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AUTOMATED BIOCHEMICAL IDENTIFICATION OF BACTERIAL FISH PATHOGENS USING THE ABBOTT QUANTUM II

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ABSTRACT: The Quantum II, originally designed by Abbott Diagnostics for automated rapid identification of members of Enterobacteriaceae, was adapted for the identification of bacterial fish pathogens. The instrument operates as a spectrophotometer at a wavelength of 492.600 nm. A sample cartridge containing 20 inoculated biochemical chambers is inserted in the path of the analyzing beam. Reactions are converted into a 7-digit octal biocode, relayed via a sensor to the memory module, and compared to biocodes preprogrammed in the memory. An identification is then printed. Presently, the Quantum II is capable of identifying human strains of *Aeromonas hydrophila* and *Edwardsiella tarda*. This study was initiated to determine the feasibility of expanding the use of the Quantum II to include identification of bacterial fish pathogens. Ten to 50 isolates of *Edwardsiella ictaluri, Serratia liquefaciens, Yersinia ruckeri, Aeromonas hydrophila,* typical *Aeromonas salmonicida,* and atypical *Aeromonas salmonicida* were utilized to determine optimal incubation conditions, relative stability of the biochemicals, and ability to obtain consistent biocode numbers.

After sorting the octal biocodes from the 169 isolates into groupings using a cluster analysis technique, it was shown by a Chi-square goodness of fit test that isolates of a given species were sorted into the same cluster group at a frequency of at least 99%. Results of this study illustrate the usefulness of the Quantum II BID system for the identification of bacterial fish pathogens not contained within the system's memory module.

Key words: Abbott Quantum II, automated bacterial identification, octal biocodes, bacterial fish pathogens, biochemical tests, experimental study.

INTRODUCTION

Automated identification systems for bacterial pathogens are currently available for human medical applications. Similar methods, however, are lacking for the rapid identification of bacterial fish pathogens. Rapid identification of fish pathogens in the genera *Aeromonas*, *Edwardsiella*, *Serratia*, and *Yersinia* is highly desirable to facilitate prompt treatment of infected fish in order to reduce stock mortality. From an economical and management standpoint, it is important to develop new methodologies for the rapid identification of fish pathogens. Therefore, it was the intent of this study to ascertain the possible adaptation of an automated system, the Quantum II, for use in identifying common fish pathogens.

The Quantum II bacterial identification system (BID) is an automated identification instrument manufactured by Abbott's Diagnostic Division (Irving, Texas 75015, USA). The instrument is capable of rapid identification of commonly encountered human disease organisms, primarily members of the Enterobacteriaceae, and some nonfermentative organisms. Identification is based upon a battery of 20 biochemical substrates contained within a plastic cartridge; following inoculation and incubation, the microorganism's identity is spectrophotometrically determined. By comparing the reaction profile to a probability matrix in a memory module, an identification of the organism along with a percent likelihood and an octal biocode number can be obtained. Fortunately, in the event of a "no identification," a reaction profile and biocode number are still obtained. Utilizing these data, one can group isolates having similar biocode numbers. It is possible to build a database for identifying bacterial species not contained within the memory module if the biocodes for a particular set of isolates are reproducible and stable under similar incubation conditions.

The objective of this study was to de-

termine the feasibility of constructing a database for the identification of commonly encountered cold and warm water bacterial fish pathogens. To achieve this goal, it was necessary to determine optimal incubation conditions for each organism and to analyze the biocodes to ascertain whether sufficient and stable differences existed to separate each isolate.

MATERIALS AND METHODS

A total of 169 clinical isolates of freshwater bacterial fish pathogens maintained in our laboratory's culture collection were used. Species used and biotypically identified by conventional means included 49 isolates of *Yersinia ruckeri*, 15 isolates of *Serratia liquefaciens*, 28 isolates of *Aeromonas hydrophila*, 60 isolates of *Edwardsiella ictaluri*, and 17 isolates, including both atypical and typical strains, of *Aeromonas salmonicida*. *Edwardsiella ictaluri* isolates included those cultured from fish of the genera *Ictalurus*, *Clarius*, and *Danio*. The regional distribution of isolates included the northwestern, midwestern, eastern, and southeastern United States, Great Britain, and the Far East (Asia).

