoms according to the following criteria:

General. A set of symptoms was counted as a separate illness when the interval between symptoms was more than three days. However, a new illness could start during or immediately after an old illness when the onset or character of symptoms warranted such a diagnosis. In practice this judgment was rarely made.

Respiratory illness. This was defined as at least 2 different respiratory symptoms occurring on the same day or a single respiratory symptom on two consecutive days. An exception was made when sneezing remained the only symptom, in which case no illness was recorded.

Gastrointestinal illness. Such an illness was defined as multiple episodes of vomiting or diarrhea or both occurring on a single day or single episodes of either symptom on two or more days.

Combined respiratory-gastrointestinal illness. This was defined as combinations of the diagnoses listed above, and was made when both respiratory and gastrointestinal symptoms were present in what appeared to be a single symptom complex. This designation could obviously include cases in which 2 illnesses, 1 respiratory and 1 gastrointestinal, due to different agents, had concurrent onsets, as well as single illnesses with manifestations in both systems.

Supplementary. Several supplementary surveillance methods were devised to evaluate the validity and reliability of the basic method of surveillance:

Respiratory absenteeism. Absentee records kept by the company's medical department were made available to the study. Diagnoses were usually made by the employees themselves and, although of limited use in calculating exact rates, were grouped by diagnostic categories to provide background information on overall illness experience.

Telephone survey. For six months beginning in March, 1964, the study nurse called 3 persons each working day from a randomized list of employees. Each was asked if he or she was currently experiencing or had had symptoms during the previous two weeks.

Prevalence sampling. On October 24, 1963, February 27, 1964, and September 21 and 23, 1965, groups of employees were called to the medical department for sampling of virus prevalence. The presence or absence of symptoms was recorded; nasal and pharyngeal swabs were collected.

Floor survey. Fifty randomly selected employees were approached personally on 5 separate occasions during a two-week period (May 16 to 29, 1964) and questioned about the presence of symptoms. Each person was reminded that he was still responsible for checking his own cards, but in addition the study nurse kept a separate card on each on the 50 workers.

Information gained from these supplementary surveillance procedures made it appear desirable to institute on-the-floor surveillance by the study nurse. Beginning in September, 1964, the study nurse began getting in touch with employees personally on a rotating schedule so that each week she visited every employee in the building. On these daily rounds, the nurse kept her own record of illness onsets, encouraged accurate symptom reporting, and urged those persons with symptoms to report for viral study.

Sampling

Employees were requested to report to the medical department at the onset of an illness; they were examined by 1 of 4 physicians or by a specially trained nurse who recorded symptoms and signs on a standardized form. Nose and throat swabs were collected, and acute-phase and convalescent-phase blood specimens drawn when possible. For the first twenty-eight months specimens were collected only from persons with illnesses of three days' duration or less. Thereafter, those with illnesses of up to seven days' duration were studied. During the first year 810 specimens were collected from subjects, selected on a random basis, who had been free of respiratory symptoms for a minimum of two weeks.

Laboratory

The laboratory materials and methods used were similar to those previously reported[6] except for modifications to be mentioned. For the first twenty-one months, the separate nasal and pharyngeal swabs obtained from each person were placed in the same 5 ml. of beef-heart infusion broth containing 1 per cent bovine serum albumin. Thereafter, nasal, pharyngeal and salivary specimens were collected and processed separately. For the latter the patient was asked to expectorate the contents of the anterior oral pool into a vial containing 1 ml. of broth. Original specimens were tested before freezing in human diploid fibroblast (strain WI 26 or 38) cultures. Four hundred and forty-nine specimens from employees with illnesses were tested in 2, 133 in 4, 267 in 6, and 176 in 9 WI tissue-culture tubes. During the first year 810 specimens from asymptomatic persons were tested in 2 WI tubes. During the first eight months 869 specimens from both ill and well persons also were tested in HEp-2 cells. Rhinoviruses were identified as previously described.[6] Herpes-simplex viruses were identified by neutralization testing, and the 1 respiratory syncytial virus isolated was identified by complement fixation. Diphasic mycoplasma medium[7] was used in attempts to isolate *Mycoplasma pneumoniae*. Paired serum specimens obtained from subjects with and without respiratory symptoms were tested for the presence of antibodies against influenza viruses A, B and C, parainfluenza viruses 1, 2 and 3, Coxsackie A-21 virus, reoviruses 1, 2 and 3, adenoviruses, herpes-simplex virus, respiratory syncytial virus

