J. King Saud Univ., Vol. 4, *Science* (2), pp. 139 – 147, Riyadh (A.H. 1412/1992)

Comparison of Heat Treatment and Ethanol Treatment Procedures Used to Isolate Sporeforming Bacteria and Identification of a Food Poisoning-Causing Bacteria as *Bacillus cereus* Using Ethanol Treatment

Ali Abdullah Salamah
Microbiology Unit, Botany and Microbiology Department, College of Science
King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

(Received 20 May 1991; accepted for publication 25 February 1992)

Abstract. Ethanol treatment and heat treatment procedures for the isolation of sporeforming bacteria were compared. Heat treatment was not able to eliminate all the vegetative cells which were present at high concentration, whereas ethanol treatment eliminated all the vegatative cells with some activation of spores germination. An application of ethanol treatment for the investigation of a food poisoning outbreak was discussed. Fifteen individuals were having a picnic. Seven of them ate fried rice with the lunch meal of the second day. The fried rice was made from a leftover boiled rice prepared during the first day. All seven individuals who had eaten from the fried rice had nausea, abdominal cramps, diarrhea and vomiting, whereas the others who did not eat had no symptoms. *Bacillus cereus* and *B. licheniformis* were isolated from the leftover boiled rice and for several discussed reasons the food poisoning causing agent was identified as *B. cereus*.

Introduction

Enrichment technique is the most effective method to date for the isolation of specific bacteria from environmental samples. The success of the enrichment technique, however, varies with the type of organism desired. The ability of the *Bacillus* and *Clostridium* species to form endospores often makes their recovery from mixed populations easier. The spore selection technique, therefore, is routinely used in the isolation of many environmental *Bacillus* and *Clostridium* species [1; pp. 159-172,2,3,4,5,6,7]. The spore selection technique depends upon resistance of the endospores to heat [2,3,7] or to chemicals [1,4,5,6] and the lack of resistance by non-sporeforming cells to these treatments. The heat resistance of spores, however, varies from species to species [7,8]. Further, the success of the heating method in

eliminating all the vegetative cells present, depends a great deal on the temperature and time of treatment as well as the number of cells/ml present in the sample. For these reasons, several authors have resorted to the use of ethanol instead of heat for the selective isolation of *Bacillus* and *Clostridium* species from environmental samples [4,5,6].

In this study, the effectiveness of a heat treatment and an ethanol treatment commonly used for isolation of bacterial spores were compared. The ethanol treatment was further applied to confirm a suspected *Bacillus cereus* food poisoning by the isolation of the organism and the confirmation of its toxicity.

Materials and Methods

Part I. Comparison of Ethanol Treatment and Heat Treatment Procedures Used Routinely for the Selection of Endosporeforming Bacteria

Organisms and growth conditions

The bacterial strains used in this study are shown in Table 1. The medium of Salamah *et al* [9], which was originally prepared to sporulate *Bacillus pumilus,* was used in this study for the preparation of vegetative cells or spores to be used for heat treatment and ethanol treatment. This medium had the following composition (in grams per liter): Xylose, 2; casamino acids, 2; $FeSO_4.7H_2O$, 5×10^{-4}; $CuSO_4.5H_2O$, 5×10^{-3}; $ZnSO_4.7H_2O$, 5×10^{-3}; $MgSO_4.7H_2O$, 4×10^{-1}; $(NH_4)_2SO_4$, 2; $CaCl_2.2H_2O$, 8×10^{-2}; $MnSO_4.H_2O$, 5×10^{-2}. The medium was buffered at pH 7 with Na_2HPO_4, 5.4; NaH_2PO_4, 7.4. It also included 10μg of biotin per liter. More than 85% of the vegetative cells of *Bacillus* strains sporulated in this medium in less than 48 h of shaking at 100 rpm and 30°C. Further, the growth yields of the nonsporulating bacterial genera were high.

Heat and ethanol selection techniques

Heat treatment and ethanol treatment were performed as described by Koransky *et al* [4] with some modifications as follows:

(i) **Heat treatment.** One-milliliter aliquots of 48 h broth cultures were heated in an 80°C water bath for 15 min and then transfered to a cool water bath at 25°C for 30 min.

(ii) **Ethanol treatment.** An aliquot (0.5 ml) of each 48 h broth culture and 0.5 ml of absolute ethanol were shaken in a screw-capped tube at 50 rpm on an environmental shaker (Precision, USA) for 1 h at room temperature.