Isolates were subcultured to 5% bovine blood agar (BAP) and incubated at 30 C for 24 hr. After incubation, sample preparation consisted of picking well-isolated colonies and transferring them to a 5 ml sterile 0.85% saline blank. The final adjusted suspension was equivalent to a 0.5 McFarland ($BaSO_4$) turbidity standard. The cytochrome oxidase reaction was determined from the BAP for each isolate.

The plastic chambers of the Quantum II BID cartridge each contain a lypholyzed biochemical substrate and appropriate pH indicator. From chamber 1 to 20, these include: glucose, lysine, ornithine, citrate, malonate, esculin, urea, adonitol, arabinose, inositol, lactose, mannose, rhamnose, sorbitol, sucrose, xylose, arginine, indole, acetamide, and polymyxin B. Position 21 of the reaction profile is the oxidase reaction. The cartridge inoculation procedure followed manufacturer's recommendations (Quantum II Dual Wavelength Analyzer Operations Manual, Abbott Laboratories, Diagnostic Division, Irving, Texas 75015, USA). Briefly, the paper strip lining the cartridge top was perforated with the punch provided. Using a pre-calibrated pipettor, 200 µl of the turbidity-adjusted bacterial suspension was dispensed into each of the 20 chambers. The top of the cartridge was sealed with an adhesive plastic strip designed with chamber 18 open to facilitate addition of Kovac's reagent for determination of indole. Following loading and sealing, each cartridge was placed under appropriate incubation conditions.

After the incubation period, the indole reaction was determined by adding 50 µl of Kovac's reagent to chamber 18 in each cartridge. Kovac's reagent was prepared by combining 5 g p-dimethylamino benzaldehyde (Eastman Kodak Company, Rochester, New York 14650, USA), 75 ml n-amyl alcohol (J. T. Baker Chemical Company, Phillipsburg, New Jersey 08865, USA), and 25 ml concentrated hydrochloric acid (J. T. Baker Chemical Company, Phillipsburg, New Jersey 08865, USA). A pink to red color was recorded as a positive result. The results of both the indole and oxidase reactions were manually entered into the instrument at the time of cartridge insertion.

The Quantum II functions as a spectrophotometer operating at a fixed wavelength of 492.600 nm. As the beam is directed through each chamber in turn, it is detected by a photocell, thus producing a reaction profile for the cartridge. Each reaction profile is applied to a probability matrix within the memory module. If the species identity has been programmed into the memory module, an identification, percent likelihood, and biocode number will be printed. If no identification is obtained, a biocode number for each isolate is still calculated. The biocode is a numeric expression of the reaction profile, with each digit representing a group of three biochemical tests. For each triplet, the first position is assigned the number 1, followed by the numbers 2 and 4 for the second and third positions, respectively. If only position 1 of a given triplet is positive, then the octal digit is recorded as 1. If positions 1 and 2 are positive, then the octal digit is 3 (1+2). If all positions are positive the assigned octal digit is 7 (1+2+4). For each triplet, there are eight possible digits (0 to 7) which can be assigned.

Each species of bacterial fish pathogen had an optimal incubation temperature which was defined as that temperature which resulted in good growth. Optimal incubation time was ascertained by determining the stability of the reaction profile and biocode number by repeated examination for 72 to 96 hr. Representative isolates of the various species were examined using the BID at time intervals of 5, 24, 48, and 72 hr. The shortest incubation time which resulted in completed reactions with no subsequent changes in the reaction profile at a later time was defined as the optimal incubation time (Table 1).

All octal biocodes were sorted into groups using a cluster analysis program (SAS; SAS Institute Inc., Cary, North Carolina 27511, USA. The cluster analysis program utilized Ward's method—a hierarchial clustering algorithm. In

TABLE 1. Optimal incubation conditions for the various species of fish pathogens.

Species	Time (hr)	Temperature (C)
Aeromonas hydrophila	5	37
Aeromonas salmonicida[a]	72	20
Edwardsiella ictaluri	24	30
Serratia liquefaciens	48	30
Yersinia ruckeri	48	30

[a] Both typical and atypical isolates.

