THE DISTRIBUTION OF THE SPORES OF AEROBIC, LACTOSE-FERMENTING, SPORE-FORMING BACILLI IN NATURE

by

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INTRODUCTION

Aerobic, spore-forming, lactose-fermenting bacilli are frequently encountered in the examination of water for the presence of coliform organisms. Since the spore-forming, lactose-fermenting organisms may be responsible for false presumptive tests in the coliform determinations, it seemed desirable to obtain more data on their distribution and numbers in nature.

A review of the literature disclosed reports of several species of lactose-fermenting bacilli isolated from water and from sources that might easily contaminate a water supply. The most commonly encountered bacilli are Clostridium perfringens, Bacillus polymyxa and Bacillus macerans. In this discussion, attention is focused only upon the aerobic spore-formers B. polymyxa and B. macerans because these organisms very often give false positive presumptive tests in the analysis of chlorinated water.

REVIEW OF LITERATURE

Bacillus polymyxa was first described by Prazmowski (1880) as an aerobic, spore-forming bacillus that produced carbon dioxide from some carbohydrates. He designated this organism as Clostridium polymyxa.

Beijerinck (1893) studied an aerobic bacillus that formed gas, was motile, formed spores and granulose, and which he called Granulobacter polymyxa (Bacillus polymyxa). He reported it to be on grains of wheat and plentiful in garden soil.

An organism, recognized today as Bacillus polymyxa, was isolated

from decaying carrots by Arthur Meyer (1897). He gave the organism the name Astasia asterosporus.

Several years later Bredemann (1909) published the results of a study of Astasia asterosporus. He examined 138 samples of soil from widely separated parts of the Earth and found Astasia asterosporus in 34 of these specimens. He stated that the bacillus was never found in uncultivated soil, such as sea sand and earth from swamps and forests, but only in cultivated soil, such as fertilized fields. He felt that the bacterium was, therefore, probably introduced through the intervention of man.

This organism has been isolated from various other sources by many other workers. Alderhold (1899) found it in canned asparagus; Wahl (1906) found the organism in decaying vegetables; and Behrens (1903) found it in fresh vegetables and vegetable products. Koser and Shinn (1927) found this spore-forming bacillus in 23 of 52 soil samples taken from cultivated fields. They found the distribution of the organism to be rather irregular. In some cases, five- or ten-gram samples yielded negative results, while in others the bacterium was obtained from relatively small amounts, often from dilutions representing 0.01 or 0.001 gram of soil. In some places this bacterium was fairly abundant, and it seemed reasonable that it might be washed into water supplies in fairly large numbers. Its presence in the intestinal tract of cows was noted by Ankersmit (1906). Havens and Dehler (1923) found a similar organism in the intestines of fish. Astasia asterosporus was encountered by Lisk (1923) in a sample of milk at Fort McCoy, Florida. Wagner (1916) isolated a spore-forming bacillus from eggs and called the organism Bacillus mycoides var. ovoethylicus,

which Porter, McCleskey and Levine (1937) considered to be synonymous with Bacillus polymyxa.

Until 1918, the presence of aerobic, spore-forming bacilli had not been specifically tested for in the examination of water. By 1917, chlorination of drinking water was becoming increasingly popular and was being used in most of the larger cities. The presence of lactose-fermenters in water remained unnoticed until E. M. Meyer (1918) described an aerobic, spore-forming, lactose-fermenting bacillus which he isolated during the course of routine water analysis at Newport and Covington, Kentucky. Eight strains were obtained from the city water supply.

Within the next few years, Bacillus polymyxa was reported to have been found in water supplies by several investigators. Ewing (1919) isolated 19 strains from the Baltimore city water supply. The organism was apparently identical with that described by Meyer (1918). Perry and Monfort (1921) reported B. polymyxa from surface waters in Illinois. The organism was also reported by Schreiner (1927) in Kansas, Raab (1923) in Minneapolis, Ginter (1927) in Oklahoma, and Ellms (1922) from the Milwaukee water supply.

Norton and Weight (1924) made an extensive study of the distribution of these aerobic, lactose-fermenting, spore-forming bacilli in the Chicago area. From 438 samples collected from a variety of sources, they isolated 25 strains of the bacillus. The highest incidence of isolation was from animal manures, garden soil, and root-vegetable washings. In most cases, the number of recorded samples was too small to serve as a basis for definite statements regarding the habitat of these organisms. However, those

organisms which they did isolate were either identical with or closely related to those isolated by Meyer (1918), Ewing (1919), Hinman and Levine (1922) and others.

Table 1 presents the sources of the samples used by Norton and Weight and the percentage frequency of isolation from each of the different sources.

Table 1. Frequency of isolation of Bacillus aerosporus (B. polymyxa) from various sources.