M pneumoniae microtitration methods of hemagglutination inhibition or complement fixation being used.

Results

Occurrence of Illness

Illnesses reported and sampled. A total of 3314 respiratory, 268 gastrointestinal and 117 combined respiratory and gastrointestinal illnesses were reported (Table 1). Of this number, 1025 respiratory and combined respiratory and gastrointestinal illnesses were studied for viral agents, a 30 per cent sample of all illnesses with respiratory symptoms.

Illness rates. Respiratory illness constituted the great bulk of all illnesses recorded (Fig. 1). Seasonal variations occurred as expected, with particularly prominent September peaks. Rates for gastrointestinal and combined respiratory and gastrointestinal illness were low and remained relatively constant throughout the year, and appeared to be unaffected by changes in respiratory illness rates.

Surveillance. Results of the supplementary surveillance studies indicated that a number of illnesses, estimated variously from 20 per cent to as much as 40 per cent of the total, were not being recorded or were being recorded inaccurately. After institution of on-the-floor surveillance in September, 1964,

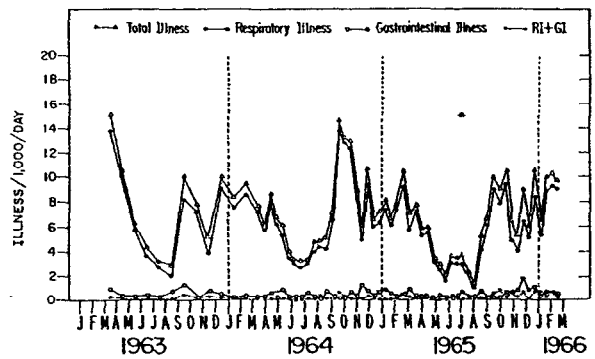

FIGURE 1. *Seasonal Occurrence of Acute Illness — Total-Illnesses with Respiratory (RI) or Gastrointestinal (GI) Symptoms or Both — in Insurance-Company Employees.*

TABLE 1. *Reporting* and Sampling of Illnesses and Rates of Rhinovirus Isolation.*

