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Antibiotic Effects on Coliform Enumeration

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Abstract

Wozniak, Keith Leonard. M.S., Eastern Illinois University, May 1987. Antibiotic Effects on Coliform Enumeration. Major Professor: Dr. William A. Weiler.

The inhibitory effects of Bacillus polymyxa on Escherichia coli were studied. Significant coliform inhibition was observed. The inhibitory agent (presumably polymyxin) was active against coliforms after a one hour holding time. Different suspending menstrua were analyzed for their effects on coliform inhibition. It was observed that Peptone water provided the highest recovery of coliforms while phosphate buffer provided less protection. Two methods of enumeration were also studied as to their ability to quantify coliforms inhibited by B. polymyxa. Comparable inhibition was shown using both methods. No significant differences exist between the Standard Plate Count and Membrane Filter techniques. Overall, wide variations in coliform inhibition independent of the experimental conditions were observed.

INTRODUCTION

An understanding of the survival of coliforms in the environment is important to the interpretation of the sanitary quality of a water system. Coliform survival is dependent on physiochemical conditions such as pH, temperature, sunlight, specific ion toxicity (eg; NaCl, ammonia, heavy metals) and high molecular weight compounds. In addition, biological factors, including predation by protozoa and inhibition by algae and antagonistic bacteria, are detrimental to coliform survival.

Antagonistic bacteria are of particular interest. Bacillus polymyxa produces an antibiotic (polymyxin) that is unique in its specificity for coliform bacteria. As a result, some coliforms are not enumerated in water quality analyses when, in fact, they are present. Suppression of coliform bacteria can result in a serious underestimation of the bacteriological quality of a water supply.

The purpose of this study was to examine the inhibitory effects of Bacillus polymyxa on Escherichia coli. This study was approached with the following questions in mind.

(1) What effect does polymyxin contact time have on coliform inhibition?

(2) What effects do different suspending media have on coliform inhibition?

(3) Are there any differences between different methods of enumeration (SPC vs MF) on coliform inhibition?

(4) At what concentration are Bacillus polymyxa cells and culture filtrates inhibitory to coliforms?

(5) What is the effect of boiling on the antibiotic factor?

Literature Review

Coliform bacteria have long been used as indicators of potential health hazards to humans and animals (4,22,23). The use of coliform bacteria as indicators originated in 1880 when Von Fritsch isolated Klebsiella pneumoniae and K. rhinoscleromatis as bacteria commonly present in fecal material. A few years later, Escherich reported Bacillus coli (later named Escherichia coli) as the predominant organism in feces. MacConkey (39) recognized the importance of these organisms in feces as indicators and devised a series of tests to separate these organisms. A differential system based upon the fermentation of sucrose and dulcitol, production of indole and acetylmethylcarbinol, and gelatin liquefaction was used to classify 128 different coliform types. A number of new developments led to the use of the IMViC differentiation procedure for coliforms which is still used today (48).

Many different indicator systems have been proposed to quantify the potential enteric pathogens in the environment. Among this large and varied group, only total coliforms, fecal coliforms, fecal streptococci, Clostridium perfringens and a few anaerobic bacteria fulfil the prerequisite of an ideal indicator. The characteristics of an ideal indicator of fecal pollution have been discussed by Berg (6) and Geldreich (24). Briefly stated, an indicator organism must be: (i) present only in the feces of warm blooded animals; (ii) easily quantifiable; (iii) present when the pathogen is present or (iv) in a constant ratio to the pathogen; (v) present in sufficient density to allow detection; (vi) equally resistant to disinfection and to noxious environments; and (vii) able to multiply, remain static or die off in

constant numbers to the pathogen. Logically, the ideal indicator must be the pathogen itself. However, using the pathogen is impractical and as a result an acceptable indicator is used. This acceptable indicator must be present when the pathogen is present and must be easy to detect and quantify. Today, a fully adequate indicator system for all pathogens in all situations simply does not exist. However, recent findings confirm earlier studies that the coliform indicator concept is adequate in assuring bacterial and viral safety of water (3).