Ward's method the distance between two clusters is the sum of squares between two clusters summed across all the variables. Using the established criteria of placing like species in the same sorted grouping at least 99% of the time, the data were analyzed by a Chi-square goodness of fit test.

RESULTS

Optimal incubation time and temperatures for each species of pathogen were determined and tabulated (Table 1). At temperatures of 37 C, the incubation time is 5 hr. This reflects conditions under which the instrument was designed. As the temperature is decreased to 30 C the incubation time is extended to 24 or 48 hr. For both *S. liquefaciens* and *Y. ruckeri* the reactions in the cartridge were incomplete at 24 hr but resulted in stable biocodes at 48 hr. For *E. ictaluri* very little change in the number sequence resulted after extending the incubation time from 24 to 48 hr. Due to the low growth temperature used for *A. salmonicida* the time was extended to 72 hr. At this temperature no significant dehydration occurred in the cartridge chambers, even for the extended period.

The biocode numbers from all 169 isolates were sorted into five groups by a cluster analysis (Table 2). Using a Chi-square goodness of fit test with our established criteria of like species being placed in the same cluster group at least 99% of the time, all species used in this study met the criteria. For example, in cluster group one (*Y. ruckeri*) the χ^2 was 0.5 (df = 1, $P > 0.05$). In cluster group three, all *Aeromonas hydrophila* were grouped without distinction as to virulent or nonvirulent strains. Similarily, cluster group five provided no subgroupings for typical and atypical *Aeromonas salmonicida*.

Table 3 illustrates the most frequently occurring octal digit for each triplet of biochemicals. The higher the percent occurrence of a digit, the more stable the triplet. By combining the most frequently occurring digit for each triplet, a theoretical optimum biocode was determined for each organism. Commonly occurring biocode numbers were tabulated in order of decreasing frequency for comparison with those numbers obtained in Table 3.

DISCUSSION

The most significant finding in this study was that common fish pathogens could be grouped into species-specific groups based on the BID biocodes. Even with observed intraspecies variations (i.e., one or more digits differing from the optimal biocode), Chi-square goodness of fit test showed that like species were placed in the same group-

TABLE 2. Results of the cluster analysis between biocode numbers and species groupings.

Species	Cluster group 1	2	3	4	5
Yersinia ruckeri	49[a] (100%)[b]				
Serratia liquefaciens		15 (100%)			
Aeromonas hydrophila			28 (100%)		
Edwardsiella ictaluri				60 (100%)	
Aeromonas salmonicida					17 (100%)

[a] Number of isolates in each group.
[b] Percentage of isolates of a particular species sorted into each cluster group.

TABLE 3. Most common octal digits and their % frequencies occurring at each biochemical triplet for each species grouping.

Species	Biochemical triplet*						
	GLO	CME	UAA	ILM	RSS	XAI	APO
Yersinia ruckeri	7 (100)	1 (89.8)	1 (38.7)	4 (100)	0 (61.2)	2 (91.8)	0 (32.7)
Serratia liquefaciens	7 (100)	5 (100)	5 (100)	5 (66.7)	6 (100)	3 (73.3)	0 (60)
Edwardsiella ictaluri	3 (56.7)	1 (93.3)	1 (51.7)	0 (100)	0 (100)	2 (83.4)	2 (78.3)
Aeromonas hydrophila	1 (68.4)	4 (89.5)	4 (36.8)	4 (63.2)	6 (42.1)	6 (73.7)	4 (73.7)
Aeromonas salmonicida	7 (70.6)	3 (76.5)	1 (100)	1 (100)	0 (94.1)	6 (70.6)	7 (82.4)

* GLO—glucose, lysine, ornithine; CME—citrate, malonate, esculin; UAA—urea, adonitol, arabinose; ILM—inositol, lactose, mannitol; RSS—rhamnose, sorbitol, sucrose; XAI—xylose, arginine, indole; APO—acetamide, polymyxin B, oxidase.

ing at least 99% of the time. Because of this, one can identify organisms which are not programmed into the memory module. A biocode obtained for a particular clinical isolate can be compared with a list of theoretical biocodes for common pathogens as obtained in the database in this study.