Period† 1963-1966	Average Employee Population	Respiratory Illnesses	Respiratory-Gastrointestinal Illnesses	Respiratory Illnesses Sampled		Rhinovirus Isolations	
	no.	no.	no.	no.	%	no.	%
Mar.	452	194	6	60	30.0	8	13.3
Apr.	465	135	3	55	39.9	13	23.6
May	505	88	1	26	29.2	8	30.8
June	500	56	1	23	40.3	4	17.4
July	516	42	3	16	35.6	2	12.5
Aug.	488	31	0	14	45.1	2	14.3
Sept.	506	128	8	55	40.4	18	32.7
Oct.	513	115	5	42	35.0	13	31.0
Nov.	506	60	7	17	25.4	0	0.0
Dec.	521	152	4	51	32.7	4	7.8
Jan.	520	121	7	36	28.1	3	8.3
Feb	511	130	0	38	29.2	6	15.8
Mar.	513	115	2	34	29.1	4	11.8
Apr. 1-May 1	503	104	0	26	25.0	1	3.8
May 2-May 29	533	88	1	16	18.0	8	50.0
May 30-July 10	541	70	2	18	25.0	7	38.9
July 11-Aug. 7	547	54	1	17	30.9	9	52.9
Aug. 8-Sept. 4	539	66	4	16	22.9	5	31.3
Sept. 5-Oct. 2	523	150	5	23	14.8	16	69.6
Oct. 3-Oct. 30	526	188	2	25	13.2	3	12.0
Oct. 31-Nov. 27	537	99	1	16	16.0	5	31.3
Nov. 28-Jan. 8	532	165	6	32	18.7	4	12.5
Jan. 9-Feb. 5	528	100	0	18	18.0	3	16.7
Feb. 6-Mar. 5	525	124	5	23	17.8	4	17.4
Mar. 6-Apr. 2	490	90	4	20	21.3	1	5.0
Apr. 3-Apr. 30	474	73	1	15	20.3	6	40.0
May 1-May 28	469	39	1	11	27.5	4	36.4
May 29-July 9	420	48	2	8	16.0	4	50.0
July 10-Aug. 6	339	25	0	13	52.0	3	23.1
Aug. 7-Sept. 3	346	26	1	11	40.7	3	27.3
Sept. 4-Oct. 1	342	73	4	53	68.8	25	47.2
Oct. 2-Oct. 29	329	81	6	46	52.9	18	39.1
Oct. 30-Nov. 26	328	42	6	32	66.7	8	25.0
Nov. 27-Jan. 7	327	93	8	47	46.5	10	21.3
Jan. 8-Feb. 4	326	65	6	27	38.0	3	11.1
Feb. 5-Mar. 4	327	84	4	45	51.1	4	8.9
Totals		3,314	117	1,025		239	
Averages	468				29.9		23.3

*Includes respiratory illnesses reported to nurse but not recorded on employee's symptom card, beginning on Sept. 19, 1964.

†Period consists of 1 mo. up to May, 1964; subsequently, it comprises 4 wk. (2 pay periods). June & Dec. intervals contain 3 pay periods.

under-reporting on the employees' symptom cards averaged 12 per cent; total surveillance was achieved since all acknowledged illnesses were recorded by the nurse.

Attack rates. The attack rate for the population as a whole was 2.3 respiratory illnesses per person per year (Table 2). Rates were slightly higher for females (2.38) than for males (2.20). The excess illness rate in females was contributed by those in the group from sixteen to twenty-four years of age. Except for this difference age did not significantly alter respiratory illness rates in this population composed primarily of young adults. Rates were not influenced by the presence of children in the home or by cigarette smoking (Table 3). Further analysis of the effect of contact with children showed that the presence of school children in the home did not influence illness rates in employees.

During a one-year period (October, 1964, to September, 1965) subjected to special analysis, 23 per cent of employees reported no illnesses, 60 per cent had 1, 2 or 3, and 17 per cent had 4 or more. Comparison of these 3 groups for age, sex, marital status, presence of children in the home, cigarette smoking and history of allergy showed no differences.

Rhinovirus Infections

Isolations. During the first twelve months, when rhinoviruses were isolated from 19.5 per cent of 433 specimens from patients with respiratory illness, they were isolated from 2.1 per cent of 810 specimens from randomly selected well subjects. Over the three years 239 rhinoviruses were isolated from 1025 respiratory illnesses, an overall isolation rate of 23.3 per cent (Table 1). Age and sex (Table 4), children in the home and cigarette consumption had no influence on the frequency with which rhinoviruses were isolated. Isolation rates fluctuated between extremes of 0 per cent and 70 per cent (Table 1), with rather consistent seasonal variations (Fig. 2). Isolation percentages were highest in September and October, fell rather sharply in the late fall, remained low until March and April and then rose and remained relatively high during the summer months.

Seasonal occurrence. Total rhinovirus illness rates (Fig. 2) were obtained by extrapolating the rhinovirus isolation percentages in the sampled portion of the ill populace to the entire ill group. This required the assumption that the rhinovirus isolation percentage would be the same for the total as it was for the sampled portion. Analysis of the characteristics of the sampled vs. the nonsampled ill population revealed that the latter contained significantly more employees in the age range from sixteen to twenty-four years. Since there were no differences in rhinovirus isolation rates according to age this bias in sampling was thought not to invalidate such extrapolation.