Coliforms have been the traditional indicators of the sanitary quality of a water supply largely because they are easy to detect and to quantify. The accumulated data over the years suggests that the absence of coliforms in water is evidence of a bacteriologically safe supply. The total coliform group comprises all aerobic and facultatively anaerobic, gram-negative, non-spore forming bacilli. This group includes E. coli, all other fecal coliforms and many non-fecal coliforms that ferment lactose and produce gas within 48 hours at 35 C.

Escherichia coli conforms to the definition of the family Enterobacteriaceae. Escherichia coli, as defined in Bergey's Manual (29), are straight rods, motile by peritrichous flagella or non motile. Gram-negative, facultatively anaerobic, oxidase negative and citrate negative are also characteristics of this genus. Acetate can be utilized as a sole carbon source. Glucose and other carbohydrates are fermented with the production of pyruvate. Most strains of E. coli can ferment lactose but fermentation may be slow or absent. Further characteristics of this genus include indole positive, methyl red negative and Voges-Proskauer negative reactions.

The ability of E. coli and other coliforms to survive in nonindigenous environments is dependent on a number of physical, chemical and biological stresses. Temperature as a factor affecting the survival of enteric bacteria was first reported in 1927 (56) and more recently in 1972 (43). Heating (16) and freezing (46,51,52) of a body of water influence the survival of coliforms. Previous studies have shown that the viability of E. coli and other coliforms was prolonged if the temperature of the surrounding water was about 12 C (25,50). In addition, seasonal temperature variation has been found to play a role in the survival of many members of the Enterobacteriaceae (12,60).

The effects of sunlight may also be detrimental to many coliform bacteria. Hollaender (28) has shown that near visible and visible light was lethal to E. coli. Subsequent studies have revealed the deadly effects of sunlight on various aquatic bacteria (17,26).

Variations in pH influences the survival of coliforms in aqueous environments. In a study by McFeters and Stuart (43), the optimum pH for the survival of coliforms occurred in the range 5.5-7.5. However, a rapid decline of population viability occurred both above and below these values. Conversely, more recently, Granai and Sjorgren (25) have found that the survival of coliforms was enhanced at lower pH values. A pH of 5 was found to be the optimum value.

Nutrient deficiency and consequent starvation may play an important role in the persistence of coliforms. Postgate and Hunter (50) reported that a population of Enterobacter aerogenes, in a buffered physiological saline, declined at a rate of 8% per hour for the first 7-10 hours. This population decreased to less than 2% of the original population in 24 hours. Nutrient starvation has been found to increase microbial

sensitivity to physical and chemical stresses (34,49). As a result, some starvation sensitive bacteria may be less able to withstand other stresses than resistant varieties (64).

A number of other chemical factors have been shown to influence coliform survival. These include carbon dioxide (40), low molecular weight fatty acids (33), low molecular weight cationic compounds (35) and high molecular weight compounds (42). In addition to organic inhibitors, heavy metals (15,32,37,41), chlorine (13,45) and ammonia (21) may cause the death of introduced species.

Biological stresses too may play an important role in the reduction of introduced coliforms into the environment. Predation by protozoa is an important factor in the elimination of bacteria from aquatic and terrestrial environments (2). Curds and Fey (14) have shown that the presence of protozoa dramatically reduced the survival time of E. coli in activated sludge and that ciliates were primarily responsible. The accumulated data suggests that protozoa are major regulators of populations of bacteria in soil and water (2).

Bactericidal activity on coliforms by algae (57) and bacteria (54) have been well documented. Bacterocins were discovered in 1925 when Andre Grattia observed that the supernatent of one broth culture of E. coli was inhibitory to the growth of another strain of E. coli even when diluted 1000 fold (44). Hutchinson and co-workers (31) isolated strains of bacteria capable of inhibiting E. coli in 8, 11 and 37% of the water samples collected from springs, ground waters and surface waters, respectively. The influence of non-coliform microorganisms on the survival and recovery of coliform bacteria has been documented.