(iii) **Quantitative determination.** Untreated, heat treated and ethanol treated cultures were plated on brain heart infusion agar plates after preparing appropriate dilutions (Merck). The colonies were counted after a 48 h incubation at 30°C.

Part II. Use of Ethanol Treatment for the Confirmation of a *Bacillus cereus* Food Poisoning Case

Thirteen individuals, aged 18–26 years had a picnic at the Riyadh desert during the month of March, 1990. This month is usually humid, windy and rainy with a temperature range of 14–34°C. Fried rice was served with a lunch meal on the second day which was prepared from boiled rice leftover from the first day and stored at the tent temperature. Seven individuals who had eaten the fried rice experienced nausea, abdominal cramps, vomiting and diarrhea 1h to 4h after eating, whereas, the remaining 6 individuals who had not eaten from the fried rice did not have any symptoms. Thinking that the leftover boiled rice was the source of the problem, the individuals brought a sample of the leftover rice to this laboratory using a sterile plastic bag. Two grams of this rice were suspended in 10 ml of sterile 0.025 M phosphate buffer at pH 7 and vortexed for 1 min. The vortexed sample was then squeezed in a sterile cloth to remove the rice particles. The filtrate was collected in a sterile beaker and assayed for bacteria. Some of the filtrate was plated on the *Staphylococcus aureus* 110– selection medium (Oxoid) and appropriate dilution of the filtrate was also plated on brain heart infusion agar for total count. Two ml aliquots of the filtrate were each mixed with 2 ml absolute ethanol and were shaken for 1 h at room temperature as described earlier for ethanol treatment. Aliquots of 0.1 ml are removed from the shaking mixtures and surface plated on brain heart infusion agar plates which were incubated at 30°C for 48 h. Morphologically two different colonies (A,B) were observed which were purified by repeated streaking for further morphological and physiological characterization as described by Gibson and Gordon [10, pp. 112-198] and by Smibert and Krieg [11, pp. 409-443].

Mouse lethal-toxin test

The identified *B. cereus* and *B. licheniformis* isolates were inoculated separately into 15 ml of brain heart infusion broth and incubated for 12 h in a shaking incubator (120 rpm) set at 32°C. The culture broths were filtered through a 0.2–μm-pore membrane filter (Millipore Corp.) and the filtrates (0.1 ml) were separately injected into the caudal veins of three outbred Female Swiss-Webster mice, 6 weeks of age, weighing 25 to 30 g (College of Pharmacy, King Saud University). The control mice were injected with a fresh brain heart infusion broth. Death of the mice injected with the filtrate within 30 min was considered to be a positive response.

Results

Part I. Comparison of Ethanol Treatment and Heat Treatment Procedures

The results of ethanol treatment and heat treatment of selected bacterial cultures are shown in Table 1. The ethanol treatment appeared to destroy all the vegetative cells, whereas, the heat treatment employed only seemed to reduce them. For *Bacillus* cultures more colony forming units were obtained after ethanol treatment than after heat treatment, which suggested that the thanol treatment is superior to the heat treatment for selecting members of the genus *Bacillus*.

Table 1. Effect of heat treatment (80°C for 15 min) and ethanol treatment (50% ethanol for 1 h) on the survival of sporeforming and nonsporeforming bacteria in mineral salts broth culture.

Bacteria	Strain no.	Untreated	Heat	Ethanol
Bacillus licheniformis	NCTC 1025	2.4×10^7	2.2×10^7	5.7×10^7
B. megaterium	CBSC 15–4900A	8.4×10^7	2.0×10^7	9.6×10^7
B. cereus	CBSC 15–4870A	3.3×10^7	1.2×10^7	5.4×10^7
B. subtilis	ATCC 6051	2.9×10^7	0.8×10^7	4.1×10^7
B. pumilus	CBSC 15–4905	6.3×10^7	4.3×10^7	7.9×10^7
Escherichia coli	ATCC E11775	7.2×10^7	2.6×10^7	0
Staphylococcus aureus	CBSC 15–5554A	6.7×10^7	4.4×10^7	0
Streptococcus faecalis	CBSC 15–5600A	5.5×10^7	2.3×10^7	0
Yersinia pseudotuberculosis	NCTC 10275	5.2×10^7	4.4×10^7	0
Shigella flexneri	ATCC 29508	4.7×10^7	1.3×10^7	0
Pseudomonas aeruginosa	ATCC E10145	4.2×10^7	1.8×10^7	0