During each of the three years there was a peak of illness in September and early October associated with a high rhinovirus isolation rate (Table 1 and Fig. 2). Since the patterns for other months were generally similar, data for the three years were

TABLE 2. *Respiratory-Illness Attack Rates — Age and Sex Specific — March, 1963, to March 4, 1966.*

AGE	AVERAGE NO. OF EMPLOYEES	NO. OF RESPIRATORY ILLNESSES*	NO. OF ILLNESSES/ PERSON/YR.
yr.			
Males:			
16-24	79.8	531	2.21
25-34	68.0	438	2.14
35-44	37.5	261	2.31
45+	7.7	51	2.19
All males	193.0	1,281	2.20
Females:			
16-24	159.4	1,253	2.61
25-34	79.2	504	2.11
35-44	27.5	174	2.10
45+	8.3	33	1.31
All females	274.4	1,964	2.38
All employees	467.4	3,245	2.30

*Does not include illnesses reported to nurse & not recorded on symptom cards (under-reporting).

TABLE 3. *Respiratory-Illness Attack Rates, May 2, 1964, to March 1966.*

POSSIBLE FACTOR	AVERAGE NO. EMPLOYEES	RESPIRATORY ILLNESS	ILLNESSES/PERSON/YR
Children in the home:			
0	225.24	878	2.11
1	111.09	407	1.98
2	81.05	322	2.15
3	25.37	89	1.90
4 & more	10.91	32	1.60
Totals	453.66	1,728	2.06
Cigarettes smoked/day			
0-5	310.16	1,223	2.14
6-20	108.63	373	1.86
21 & more	34.87	132	2.04
Totals	453 66	1,728	2.06

TABLE 4. *Rhinovirus Isolation Rates According to Age and Sex, March, 1963, to March 4, 1966.*

AGE	ILLNESSES SAMPLED	RHINOVIRUS ISOLATED	
yr.	no.	no.	%
Males:			
16-24	104	22	21.2
25-34	166	37	22.3
35-44	123	32	26.0
45+	27	5	18.5
All males	420	96	22.9
Females:			
16-24	315	72	22.9
25-34	192	47	24.5
35-44	88	22	25.0
45+	10	2	20.0
All females	605	143	23.6
Totals Average	1,025	239	23.3

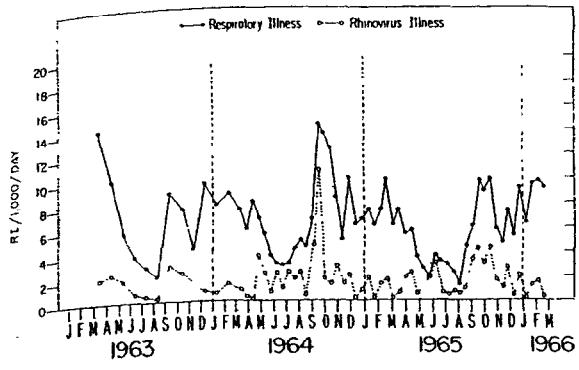

FIGURE 2. *Seasonal Occurrence of Respiratory Illness and Rhinovirus Illness in Insurance-Company Employees.*

pooled to obtain the curves in Figure 3. The total occurrence of rhinovirus illness, even more than the percentage of illnesses yielding rhinoviruses, increased markedly in September. Rhinovirus illness occurred during all months of the year, but 40 per cent of all such illness occurred during this eight-week period. Rhinovirus infection was responsible for a high percentage of the few illnesses that occurred during the summer months.

Results of the mass samplings (Table 5) confirmed the association of rhinoviruses with illness, although the prevalence of rhinovirus infection was low in both late October, 1963, and February, 1964.