Various studies have shown that antibiotic or inhibitory substances produced by various aquatic bacteria are active against E. coli and a variety of gram-negative bacteria (31,53). Several genera were shown to be "antagonistic" toward coliform bacteria. These include Pseudomonas, Sarcina, Micrococcus, Flavobacterium, Proteus, Moraxella and Bacillus.

A number of Bacillus spp. with antibiotic producing characteristics have been isolated from water (62). Bacillus polymyxa produces an antibiotic substance (polymyxin) active against coliform bacteria. Bacillus polymyxa, as defined by Holt and Sneath (30), are aerobic or facultatively anaerobic, gram-positive, spore-forming, straight rods. The spores are very resistant to adverse conditions and are not repressed by exposure to air. The spore has parallel longitudinal surface ridges that gives it a star-like appearance in cross section. Further characteristics of B. polymyxa include the production of acid and gas from glucose and hydrolysis of starch and gelatin. The catalase and Voges-Proskauer tests are positive and the indole test is negative. B. polymyxa cannot utilize citrate or urea, but most strains do have the ability to fix molecular nitrogen under anaerobic conditions.

Antibiotic substances produced by Bacillus polymyxa were discovered in 1947 by three different groups of workers. Stansley, Shepherd and White (57) and Benedict and Langlykke (5), working independently, isolated the antibiotic polymyxin. At the same time Ainsworth, Brown and Brownlee (1) described 'aerosporin' isolated from a strain first thought to be Bacillus aerosporin, but later shown to be identical to B. polymyxa. Antibiotics of the polymyxin group are produced by various species of Bacillus and are characterized as cyclic peptides, of molecular weight about 1200, containing threonine, alpha-gamma-

diaminobutyric acid and a fatty acid residue which may be 6-methyloctanoic acid, 6-methylheptanoic acid or n-octanoic acid. There are five chemically distinct peptides produced by different strains of Bacillus polymyxa (11). Polymyxin A,B,C,D and E, isolated in 1947, are the only known antibiotics derived from B. polymyxa. These five polymyxins possess similar antibacterial spectra and are all active at about the same concentration (10). Polymyxins are more active against gram-negative bacteria than gram-positive bacteria and have also been found to be bacteriocidal rather than bacteriostatic (63). Bliss and co-workers (7) studying the effects of polymyxin on the growth of Bacterium coli (E. coli) found that the minimum concentration required to inhibit growth depended on the size of the inoculum used in the growth test. This suggested considerable absorption of the antibiotic by the bacteria. The nature of the growth medium did not affect the limiting inhibitory concentration of polymyxin for E. coli, indicating that the growth medium used was not detrimental to either E. coli or the antibiotic polymyxin.

The mode of action of polymyxin has been well documented. Polymyxin is thought to interact with phospholipids in the cell envelope by rapid attachment to the cytoplasmic membrane sites (59). This causes a disorganization of the cytoplasmic membrane that results in disruption of the osmotic equilibrium of the cell (47,55). Autolysis follows due to the breakdown of cytoplasmic constituents like ribosomes (59). Whan and co-workers (61) have shown that high concentrations of polymyxin produces pertuberences from the cell surface and rapid cytoplasmic destruction in E. coli. It is well known that lipoproteins and

lipopolysaccharides (LPS) are the major components of the outer layer of the cell wall of gram-negative bacteria. Lopes and Inniss (38) have shown by electron microscopy that the (LPS) extracted from E. coli was broken down into short sections when it was exposed to polymyxin. Many of the environmentally induced changes which take place in the gram-negative envelope are in the outer membrane and it is therefore not surprising that they markedly affect sensitivity of an organism to polymyxin (9,21). Resistance to polymyxin depends to some extent on the chemical composition and structure of the cell wall (18). In addition, the access of the antibiotic to the membrane and the presence of certain density target sites in the membrane is critical to the resistance of a bacterium (20).