Part II. Use of Ethanol Treatment for the Confirmation of a *B. cereus* Food Poisoning Case

No growth after incubation on the *Staphylococcus* 110– medium eliminated the possibility of *Staphylococcus aureus* being the cause of the food poisoning observed. However, two isolates (A,B) were obtained from the alcohol treated filtrates. One isolate (A) corresponded to a cell count of 2×10^7/g of boiled rice, whereas, the other isolate (B) corresponded to a cell count of 4×10^4/g of boiled rice. According to the morphological and chemical studies shown in Table 2, the isolates were identified as *Bacillus cereus* (A) and *B. licheniformis* (B). This identification was later confirmed by the American Type Culture Collection.

Table 2. Characterization of the boiled rice isolates (A,B).

Characterization	Isolate A	Isolate B
Rods strait.	+	+
Rods curved.	–	–
Length 2.1–3.0.	–	+
Length 3.1–4.0	+	–
Width 0.5–1.0.	–	+
Width 1.1–2.0.	+	–
Cells single.	+	+
Cells chained.	+	+[a]
Ends rounded.	+	+
Endospore formed.	+	+
Sporangium swollen.	–	–
Spore cylindrical.	+	+
Spore oval.	+	+
Sore central.	–	+
Spore terminal.	–	–
Spore subterminal.	+	+
Parasporal crystal.	–	–
Gram positive.	+	+
Gram negative.	–	–
Gram variable.	–	–
Colony opaque.	+	+
Colony entire.	–	+
Colony erose.	+	–
Colony irregular.	–	+[b]
Colony low convex.	+	–
Colony high convex.	–	+
Colony disassociates.	–	+
Colony glistening.	–	+
Colony dull.	+	+[c]
colony dry.	+	+[c]
Colony smooth.	–	+
Colony rough.	+	–
Colony pigmented.	–	–
Cells motile.	+	+
Flagella peritrichous	+	+
Optimum temperature 21–30°C	+	+
Growth at 37°C.	+	+
Growth at 45°C.	+	+
Growth at 50°C.	–	+
Growth at 55°C.	–	+
Growth at 60°C.	–	+
Growth at 65°C.	–	–
Growth in 5% NaCl.	+	+
Growth in 7% NaCl.	+	+
Growth in 10% NaCl.	–	+
Acid from L-arabinose.	–	+
Acid delayed ≥ 14 days.	–	+
Gas from L-arabinose.	–	–
Acid from D–xylose.	–	–
Acid delayed ≥ 14 days.	–	–
Gas from D–xylose.	–	–
Acid from D–glucose.	+	+
Acid delayed ≥ 14 days.	–	–
Gas from D–glucose.	–	–
Acid from lactose.	–	–
Acid delayed ≥ 14 days.	–	–
Gas from lactose.	–	–
Acid from sucrose.	+	+
Acid from D–mannitol.	–	+
Propionate utilization.	+	+
Starch hydrolyzed.	+[d]	+
Polysaccharide hydrolyzed.	+	+
Citrate utilization.	+	+
Hippurate hydrolyzed.	–	–
Gelatin liquified.	+	+
Casein hydrolyzed.	+	+
Methylene blue reduced.	+	+
Methylene blue reoxidized.	–	–
Nitrate reduced.	+	+
Nitrite reduced.	–	–
H_2O_2 decomposed.	+	+
Indole.	–	–
Tyrosine decomposed.	+	–
Dihydroxyacetone.	–	–
Litmus milk acid.	–	–
Litmus milk alkaline.	+	+[d]
Litmus milk peptonized.	+	+
Litmus milk reduced.	+	+
Growth at pH 6.0.	+	+
Growth at pH 5.7.	+	+
pH VP 5198 6.0 or less.	–	–
pH VP 5198 7.0 ± 0.5.	+	–
pH VP 5198 8.0 or more.	–	+
Aerobe.	+	+
Facultative.	+	+
Microaerophil.	–	–
Anaerobe.	–	–
Gas from sealed nitrate.	+	+
Lecithinase.	+	–
Growth in 0.02% azide.	+[e]	–

[a] Only a few cells were in chains.

[b] Mucoid eruptions.

[c] Delayed.

[d] Weak.

[e] Trace.