The seven digit biocodes from each of the five cluster groupings were used to calculate a theoretical biocode for each of the species. These calculations were based on the frequency of octal digits occurring for each triplet (Table 3). The digit with the highest percent frequency occurring for a given triplet was assigned as the optimal number for that triplet. As an example, all 49 isolates of *Y. ruckeri* had 7 at the first triplet (i.e., consisting of positive reactions for glucose, lysine, and ornithine). Therefore 7 was selected as the optimal digit at that triplet for that species. For *A. salmonicida* the digit occurring at the highest frequency for the first triplet was 7 (i.e., 70.6% of the 17 isolates had 7 as the first triplet digit). Therefore 7 was selected as the optimal digit for that triplet and species. Optimal digits at respective triplets for all species were calculated in a similar manner (Table 3). These artificially derived digits were compared to observed biocodes (Table 4). It can be seen that the theoretical biocode based on triplet frequencies was not always experimentally observed in high frequency. This was due to variations existing among isolates of a given species with respect to utilization of particular biochemical substrates. For example, *Y. ruckeri* isolates differing only in ability to utilize sorbitol resulted in two different biocodes; one number matching the theoretical biocode and the other deviating by one digit. As the number of variable reactions for a given species increases, the chances of the experimental biocode matching the theoretical biocode decrease.

Very few biochemical substrates appeared to be unstable at the incubation conditions used in this study. The least stable reactions were hydrolysis of urea (chamber number 7) and arginine dihydrolase (chamber number 17). Murray et al. (1984) have previously reported unstability of urea and arginine along with inhibition of polymyxin B and esculin. Other previously reported unstable reactions include acetamide, malonate and polymyxin B (Pfaller et al., 1986).

Biochemical results which deviated from the expected for *Edwardsiella ictaluri* included ornithine, citrate and urea. The octal digit for the first triplet containing glucose, lysine and ornithine changed from 3 to 7 with time. This was due to changes in chamber number 3. At 24 hr each of the two digits appeared an approximately equal number of times: 3 at 57% and 7 at 43%. Therefore, it was concluded that at a specific time interval, either 3 or 7 was an acceptable digit. If the cartridge is allowed to incubate for 48 hr, this digit will be a 7 (i.e., at 48 hr, ornithine is positive for 100% of the isolates). For *Serratia li-*

TABLE 4. A sample of commonly observed biocodes and their frequency of occurrence for the different species groups.

Species	Commonly observed biocodes	Frequency (%)
Yersinia ruckeri	7114020*	18
	7114223	16
	7114022	14
	7114221	12
	7114220	2
Serratia liquefaciens	7557630	27
	7555630	20
	7555631	20
	7555610*	13
	7555611	13
	7557631	6
Edwardsiella ictaluri	3000022	20
	7010022	20
	3010022	15
	3000020	10
	7010020	5
	3000002	3
	7010002	3
	3110022*	2
Aeromonas hydrophila	1444664*	14
	1454464	4
	1454264	4
	1454666	4
Aeromonas salmonicida	7310067*	41
	3310067	12
	7710027	12
	7710025	12
	7310065	6
	5310067	6

* Theoretical optimal biocode, see Table 3.

quefaciens, the biochemical substrates which occurred at a higher frequency than expected were lactose and arginine.

The *Aeromonas salmonicida* isolates included both typical and atypical strains. Based on biocodes, all *A. salmonicida* were recognized as a single group distinct from other species, but subgroups were not distinguished. This may be due to the small number of isolates studied and/or the fact that distinguishing characteristics for these subgroups were not contained in the characteristics available in the BID cartridge.

The identity of the isolates used in this study was not contained in the memory module. Consequently, the majority of the isolates examined printed out a "no identification"; in a few cases, a possible identification with a low percentage likelihood (<30%) appeared. In no case did the BID falsely identify an isolate with a high likelihood (>80%). This would indicate that the reaction pattern obtained in the BID cartridge for these bacterial fish pathogens did not overlap significantly with any species contained in the memory module. Further, this study illustrates the flexibility of the Quantum II BID system for use in the identification of organisms not in the memory module, and also a potential methodology for the extension of the existing memory module to accommodate the needs of a particular diagnostic laboratory.

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