(Norton and Weight, 1924)

Source	No. Samples	No. Strains	Percentage
Tap water	128	2	1.6
Drinking fountains	50	1	2.0
Raw lake water	32	1	3.1
Human urine	6	0	0
Human feces	36	1	2.5
Street drainage	44	2	4.5
Sidewalk drainage	10	2	20.0
Snow	4	0	0
Mat dirt	18	2	11.1
Floor dirt	8	1	12.5
Street mud	6	2	33.3
Horse manure	8	3	37.5
Rabbit manure	6	0	0
Sheep manure	6	1	16.6
Sidewalk soil	8	0	0

Table 1. (concl.)

Source	No. Samples	No. Strains	Percentage
Garden soil	10	3	30.0
Carrot washings	8	4	50.0
Oat washings	8	0	0
Swimming pools	36	0	0
Raw sewage	6	0	0
	438	25	5.7

Greer (1928 a) isolated over 60 strains of aerobic, spore-forming, lactose-fermenting bacilli from Chicago city water. Although these organisms were similar or identical to those isolated by other workers, Greer gave them the name of Bacillus aerosporus (B. polymyxa), which he stated was suggested by Dr. John Norton of the University of Chicago in correspondence on the subject.

Greer (1928 b) noted that this type of organism (B. polymyxa) varied in numbers according to the season of the year and whether the water was treated or untreated. In raw water, the greatest number of these bacilli was found in February during which time there were about 150 organisms per ml. of water. B. aerosporus (B. polymyxa) constituted 7.5 percent of the microbial population and was the third most common organism found in raw water. In treated water, B. aerosporus (B. polymyxa) was the second most frequent organism found and constituted 28.4 percent of the total organisms. During the winter months of the year there were as many as 300 of these organisms per ml. of chlorinated water.

In order to determine the sources of B. aerosporus (B. polymyxa) other than in water, Greer (1928 d) collected samples of manures, sewage, soils and well waters. He found the bacilli prevalent in horse and cow manures and in fertilized soils.

Table 2 shows the sources of the samples used by Greer and the percentage of organisms isolated from each of the different sources.

Table 2. Frequency of isolation of Bacillus aerosporus (B. polymyxa) from various sources.

(Greer, 1928)

Source	: No. Samples	: No. Strains	: Percentage
Horse manure	18	17	94.4
Cow manure	14	11	78.5
Human feces	18	1	5.5
Sewage	44	3	6.8
Fertilized soil	9	7	77.7
Well water	70	0	0

When Greer's results were combined with Norton and Weigh's, one had a much better sampling of sources from which to draw conclusions about the distribution of Bacillus polymyxa. It seemed to most prevalent in the manure of farm animals and in manure-fertilized soils.

Schaut (1929) isolated aerobic, lactose-fermenting, spore-forming bacilli from Philadelphia drinking water. He believed that the number of these organisms is greatest in chlorinated water during January and February because the filters and chlorine are least efficient when the water is cold. His publication fails to reveal whether he attempted to identify the organism.

Corbet (1930), while concerned with the cause of coagulation of raw, liquid latex, isolated an aerobic, lactose-fermenting, spore-forming bacillus from the latex which he designated Bacillus pandora. Bergey's Manual of Determinative Bacteriology (6th Ed.) lists this organism as probably a variant of B. polymyxa.

Bacillus macerans was described by Schardinger (1905) when he isolated the organism from decaying potatoes. Weizmann (1915) isolated an organism from soil samples and from cereals which he designated as Bacillus granulobacter pectinovorum. This is probably a variant of B. macerans since the characteristics of the two organisms are very similar.

B. macerans was first noticed in water when Hinman and Levine (1922) isolated it from chlorinated surface water supplies in Iowa.

In an attempt to find a cheap method for the production of acetone, Northrup, et al. (1919) isolated from decaying potatoes an organism which they named Bacillus acetoethylicum. Porter, et al. (1937) claimed that this organism is a variant of B. macerans.

Organisms that differed from those isolated by Hinman and Levine (1922) only in that they liquified gelatin, were isolated from corn stalks by Burkey (1928).

Porter, et al. (1937) worked with 87 strains of these two spore-forming bacilli that had been isolated from various sources to determine their systematic relationships. Included in the species observed were all the available organisms of this group, e.g., the original strains of A. Meyer, Bredemann, Wagner, Schardinger, and Northrup. Cultural and serological criteria were employed and the cultures fell into two distinct groups -

B. polymyxa and B. macerans.

From the descriptions given by the various workers, it was obvious that they were dealing with the same or very similar organisms. All workers agreed remarkably as to the properties of the organisms though isolated from a variety of sources and localities. No disagreements on sugar reactions were reported by any author. Some minor differences were noted in the rate of gelatin liquefaction. Some of the organisms were reported as Gram-positive and others as Gram-negative.

On agar slants, all authors agreed that this organism produced a fine-beaded, translucent growth which adhered to the agar. The spores have been observed by many workers. They are oval and may be central, eccentric or subterminal.