Mouse lethal-toxin test

The three mice injected with isolate A (*B. cereus*) died within 30 minutes of injection, whereas, the three mice injected with culture filtrate from isolate B (*B. licheniformis*) and the 3 control mice did not die even after 10 days post injection. This indicated that an enterotoxin was produced by *B. cereus* only.

Discussion

Endospores are more resistant to heat than vegetative cells producing them [7,8]. Therefore, heat treatment is often used by investigators for the selection of the sporeforming bacteria from nature. The standard heat treatment of 80°C for 15 minutes, however, does not seem to be adequate to eliminate all vegetative cells, particularly if the vegetative cells are present in high numbers, as indicated by this study. On the other hand, bacterial spores may be up to 100,000 times more resistant to chemicals [1] than the vegetative cells of bacteria. Ethanol treatment, therefore, eliminated all the vegetative cells as indicated by this study. Treatment with ethanol has been shown to increase the germination rate of spores [4]; this explains the increase of the colony forming units for those samples treated with ethanol as compared with untreated and heat treated samples.

B. cereus is widely distributed, having been isolated from rice, spices, meat and egg and dairy products [12,13]. Those which are toxigenic have been shown to produce two different clinical syndromes, one similar to *Clostridium perfringens* food poisoning with an average incubation period of 10–12h, while the other syndrome is similar to *Staphylococcus aureus* food poisoning with an average incubation period of 1–6h [14]. The fact that only the individuals who ate the fried rice became ill suggested that the leftover boiled rice was probably the cause. It is also clear from the results of this investigation that the cause of the outbreak was *B. cereus* for the following reasons:

(i) The symptoms fit the classic description of *B. cereus* food-borne illness which resembles the one caused by *S. aureus.*

(ii) *S. aureus* was not detected.

(iii) The food poisoning syndrome caused by *B. cereus,* and resembling *S. aureus* syndrome, is commonly associated with the consumption of improperly stored boiled rice [12,15,16,17].

(iv) *B. licheniformis* has not been ever implicated in food poisoning.

(v) The estimated number of *B. cereus*/g of rice was not only higher than that of *B. licheniformis* but in the range often associated in outbreaks reported by others [18, pp.349-375].

(vi) Animal inoculations of filtrates from both isolates have shown that an enterotoxin was produced by the isolate identified as *B. cereus*.

Finally, the ethanol treatment method has proven its value as a means for the selection of endosporeforming bacteria. More studies however, are needed for the isolation of *B. cereus* from other environments using this method.

Acknowledgement. This research (Bot/1407/09) was supported by the Research Center, College of Science, King Saud University, Riyadh, Saudi Arabia. I would like to thank Mr. Shamsher Khan for his vauable suggestions and for reading the manuscript.

Reference

[1] Waites, W.M. and Bayliss, C.E. "The Preparation of Bacterial Spores for Evaluation of the Sporicidal Activity of Chemicals." In *Microbial Growth and Survival in Extremes of Environment* (Gould, G.W. and Corry, J.E.L. eds.). London: Academic Press, 1980.

[2] Travers, R.S.; Martin, P.A.W. and Reichelderfer, C.F. "Selective Process for Efficient Isolation of Soil *Bacillus spp.*" *Appl. Environ. Microbiol.*, 53 (1987), 1263-1266.

[3] Mahler, I.; Levinson, H.S.; Wang, Y. and Halvorson, H.A. "Cadmium-and Mercury-Resistant *Bacillus* Strains from a Salt Marsh and from Boston Harbor." *Appl. Environ. Microbiol.* 52 (1986), 1293-1298.

[4] Koransky, J.R.; Allen, S.D. and Dowell, V.R. "Use of Ethanol for Selective Isolation of Sporeforming Microorganisms." *Appl. Environ. Microbiol.* 35 (1978), 762-765.

[5] Hyatt, M.T. and Levinson, H.S. "Water Vapor, Aqueous Ethyl Alcohol, and Heat Activation of *Bacillus megaterium* Spore Germination." *J. Bacteriol.* 95 (1968), 2090-2101.

[6] Craven, S.E. and Blankenship, L.C. "Activation and Injury of *Clostridium perfringens* Spores by Alcohols." *Appl. Environ. Microbiol.* 50 (1985), 249-256.

[7] Gombas, D.E. "Bacterial Spores Resistance to Heat." *Food Technol.* 37 (1983), 105-110.