Materials and Methods

Bacterial Strains

Bacillus polymyxa (ATCC 842) was obtained from a stock culture maintained routinely at the Eastern Illinois University Botany Department. *Escherichia coli* was isolated from secondary effluent at the Charleston Wastewater Treatment Plant (Charleston, IL). The isolate was identified by using the ENCISE II™ Identification system (Roche Diagonostics, Nutley, NJ). Traditional test media were substituted for the Entertube™ procedure. Both cultures were maintained on Plate Count agar (PCA) (Difco Laboratories, Detroit, MI) at 4 C. The cultures were transferred to fresh PCA slants and incubated at 35 C for 24- 48 hours prior to use.

Media

All media were prepared using laboratory pure water processed through a Milli-Q ™ reagent grade water system. Tryptone Glucose Yeast extract (TGY) broth (50% Plate Count Broth, Difco Laboratories) and MacConkey Agar (Difco Laboratories) were prepared according to the manufacturer's instructions. Two different diluents, Standard Methods Phosphate Buffer solution (pH=7.2) and 0.1% Peptone (Difco Laboratories) water (4), were also prepared. All media and diluents were sterilized at 121 C at 15 psi for 15 min in an autoclave.

Methods

The cultures were inoculated into 250 ml flasks containing 100 ml of TGY broth. *E. coli* and *B. polymyxa* were incubated using a rotary water bath shaker at 32 C, operating at 140 cpm for 24 and 48 hours,

respectively. A 50 ml portion of the B. polymyxa broth suspension was filtered through sterile Gelmann 0.45 micrometer pore size membrane filters (Type GN-6) to remove the cells. The filtrate was collected in a sterile 25 mm diameter test tube. The broth culture containing E. coli was serially diluted using Standard Methods Phosphate Buffer Solution or 0.1% Peptone water. Ten milliliters of the B. polymyxa broth culture or culture filtrate was added to the E. coli suspension. The unused portion of the filtrate was stored at 4 C for less than one week. In addition, a control consisting of 10 ml of the appropriate suspending medium was also added to the E. coli suspension. The suspension was than plated on MacConkey agar. Three to five replicate plates were performed for each plating scheme. The plates were incubated inverted at 35 C for 24 hours. Any pink to red colonies that developed were enumerated and recorded as E. coli. The geometric mean was then calculated for each set of replicate plates.

Results and Discussion

During early testing, difficulty was experienced in demonstrating inhibition of Escherichia coli by Bacillus polymyxa. Results were extremely variable. Contact time between E. coli and the cells and culture filtrate of B. polymyxa was thought to be the must logical reason for this variability. A longer contact time allows a greater association period of the test bacterium with the antimicrobial agent. Under optimal conditions, researchers have found that polymyxin is active against coliforms only after 0.5-1.0 hour contact time (8,27,63). The effects of holding time on coliform inhibition by B. polymyxa were analyzed (Table 1). Coliform recovery, without a holding time, ranged from 44-60 cells. Conversely, using a 1 hour holding time, considerable inhibition was shown as only 1-4 coliforms were recovered. A one hour holding time was found sufficient to demonstrate coliform inhibition. The occurrence of coliform inhibition after a period of contact can be explained by the longer contact time which allows a greater time for association of the inhibitory substance with cytoplasmic membrane sites on E. coli (59). Reduction in viability estimates of the cells is the end result.

The type of diluent used may have an effect on coliform recovery. A "deficient" suspending diluent may cause changes in the cell structure which could affect the results of chemical or antibactericidal action (19). Different diluents (Standard Methods Phosphate Solution and 0.1% Peptone water) (4), were studied for their effects on coliform inhibition by cultures and culture filtrates of B. polymyxa (Table 2). Coliform recovery was approximately the same using either the cells or

Table 1: The effects of holding time on coliform inhibition by culture and culture filtrates of Bacillus polymyxa.