EXPERIMENTAL METHODS AND MATERIALS

Isolation of Lactose-Fermenting, Spore-Forming Bacilli

In order to determine qualitatively the distribution of Bacillus polymyxa, samples of soil, sewage, compost, surface waters and dungs of cattle, horses, sheep and chickens were studied. An approximately two-gram portion of the sample to be tested was placed in a tube containing 10 ml. of lactose broth. The composition of the lactose broth employed was as follows:

Beef extract - - - - - - - - - - - - - - 1 g.
Proteose peptone No. 3 - - - - - - - - - 10 g.
Lactose - - - - - - - - - - - - - - - - 10 g.

Sodium chloride - - - - - - - - - - - - - - - 5 g.

Brom thymol blue (0.2% alcoholic Sol.)- - - 17.5 ml.

Distilled water - - - - - - - - - - - - - - - 1000 ml.

The medium was adjusted to pH 7.0 with Normal sodium hydroxide. If the sample was of a solid nature, such as the dungs and soil, it was heated to 80° C. for 20 minutes. The water and sewage samples were heated to 80° C. for only ten minutes. After heating, the tubes were cooled rapidly to about 35° C. in a water bath.

After the cultures were cooled, they were then transferred to 125 ml. Erlenmeyer flasks to permit the cultures to grow under aerobic conditions for 24 hours at 37° C. After incubation, inoculations were made from each flask into lactose fermentation tubes of the same composition as described above. All subsequent incubations were at 37° C. The fermentation tubes were observed at 24 and 48 hours for the formation of acid and gas. If acid and gas were formed, these cultures were then streaked onto Bacto-Endo agar plates. Colonies representative of the types that grew on the Endo agar were then transferred to lactose fermentation tubes and observed after 24 and 48 hours for the formation of acid and gas. If acid and gas were formed within 48 hours, the cultures were then transferred to nutrient agar slants, the composition of which was as follows:

Beef extract - - - - - - - - - - - - - 3 g.

Bacto-peptone - - - - - - - - - - - - - 5 g.

Sodium chloride - - - - - - - - - - - - 5 g.

Agar agar - - - - - - - - - - - - - - - 15 g.

Distilled water - - - - - - - - - - - - 1000 ml.

After heating to dissolve, the medium was then adjusted to pH 7.0 with

Normal sodium hydroxide using brom thymol blue as an indicator.

Identification of Organisms

Identification of the organisms isolated from the various sources was made following the methods of Smith, et al. (1952). Group 2 of the genus Bacillus, according to the above authors, has sporangia definitely swollen by oval spores. Spore stains were made by the Snyder modification of the Dorner method in which the fixed smear is covered with a piece of blotting paper and then saturated with Ziehl's carbol fuchsin stain. It is then heated gently for five to seven minutes, decolorized with 95 percent ethyl alcohol and washed in water. A small drop of saturated aqueous nigrosin solution is then spread over the entire film and dried quickly without washing. This method of spore staining produces red spores and colorless vegetative cells against a purplish-black background. Forty-eight hour cultures grown on nutrient agar slants at 37° C. were used for the spore stain.

Gram stains were made with 24 hour cultures grown at 37° C. to demonstrate the position of the spores in the sporangia. These smears were also used to determine whether the cells were Gram-positive or Gram-negative. Gram stains were made of 12 hour cultures grown at 37° C. to determine the size of the vegetative cells.

All the cultures studied were selected on the basis of the fermentation of lactose with the formation of acid and gas. The only other sugar tested for fermentation was salicin.

The composition of the basal media used for fermentation tests was that recommended by Smith, et al. (1952) and was as follows:

Dibasic ammonium phosphate	1 g.
Potassium chloride	0.2 g.
Magnesium sulphate • $7H_2O$	0.2 g.
Yeast extract	0.2 g.
Agar agar	15 g.
Brom cresol purple (0.04% solution)	20 ml.
Distilled water	1000 ml.

The medium was tubed in seven ml. amounts and autoclaved at 121° C. for 15 minutes.

A ten percent aqueous solution of salicin, sterilized separately by autoclaving, was pipetted into each tube in 0.35 ml. amounts so as to result in a 0.5 percent concentration of salicin. The tubes were incubated at 28° C. for 24 hours to check for sterility. The medium was inoculated by stab and streak and observed for growth, acid, and gas at intervals up to four days.

The medium used in testing for the production of acetylmethylcarbinol was that used by Smith, et al. (1952). The composition was as follows:

Proteose peptone	7 g.
Glucose	5 g.
Sodium chloride	5 g.
Distilled water	1000 ml.

The test was made by mixing 0.6 ml. of five-percent alcoholic alpha naphthol solution with one ml. of the culture. Four-tenths ml. of

40 percent potassium hydroxide was then mixed with this solution and let stand for 30 minutes. A cherry-red layer at the top of the mixture indicated a positive test.

The presence of fat globules was demonstrated by inoculating glucose nutrient agar slants with each of the cultures. The slants were incubated at 28° C. for three days, after which time smears were made of each of the cultures. A simple stain was made with aqueous fuchsin, after the technique of Smith et al. (1952).