[8] Beaman, T.C. and Gerhardt, P. "Heat Resistance of Bacterial Spores Correlated with Protoplast Dehydration, Mineralization and Thermal Adaptation." *Appl. Environ.Microbiol.* 52 (1986), 1242-1246.

[9] Salamah, A.A. *Purification, Characterization and Immunochemical Neutralization of Bacillus pumilus Cytoplasmic and Endospore Enolase.* Fresno, USA: Fresno State University, 1980.

[10] Gibson, T. and Gordon, R.E. "Genus 1. Bacillus." In *Bergey's Manual of Systematic Bacteriology,* Vol.1, (Peter H.A. and Sneath, M.D. eds.). Baltimore: Williams and Wilkins Co., 1986.

[11] Smibert, R.M. and Krieg, N.R. "General Characterization." In *Manual of Methods for General Bacteriology* (Krieg, N.R. ed.). USA: American Society of Microbiology, 1981.

[12] Johnson, K.M. "*Bacillus cereus* Foodsborne Illness-an Update." *J. Food Prot.*, 47 (1984), 145-153.

[13] Wong, H.C. "Incidence and Characterization of *Bacillus cereus* Isolates Contaminating Dairy Products." *Appl. Environ. Microbiol.*, 45 (1988), 699-702.

[14] Thompson, N.E.; Ketterhagen, M.J.; Bergdoll, M.S. and Schantz, E.J. "Isolation and Some Properties of an Enterotoxin Produced by *Bacillus cereus*." *Infect. Immun.*, 43 (1984), 887-894.

[15] Chung, K.T. and Sun, H.L. "Distribution and Characteristics of *Bacillus cereus* Isolated from Rice in Taiwan." *J. Food Sci.*, 41 (1986), 1208-1212.

[16] Schiemann, D.A. "Occurrence of *Bacillus cereus* and the Bacteriological Quality of Chinese (take-out) Foods." *J. Food Prot.*, 41 (1978), 450-454.

[17] Terranova, W. and Black, P.A. "*Bacillus cereus* Food Poisoning." *N. Engl. J. Med.*, 298 (1978), 143-144.

[18] Jay, J.M. "Food Poisoning Caused by Gram Positive Sporeforming Bacteria." In *Modern Food Microbiology* (Jay, J.M. ed.). New York: D. Van Nostrand Co., 1978.

مقارنة طريقتي المعاملة بالحرارة والمعاملة بالإيثانول المستخدمتين في عزل البكتيريا المكونة للأبواغ، وتعريف بكتيريا مسببة لتسمم غذائي على أنها بسلس سيريس بوساطة المعاملة بالإيثانول

علي عبدالله السلامة

وحدة الأحياء الدقيقة، قسم النبات والأحياء الدقيقة، كلية العلوم، جامعة الملك سعود، ص.ب ٢٤٥٥، الرياض ١١٤٥١، المملكة العربية السعودية

(استلم في ١٤١١/١١/٧هـ؛ قبل للنشر في ١٤١٢/٨/٢٢هـ)

ملخص البحث. قورنت طريقتي المعاملة بالحرارة والإيثانول المستخدمتين في عزل البكتيريا المكونة للأبواغ. المعاملة بالحرارة غير قادرة على التخلص من كل الخلايا الخضرية الموجودة بتركيز عالٍ، بينما المعاملة بالإيثانول أدت إلى التخلص من كل الخلايا الخضرية مع تنشيط إنبات الأبواغ. تمت مناقشة تطبيق فعلي للمعاملة بالإيثانول وذلك بتقصي تسمم غذائي بكتيري. خمسة عشر شخصًا كانوا في رحلة حيث أكل سبعة منهم أرز محمر مع وجبة الغداء لليوم الثاني من الرحلة، هذا الأرز المحمر عمل من أرز مسلوق متبقي من اليوم الأول للرحلة. جميع الأشخاص السبعة الذين أكلوا من الأرز المحمر كان لديهم غثيان، تقلصات معدية، إسهال، قيء، بينما لم تظهر أيًا من هذه الأعراض على الأشخاص الذين لم يأكلوا. لقد عزلت كلًا من بسلس سيريس وبسلس ليشنيفورمس من الأرز المسلوق المتبقي من اليوم الأول، ولأسباب تمت مناقشتها عرف المسبب البكتيري للتسمم على أنه بسلس سيريس.