	No Hold		One Hour Hold	
	Trial I[a]	Trial II	Trial I	Trial II
Control[b]	60	48	39	28
Culture	60	50	1	3
Culture Filtrate	67	44	1	4

[a] Values represent the geometric mean of coliform counts on five replicate plates of MacConkey Agar.

[b] Control bottles included 10 ml of the appropriate suspending medium instead of culture or culture filtrate ("see methods").

Table 2: Effects of suspending medium on coliform inhibition by culture and culture filtrates of Bacillus polymyxa.

	Standard Methods Phosphate Diluent[a]		0.1 % Peptone Water	
	Trial I	Trial II	Trial I	Trial II
Control[b]	400	400	350	410
Culture	12 (97)	11 (97)[c]	94 (73)	239 (42)
Culture Filtrate	5 (>99)	10 (97)	34 (90)	239 (42)

[a] All values represent geometric means of coliform counts on five replicate plates of MacConkey Agar.

[b] Control bottles included 10 ml of the appropriate suspending medium instead of culture or culture filtrate ("see methods").

[c] Values in parenthesis are percent coliform inhibition.

culture filtrate of B. polymyxa. Comparing the different diluents, wide variations in the percent inhibition of coliforms were observed. The degree of coliform inhibition using Standard Methods Phosphate Solution ranged from 97% to greater than 99%. Conversely, using 0.1% Peptone water, only 42-90% coliform inhibition was observed. The wide variation in coliform recovery using the different suspending menstrua might be due to site competition. Peptones may act by directly competing with the inhibitory agent for the site of action on the E. coli cell membrane, since it seems to provide protection for the cell against the inhibitory agent. Practical selection of a diluent should be aimed towards the maintenance of the cells in a constant, stable condition. Since peptone water exhibited better recovery, this diluent was used for further experimentation.

Different methods of coliform enumeration in the presence of B. polymyxa yielded basically similar results (Table 3). Using the Membrane Filtration (MF) technique (4), on m-Endo broth MF (Difco Laboratories), greater than 97% coliform inhibition was shown. Similarly, coliform inhibition using the Standard Plate Count (SPC) technique (4) on McConkey Agar, ranged from 97% to greater than 99%. These results suggest that no significant difference exists between the MF and SPC techniques. Thus the inhibitory agent has minimal, if any, additional effects after plating. Since the unabsorbed portion of the inhibitory substance is lost through filtration, the inhibitory agent most likely became attached to all the available sites on the E. coli cells prior to plating. In addition, as shown earlier, the culture and culture filtrate showed comparable inhibition for both methods of enumeration.

Table 3: Method of coliform enumeration: The effects of culture and culture filtrates on coliform recovery using two methods of enumeration.

	Trial I		Trial II	
	SPC[a]	MF[b]	SPC	MF
Control[c]	400	520	400	470
Culture	12 (97)[d]	7 (99)	11 (97)	<10 (>97)
Culture Filtrate	5 (>99)	10 (98)	10 (97)	<10 (>97)

[a] Value represent the geometric mean of coliform counts on five replicate plates of MacConkey Agar.

[b] Values represent the geometric mean on three replicate membranes on m-Endo Broth MF.

[c] Control bottles included 10 ml of the appropriate suspending medium instead of culture or culture filtrate. ("see methods").

[d] Values in parenthesis are percent coliform inhibition.

Therefore, the culture filtrate was deemed sufficient for assessing coliform inhibition in subsequent experiments.