Simple motility mounts were made by placing a drop of 24 hour nutrient broth culture on a slide, covering with a cover slip and observing under 440x magnification.

Quantitative Determination of Spores

The quantitative determination of the number of lactose-fermenting, spore-forming bacilli was made by collecting a number of samples of soils, dungs, sewage and surface waters from various sources as in the qualitative determination of the presence of lactose-fermenting, spore-forming organisms. Duplicate dilutions were made which may be illustrated diagrammatically as shown in Fig. 1.

One gram or one milliliter of the sample was transferred to a nine-ml. water blank. The tubes were shaken to disperse the sample in the water and were then heated to 80° C. for ten or twenty minutes, depending upon whether the sample was a liquid or a solid. Immediately after heating, the tubes were shaken and cooled to about 35° C. and serial

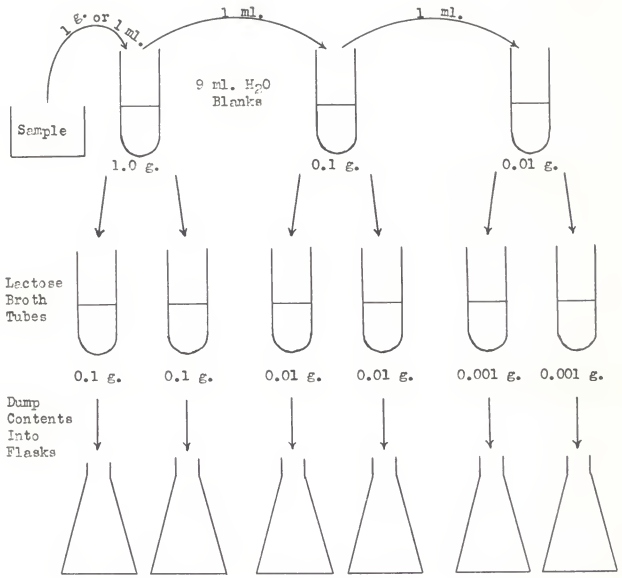

Fig. 1. Diagram illustrating method of making dilutions to determine most probable number of spores.

dilutions were made from the samples in the water blanks to two additional nine ml. water blanks. The three water blanks then contained 1.0, 0.1, and 0.01 grams of sample. Two one-ml. inoculations were then drawn from each of the serial dilutions and inoculated into tubes of lactose broth, thereby giving duplicate inocula of 0.1, 0.01, and 0.001 grams. The six inoculated tubes of lactose broth were then transferred to 125 ml. Erlenmeyer flasks to grow aerobically at 37° C. There were then six flasks containing duplicate amounts of 0.1, 0.01, and 0.001 grams of sample. After 24 hours' aerobic incubation, lactose fermentation tubes were inoculated with one loopful of culture from the flasks. The lactose fermentation tubes were then incubated at 37° C. and observed at 24 and 48 hours for the formation of acid and gas. If acid and gas were formed, then the culture was streaked onto an Endo agar plate which was of the same composition as that used in the isolation of lactose-fermenting, spore-forming bacilli. The streaked Endo plates were incubated at 37° C. for 48 hours, after which time colonies were picked from the plates and transferred to lactose fermentation tubes. These tubes were incubated at 37° C. and observed at 24 and 48 hours for the formation of acid and gas. If acid and gas were formed, the cultures were then transferred to nutrient agar slants.

Duplicate inocula of each dilution were made so as to use the Tables of Most Probable Numbers[1] in determining the number of spores present in each gram of sample.

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[1] American Public Health Association, C. E. Symons, Ed. Standard Methods for the Examination of Water and Sewage. 9th ed.,p.204. New York: American Public Health Association, 1946.

RESULTS

Isolation of Lactose-Fermenting, Spore-Forming Bacilli

As previously mentioned, samples of soil, sewage, compost, surface waters and dungs of cattle, horses, sheep and chickens were tested for the presence of aerobic, lactose-fermenting, spore-forming bacilli. The results obtained from the isolation of these organisms showed them to be very prevalent in the sources studied. Table 3 is a compilation of the results obtained.

Table 3. Frequency of isolation of Bacillus polymyxa from various sources.

Source	No. of samples used from each source	No. of samples yielding B. polymyxa	Percent of samples from each source containing B. polymyxa
Soil	38	30	79.0
Compost	28	24	85.0
Surface waters	25	3	12.0
Raw sewage	35	0	0
Horse dung	33	20	60.5
Sheep dung	37	28	76.0
Chicken dung	40	17	42.5
Cow dung	31	19	61.0

Identification of Organisms

The criterion employed for the isolation of these organisms was the production of acid and gas from lactose in 48 hours. Therefore, all the organisms studied possessed this characteristic.