Virus shedding. Duration of illness up to five days did not alter rhinovirus-isolation percentages (Table 6). Comparison of specimens collected from multiple sites and processed separately indicated that the success of isolation varied with the site from which the specimen was obtained (Table 7). Although they could be cultured from pharyngeal secretions and saliva rhinoviruses were isolated with greatest frequency from the nose. In addition, more virus appeared to be present in the nose as judged by a higher percentage of positive tubes and the fact that cytopathic effect often was noted earlier in tubes inoculated with nasal secretions than in those inoculated with pharyngeal secretions or saliva.

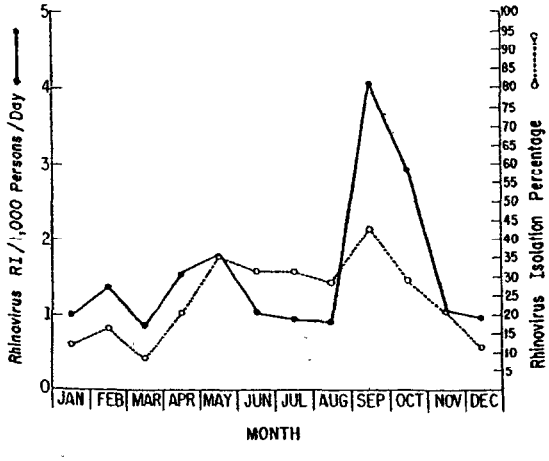

FIGURE 3. *Combined Data for the Three-Year Period, March, 1963, to March, 1966. Depicting the Seasonal Variation in the Percentage of Sampled Respiratory Illnesses Yielding Rhinoviruses and in the Rate of Rhinovirus Illness Derived by Application of This Percentage to the Total Rate of Respiratory Illness.*

TABLE 5. *Rhinovirus Isolations from Prevalence Samplings.*

DATE OF SAMPLING	RESPIRATORY ILLNESS			NO ILLNESS		
	NO. TESTED	VIRUS ISOLATED		NO TESTED	VIRUS ISOLATED	
		no.	%		no.	%
Oct. 24, 1963	14	1	7.1	129	1	0.8
Feb. 27, 1964	16	1	6.2	84	2	2.4
Sept. 21 & 23, 1965	10	8	80.0	42	1	2.4
Totals	40	10		255	4	
Averages			25.0			1.6

Absenteeism. Self-diagnosed respiratory illnesses accounted for 36 per cent of the total absenteeism recorded by the company's medical department. Ill defined constitutional illnesses, many with gastrointestinal symptoms, ranked next (17 per cent) as a cause of such absenteeism. Other categories in order of importance were surgical-dental (15 per cent), obstetric-gynecologic (13 per cent), neuromuscular (8 per cent) and all other (11 per cent). When respiratory absenteeism calculated from data reported to the study was compared to that provided by company absentee records, it was apparent that employees were more apt to report official absences to the company than they were to check "stayed home" on the study cards (Fig. 4). However, the general trends were similar. Respiratory absenteeism paralleled total absenteeism in most cases although in September and October, 1964, at the time of a peak of rhinovirus illness, there was relatively low respiratory absenteeism despite high total absenteeism. The high total absenteeism during this period was due partially to the occurrence of cases of myalgia and malaise of unknown cause and to prolonged postsurgical morbidity affecting 2 employees. The amount of absenteeism reported to the study for the first nine days of either rhinovirus or

TABLE 6. *Rhinovirus Isolations by Day of Illness when Sampled.*

INTERVAL FROM ONSET TO SAMPLING	ILLNESSES SAMPLED	RHINOVIRUS ISOLATED	
days	no.	no	%
−3	2	0	0
−2	0	0	0
−1	3	0	0
0	75	15	20
1	493	116	23.5
2	255	55	21.6
3	114	33	28.9
4	36	9	25.0
5	28	9	32.1
6	12	1	8.3
7	4	0	0
Unknown	3	1	33
Totals	1,025	239	
Average			23.3