Culture filtrates of B. polymyxa were serially diluted and tested for their inhibitory qualities (Table 4). Very low coliform recovery was observed at all of the concentrations tested. The lowest concentration of B. polymyxa culture filtrate tested showed 98% and 94% coliform inhibition for trials 1 and 2, respectively. The inhibitory agent was still very active at 1/100th of the original (10 ml) of B. polymyxa filtrate. An additional trial was performed to establish a minimal level at which the inhibitory agent was still active. B. polymyxa filtrate concentrations (0.01-5.0 ml) were tested for their effects on coliform inhibition (Table 5). The results show coliform inhibition ranging from 18-59%. The inhibitory agent was still active at a 0.01 ml (.001% of the original) concentration of B. polymyxa. However, wide variations in coliform recovery were exhibited between the results in Table 4 and 5. Even though care was taken in the growth of the E. coli cells, no effort could be made to standardize the density and physiological state of the cells. Moreover, during the growth of E. coli, a large and varied number of interactions can occur which may cause large changes both biochemically and physiologically in the absence of a change in the growth phase (19). Hence, the effects of inhibitory agents on the viability of E. coli can differ widely.

In an attempt to partially characterize the antibiotic agent, the culture filtrate was boiled for 30 min. in a water bath to assess the effects of heat on its activity (Table 5). Comparable coliform recoveries were exhibited by both boiled and unboiled filtrates,

Table 4: The effects of Bacillus polymyxa culture filtrate concentrations on inhibition of coliforms.

Filtrate Conc.	Trial I	Trial II
0.0ml[a]	2780[b]	4500
0.1ml	49 (98)[c]	289 (94)
0.5ml	5 (>99)	89 (98)
1.0ml	2 (>99)	70 (98)
5.0ml	2 (>99)	15 (>99)
10.0ml	22 (>99)	13 (>99)

[a] Control bottles included 10 ml of the appropriate suspending medium instead of culture filtrate ("see methods").

[b] Values represent the geometric mean of coliform counts on five replicate plates of MacConkey Agar.

[c] Values in parenthesis are percent coliform inhibition.

Table 5: The effects of boiling on the coliform inhibition activity of Bacillus polymyxa culture filtrate.

Filtrate Conc.	Unboiled Filtrate[a]	Boiled Filtrate
0.0ml[b]	460	460
0.01ml	361 (22)[c]	372 (19)
0.05ml	376 (18)	354 (23)
0.1ml	342 (26)	334 (27)
0.5ml	316 (31)	270 (41)
1.0ml	206 (55)	188 (59)
3.0ml	188 (59)	163 (65)
5.0ml	182 (59)	170 (63)

[a] Values represent the geometric mean of coliform counts on three replicate plates of MacConkey Agar.

[b] Control bottles included 10 ml of appropriate suspending medium instead of culture filtrate ("see methods").

[c] Values in parenthesis represents percent coliform inhibition.

suggesting that boiling of the filtrate has no effect on the inhibitory agent. Thus, the evidence suggesting that the inhibitory agent is, in fact, polymyxin includes the facts that the inhibitory agent is (i) associated with B. polymyxa, (ii) active against E. coli and, (iii) insensitive to heat, as is polymyxin (36). Agents such as polymyxin, if present in a water supply, may suppress coliform recovery. This in turn can result in a serious underestimation of the bacteriological quality of a water supply. Interpretation of the results of this or any similar study is inevitably complicated by the fact that the organism and its environment are constantly interacting independently of the presence of a particular antimicrobial agent. Such interaction effects are difficult to predict and often lead to variability even under highly controlled experimental conditions.

CONCLUSIONS

1. A one hour holding time of the test organism (Escherichia coli) with culture and culture filtrate of Bacillus polymyxa was found sufficient to demonstrate coliform inhibition.

2. Coliform inhibition was affected by differing suspending media. Using Standard Methods Phosphate Solution, 97% to greater than 99% coliform inhibition was observed. Conversely, only 42% to 90% coliform inhibition was shown using 0.1% Peptone water.

3. The Standard Plate Count and Membrane Filter technique for enumeration showed comparable coliform inhibition.

4. Dilutions of the culture filtrate of B. polymyxa as high as 1-1000 still show some inhibitory qualities.

5. Boiling of the B. polymyxa culture filtrate has no demonstratable effect on the inhibitory agent.

6. Wide variability in coliform inhibition was observed even under controlled experimental conditions.

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