Spore stains showed that all the colonies studied produced oval spores the sizes of which were 1.2 microns to 1.5 microns at the widest point by 1.5 microns to 2.5 microns in length. The spores were located centrally to terminally. All 24 hour cultures grown at 37° C. were Gram-positive. Twelve hour vegetative cells grown at 37° C. measured 0.6 microns to 0.8 microns by 3.0 microns to 7.5 microns.

In addition to fermenting lactose with acid and gas, all the cultures fermented salicin with acid and gas within 48 hours when grown at 28° C.

Smith, et al. (1952) stated that one of the most reliable tests in the species identification of the genus Bacillus was the production of acetylmethylcarbinol. All except two of the cultures studied produced acetylmethylcarbinol within 48 hours when grown at 28° C. and those two strains produced acetylmethylcarbinol within 96 hours.

The aqueous fuchsin fat stain showed the presence of fat globules in most of the cultures. The fat globules appeared as unstained bodies surrounded by the pink stained cell. The globules were about 0.5 microns in diameter and gave the cell a mottled appearance. Approximately five percent of the cultures did not show the presence of fat globules in three days.

The simple motility mounts showed that all the strains were actively motile.

On the basis of the results of the foregoing tests, each strain was identified as Bacillus polymyxa.

Quantitative Determination of Spores

The distribution of Bacillus polymyxa from any one source is irregular. In some cases the organism could not be isolated from one gram of sample while in other cases, it was isolated from 0.001 gram of sample. B. polymyxa could not be isolated from one ml. samples of raw sewage; nor could it be isolated from the two to three ml. samples used in the qualitative determination.

By use of Tables of Most Probable Numbers, it is possible to calculate the number of Bacillus polymyxa spores per one gram of sample. Table 4 indicates the number of spores present in each of the samples from the different sources.

Table 4. Most Probable Number of Bacillus polymyxa in one gram samples from various sources.

Source	Sample No. 1	Sample No. 2	Sample No. 3	Sample No. 4	Sample No. 5	Logarithmic Average No. per sample
Sheep dung	62	50	29	28	50	41.7
Horse dung	4.6	29	130	0	0	6.39
Human feces	0	0	0	0		0
Soil	29	20	700	37	29	53.4
Cow dung	13	6	28	95	21	21.2
Surface waters	0	0	0	23	0	0.89
Compost	29	0	240	62	240	39.6
Chicken dung	62	13	23	0	4.6	9.44
Raw sewage	0	0	0	0	0	0

DISCUSSION

At the start of this survey, the samples were put into lactose fermentation tubes, heated and then incubated at 37° C. for 48 hours and observed for the formation of acid and gas. Most of the tubes showed acid and gas within 48 hours but the percentage of cases of isolation of the aerobic, lactose-fermenting, spore-forming bacilli on Endo agar was very small. Greer (1928 c) has shown that Clostridium perfringens, which is very prevalent in soil and manures, will inhibit the growth of Bacillus polymyxa. Therefore, it appeared that Cl. perfringens was able to grow well in the bottom of the fermentation tube and as the oxidation-reduction potential was lowered, it grew more uniformly throughout the tube and inhibited the growth of B. polymyxa. For this reason, the samples were incubated for 24 hours at 37° C. in 10 ml. of lactose broth in a 125 ml. Erlenmeyer flask. This procedure exposed a large surface of the medium to the air; the medium was only a few millimeters deep and the Cl. perfringens present was inhibited by the high oxidation-reduction potential.

It is known that the aerobic, lactose-fermenting, spore-forming bacilli are an important cause of false positive presumptive tests in water testing. This survey was conducted to accumulate more accurate information on the distribution of these organisms in nature. Standard Methods for the Examination of Water and Sewage (American Public Health Association, 1946) stated that the presumptive test shall be incubated at 37° C. Since water samples are incubated at 37° C., it was thought that this incubation temperature should be used in the isolation of those organisms that cause

false positive presumptive tests instead of a lower, more optimum temperature for Bacillus species.

Bacillus polymyxa and Bacillus macerans are widely distributed in the soil. It was for this reason that care was exercised in the selection of dung samples. The samples were taken only from the inside of fresh dung that had not been exposed to the air or soil and were put into sterile test tubes. The soil samples were secured from farms surrounding Manhattan, Kansas. The surface water samples were taken from creeks and rivers in the same locality. The raw sewage was collected from the Manhattan, Kansas sewer outfall on the Kansas River.

The samples tested that were of a more solid nature such as soil or dung were heated to 80° C. for 20 minutes to kill all vegetative cells. The liquid samples were heated to 80° C. for only 10 minutes since it was felt that there is a much better penetration of heat through a liquid than there is through a solid.

It was found that if a lactose fermentation tube showed acid and gas in 24 hours but was not streaked on Endo medium until 24 hours after the appearance of acid and gas, the chances of recovering B. polymyxa were less than if the culture was streaked immediately after the formation of acid and gas. Apparently, Cl. perfringens overgrew and inhibited the growth of B. polymyxa. When the cultures were streaked onto Endo agar, there was no growth on the plate because the anaerobic Cl. perfringens would not grow under the prevailing aerobic conditions. All strains of B. polymyxa would oxidize the basic fuchsin in the Endo medium, but the rate of oxidation was a function of time. Some strains

oxidized the basic fuchsin within 24 hours while others oxidized it in 48 or 72 hours. Therefore, it may be said that some colonies were red while others were pink at the end of 48 hours - the time the pure cultures were inoculated into lactose fermentation tubes. Some pink colonies inoculated into lactose fermentation tubes fermented with acid and gas while others formed neither acid nor gas in 48 hours. The slow lactose fermentations on Endo agar may be due to the toxicity of the basic fuchsin contained in the media. In many cases, 48 hour pink colonies picked from Endo agar produced acid and gas within 24 hours when inoculated into lactose fermentation tubes.

A simple method was sought to isolate and determine quantitatively the number of Bacillus polymyxa spores present by making "poured" plates of the organism with Endo agar and with brom thymol blue lactose agar. Dilutions were made of the sample and duplicate plates were made of each dilution. One series of dilutions was mixed with Endo agar and the duplicate series with brom thymol blue lactose agar. It was originally thought that by employing this method, one would be able to determine the number of spores present in one gram of sample. As discussed above, not all strains of B. polymyxa produced red colonies on Endo agar within 48 hours. If a plate was counted that contained between 30 and 300 colonies, it could not be determined which colonies were B. polymyxa because all strains did not form red colonies within 48 hours. The red colonies were not necessarily B. polymyxa because there are several species of the genus Bacillus that ferment lactose with only the production of acid. For these reasons, each colony on the plate would have to be inoculated into lactose

fermentation tubes to determine the production of acid and gas and this would be impractical.

When brom thymol blue lactose agar was used instead of Endo agar in the pour plates, other difficulties were encountered. Since brom thymol blue is not inhibitory like basic fuchsin, the colonies spread profusely over the plates in many cases. This made counting of the colonies virtually impossible. It was observed that a colony may become yellow within 24 hours, indicating that lactose is fermented. However, after an additional 12 hours, the colony may become blue. This phenomenon was believed to be due to the complete utilization of the lactose and the deaminization of amino acids present. If the colony is not observed and picked from the plate when it is yellow in color, it may be mistaken for a non-lactose-fermenting strain.

The only practical method found to determine quantitatively the number of spores present in a sample was that described in the section entitled "Quantitative Determination of Spores". A simpler method of quantitative determination awaits the development of a medium upon which only lactose-fermenting organisms will grow after the sample is heated to 80° C. for 20 minutes, or one which gives a characteristic reaction with B. polymyxa and B. macerans.

It should be noted that B. polymyxa was found in three of 25 samples of surface waters (Table 3). Greer (1928 b) stated that the number of these organisms in water supplies varied with the season of the year. It was observed that the presence of B. polymyxa in surface waters may also depend upon the amount of rainfall during the year. The first 15 samples

of surface waters were collected during a period in which there had been very little or no rainfall in the area. B. polymyxa was not isolated from any of these 15 samples. The last ten samples were collected after rain had fallen in the area and B. polymyxa was isolated from three of ten samples. This seems to indicate that spores of the organism are washed into the water by drainage from the soil.

The cultures isolated during the quantitative determination of the number of B. polymyxa present in each sample were not identified as in the Results section under the sub-section entitled "Identification of Organisms". However, Gram stains were made of 24 hour cultures grown on nutrient agar to ascertain that the isolates were spore-forming bacilli with swollen sporangia. Due to the method of isolation, the organisms elicited were those that ferment lactose with acid and gas. The only two species of Bacillus that ferment lactose with acid and gas are B. polymyxa and B. macerans. All isolates in the quantitative determination were assumed to be B. polymyxa. This assumption was based on the fact that all of the organisms identified in the identification tests were B. polymyxa. The main difference between B. polymyxa and B. macerans, as seen in the following key according to Smith, et al. (1952) is that B. polymyxa produces acetylmethylcarbinol while B. macerans does not produce acetylmethylcarbinol.

Key to the Classification of the Genus Bacillus

Group 1. Sporangia not definitely swollen.

Group 2. Sporangia definitely swollen by oval spores.

a. Spores oval, rarely cylindrical, central to terminal;

spore wall thick, remnants of sporangium sometimes adhering; sporangia definitely swollen; Gram-variable.

b. Gas formed from carbohydrates.

c. Acetylmethylcarbinol produced; crystalline dextrins not formed from starch - - - B. polymyxa

cc. Acetylmethylcarbinol not produced; crystalline formed from starch - - - - - - B. macerans

Group 3.* Sporangia swollen by round spores.

Table 3 shows the frequency of isolation of B. polymyxa from various sources. The organism is prevalent in soil, compost and animal dungs. Apparently, this organism is washed into surface waters from these sources by runoff of rain water. It was not isolated from raw sewage nor was it isolated from four samples of human feces that were tested in the quantitative determination of spores. B. polymyxa was isolated from human feces by Norton and Weight (1924) and Greer (1928 d) and from sewage by Greer (1928 d). However, the percentage of isolation from these sources reported by those workers was so small that B. polymyxa does not appear to constitute a significant part of the normal flora from these sources. Therefore, the presence of B. polymyxa does not seem to be indicative of contamination by human sources, but rather from soil or animal sources.

The highest frequency of isolation of Bacillus polymyxa was from soil and animal dung. Bredemann (1909) stated that this organism was never found in uncultivated soil. It seems strange, however, that there was a greater frequency of isolation from soil and compost than there was

* See page 15

from the dung of most animals. This may possibly indicate that the organism is native to the soil and is ingested by man and animal with his food.

The use of 125 ml. Erlenmeyer flasks for aerobic growth of the organisms in the sample greatly facilitated the isolation of lactose-fermenting, spore-forming bacilli that might have been present in the sample. The original method used was simply to heat and incubate the sample in lactose fermentation tubes, but the percentage of isolation of lactose-fermenting Bacilli was small probably due to inhibition by Clostridium perfringens. The 24-hour aerobic incubation in the Erlenmeyer flasks, inhibited the Cl. perfringens and permitted even small numbers of the aerobic bacilli to reproduce and ferment lactose with acid and gas.

Further research with Bacillus polymyxa might be directed toward devising a medium to be used for the examination of water for coliform bacilli to replace lactose fermentation tubes. This medium should be one which would be inhibitory to B. polymyxa but would permit the growth of coliform organisms with a characteristic reaction. The use of such an improved medium would eliminate bothersome, time-consuming, false positive presumptive tests in the coliform determination caused by B. polymyxa and B. macerans.

Another useful medium which might be devised would be one selective for aerobic, lactose-fermenting, spore-forming bacilli to be used in the isolation of these organisms from their natural habitats.

This study of Bacillus polymyxa has shown that the presence of B. polymyxa in water is not indicative of contamination by man. The presence

of these organisms in water is indicative of contamination by soil or animal dung.

SUMMARY

One hundred forty-one cultures of Bacillus polymyxa were isolated from samples of soil, sewage, compost, surface waters and dungs of cattle, horses, sheep and chickens. The use of an aerobic culture medium for the primary isolation of the organism resulted in a greater percentage of the samples showing the presence of an aerobic, lactose-fermenting, spore-forming Bacillus.

The organisms were identified following the methods of Smith, et al. (1952). All the isolates were identified as Bacillus polymyxa. Duplicate dilutions of 0.1, 0.01, and 0.001 grams of sample were made and the logarithmic averages of the most probable number of spores per gram of sample were determined. In some cases, the organism could not be isolated from a one-gram sample, while in other cases it was isolated from a 0.001 gram sample. The logarithmic averages were used rather than the arithmetic averages in order to minimize the effect that the occasional sample with a very large number of spores per gram of sample might have on the average.

Soil and compost generally contain a larger number of spores per gram of sample than do the animal dungs. This fact might possibly warrant a reversal of thought regarding the natural habitat of Bacillus polymyxa. Since the organism was found in greater numbers in soil and compost than in animal dungs, it is felt that the organism might be ingested by the animal with its food.

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THE DISTRIBUTION OF THE SPORES OF AEROBIC, LACTOSE-FERMENTING, SPORE-FORMING BACILLI IN NATURE

by

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Bacillus polymyxa is an aerobic, lactose-fermenting, spore-forming organism that is often the cause of false positive presumptive tests in the analysis of chlorinated water for coliform bacteria. Therefore, it seemed desirable to obtain more data on the distribution and numbers in nature of this organism. In the past, this organism has been designated as Clostridium polymyxa, Granulobacter polymyxa, Astasia asterosporus, Bacillus mycoides var. ovoethylicus and Bacillus aerosporus. B. polymyxa has been isolated by many workers from a number of sources such as soil, surface water and the dung of farm animals.

PROCEDURE

In order to determine qualitatively the distribution of B. polymyxa, samples of soil, sewage, compost, surface waters and dung of cattle, horses, sheep and chickens were studied. Samples were inoculated into tubes of lactose broth and heated to 80° C. for 20 minutes. After the cultures were cooled, they were then transferred to 125 ml. Erlenmeyer flasks to permit the cultures to grow under aerobic conditions for 24 hours at 37° C. After incubation, inoculations were made from each flask into lactose fermentation tubes and were observed for the formation of acid and gas within 48 hours. Those cultures showing acid and gas were streaked onto Bacto-Endo agar plates and incubated at 37° C. for 48 hours. Colonies representative of the types that grew on the Endo agar were then transferred to lactose fermentation tubes and observed after 24 and 48 hours for the formation of acid and gas. If acid and gas were formed within 48 hours, the cultures were then transferred to nutrient agar slants.

Identification of the organisms isolated from the various sources was made following the methods of Smith, et al. (1952). Gram stains and spore stains were made of all isolates. The isolates were tested for the formation of acid and gas from salicin, the production of acetylmethylcarbinol, the presence of fat globules and for motility.

A quantitative determination of the number of spores of B. polymyxa present in nature was made by making duplicate serial dilutions of the sample to be tested into lactose fermentation tubes. This procedure was similar to the qualitative determination except that the samples were measured and duplicate serial dilutions were made. The dilutions used contained 0.1, 0.01 and 0.001 grams of sample. The number of spores present in the sample was determined from the Tables of Most Probable Numbers based upon the number of fermentation tubes showing the presence of acid and gas.

RESULTS

The results obtained in the qualitative determination of B. polymyxa spores present showed them to be prevalent in most of the sources studied. The percent of samples yielding B. polymyxa was: soil, 79 percent; compost, 85 percent; surface waters, 12 percent; raw sewage, none; horse dung, 60.5 percent; sheep dung, 76 percent; chicken dung, 42.5 percent; and cow dung, 61 percent.

Spore stains of each isolate showed the cells to be definitely swollen by oval spores, and each Gram stain was Gram-positive. All the isolates

fermented salicin with acid and gas, produced acetylmethylcarbinol and were motile. Ninety-five percent of the isolates showed the presence of fat globules. On the basis of these results, all the isolates were identified as B. polymyxa.

The number of spores present in each gram of sample was determined from the Tables of Most Probable Numbers. The logarithmic average of the number of spores per gram of sample was: sheep dung, 41.7 spores; horse dung, 6.39 spores; human feces, none; soil, 53.4 spores; cow dung, 21.2 spores; surface waters, 0.89 spores; compost, 39.6 spores; chicken dung, 9.44 spores; and raw sewage, none. The logarithmic averages were used rather than the arithmetic averages in order to minimize the effect that the occasional sample with a very large number of spores per gram of sample might have on the average.

One hundred forty-one cultures of B. polymyxa were isolated from samples of soil, sewage, compost, surface waters and dungs of cattle, horses, sheep and chickens. The use of an aerobic culture medium for the primary isolation of the organism resulted in a greater percentage of the samples showing the presence of an aerobic, lactose-fermenting, spore-forming Bacillus. Previous experimentation showed that B. polymyxa could be isolated from only a very small percentage of the samples when the samples were inoculated directly into lactose fermentation tubes. Apparently Cl. perfringens overgrew and inhibited the growth of B. polymyxa.

A simple method was sought to isolate and determine quantitatively the number of B. polymyxa spores present in a sample by making "poured" plates of the organism with Endo agar and with brom thymol blue lactose

agar. Dilutions were made of the sample and duplicate plates were made of each dilution. One series of dilutions was mixed with Endo agar and the duplicate series with brom thymol blue lactose agar. It was originally thought that by employing this method, one would be able to determine the number of spores present in one gram of sample. If a plate was counted that contained between 30 and 300 colonies, it could not be determined which colonies were B. polymyxa because all strains did not form red colonies within 48 hours. The red colonies were not necessarily B. polymyxa because there are several species of the genus Bacillus that ferment lactose with only the production of acid. For these reasons, each colony on the plate would have to be inoculated into lactose fermentation tubes to determine the production of acid and gas and this would be impractical.

When brom thymol blue lactose agar was used instead of Endo agar in the pour plates, other difficulties were encountered. Since brom thymol blue is not inhibitory like basic fuchsin, the colonies spread profusely over the plates in many cases. This made counting of the colonies virtually impossible. It was observed that a colony may become yellow within 24 hours, indicating that lactose is fermented. However, after an additional 12 hours, the colony may become blue. This phenomenon is believed to be due to the complete utilization of the lactose and the deaminization of amino acids present. If the colony is not observed and picked from the plate when it is yellow in color, it may be mistaken for a non-lactose-fermenting strain.

Further research with B. polymyxa might be directed toward devising a medium to be used for the examination of water for coliform bacilli to

replace lactose fermentation tubes. This medium should be one which would be inhibitory to B. polymyxa but would permit the growth of coliform organisms with a characteristic reaction. The use of such an improved medium would eliminate bothersome, time-consuming, false positive presumptive tests in the coliform determination caused by B. polymyxa and B. macerans.

Another useful medium which might be devised would be one selective for aerobic, lactose-fermenting, spore-forming bacilli to be used in the isolation of these organisms from their natural habitats.

Soil and compost generally contain a larger number of spores per gram of sample than do the animal dungs. This fact might possibly warrant a reversal of previous thought regarding the natural habitat of B. polymyxa. Since the organism was found in greater numbers in soil and compost than in animal dungs, it is felt that the organism might be ingested by the animal with its food and is native to the soil rather than the intestinal tract of the animal.

This study of Bacillus polymyxa has shown that the presence of this organism in water is not indicative of contamination by man, but is indicative of contamination by soil or animal dung.