Commercial phenotypic tests (API 20E) in diagnosis of fish bacteria: a review

N. Topic Popovic, R. Coz-Rakovac, I. Strunjak-Perovic

Rudjer Boskovic Institute, Zagreb, Croatia

ABSTRACT: The available data concerning rapid identification of fish bacteria via commercial phenotypic tests demonstrate that there is no agreement regarding the choice of the tests. However, API 20E, an identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods developed for clinical specimens, seems to be increasingly used for the identification of fish pathogens. In this review, adaptation of API 20E for fish bacterial isolates and its distinctiveness for fish bacteria was assessed. Some strains are wrongly identified because they are not included in the database of API 20E system. API 20E reactions should be compared with the diagnostic schemes based on reactions in conventional phenotypic tests. Due to their significance for fish health and impact on the aquaculture, and because of the need for their rapid identification, some important fish bacteria should be included in the API 20E system, such as *Yersinia ruckeri*, *Edwardsiella ictaluri*, *Vibrio anguillarum*.

Keywords: API 20E; bacteria; diagnostics; fish

Contents

1. Introduction

The effective control and treatment of aquatic animal diseases requires an access to diagnostic tests that are rapid, reliable and highly sensitive. Automated identification systems for bacterial pathogens are currently available for human medical and some veterinary applications. Similar specific methods for rapid identification of bacterial fish pathogens are missing. Still, for many fish pathogens, some of these conventional phenotypic tests are used for their identification.

Some commercially available diagnostic kits have been introduced into routine laboratory diagnostics of fish pathogens, such as API 20E, API ZYM, API 20NE, API 50 CH, API Rapid ID 32 (bioMerieux, Marcy-l'Etoile, France), Biolog MicroPlates GN2, GP2, AN (Biolog, Inc., Hayward, CA, USA), Enterotubes, BBL Crystal E/NF (Becton-Dickinson & Company, Franklin Lakes, NJ, USA), Minitek (no longer available from the manufacturer, Becton-Dickinson & Company), Bionor Aqua (Bionor, Skien, Norway) systems and some others. Of these, API 20E rapid identification system has been the most widely used for identification of fish pathogenic bacteria.

The API 20E kit is an identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods, which uses 21 standardized and miniaturized biochemical tests and a database. It consists of 21 microtubes containing dehydrated substrates. These tubes are inoculated with a bacterial suspension which reconstitutes the media. During incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The reactions are read

according to the table provided and the identification is done using the software provided by the manufacturer on the Internet, the apiweb. A seven-digit profile is obtained. API 20E ratings are based on three parameters, including the likelihood of a match between the unknown organism's profile and the computer profile, the relative value between the likelihood of the first and the likelihood of the second choice, and the number of tests against the first choice (Brown and Leff, 1996).

2. Adaptation of API 20E for fish bacterial isolates

According to the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2003), bacteriology of fish is generally conducted at temperatures between 20 and 26°C. However, some bacteria need 15°C for optimal growth and many of the bacteria isolated from warm water fish may be incubated at 30°C or 37°C to accelerate the diagnostic steps. The manufacturer of API 20E suggests the incubation of strips at 36°C ± 2°C for 18–24 hours and the use of API NaCl 0.85% Medium or API Suspension Medium (saline) or any tube containing sterile saline or sterile distilled water, without additives. However, since most *Vibrio* species are halophilous, the manufacturer suggests suspending the bacteria in API NaCl 0.85% medium if a *Vibrio* is suspected.

There have been several interpretations as to the adaptation of API 20E for fish bacteria. Some authors proceeded with API 20E identification according to the manufacturer's suggestions, be it freshwater or sea fish (Athanassopoulou et al., 1999; Li et al., 1999; Zorilla et al., 1999; Jaksic et al., 2002; Villamil et al., 2003), while some recommended the incubation of API 20E strips for fish isolates at 22°C for 36 hours (Santos et al., 1993), at 27°C for 24 hours (Kozinska et al., 2002), and others (Biosca et al., 1993; Doukas et al., 1998; Arias et al., 2003; Austin et al., 2003; Padros et al., 2006) incubated strips at 25°C and performed readings in 24 and 48 hours. For the identification of sea fish isolates, Grisez et al. (1991) suggested the following modifications of the prescribed method for the inoculation of the test strip: increasing of the incubation time to 48–72 hours, lowering the incubation temperature to 26°C, using a suspension of 1.5% saline as inoculum, and allowing only fermentation of sugars by sealing these cups with sterile mineral oil. In our previous study we described sea fish bacteria determination according to Grisez et al. (1991) additionally lowering the incubation temperature to 22°C (Coz-Rakovac et al., 2002; Topic Popovic et al., 2004). Bertone et al. (1996) used modified API 20E for sea fish bacteria, particularly a 2% NaCl modified diluent, and incubation at 25°C for 24–72 hours, according to MacDonnel et al. (1982).

3. Distinctiveness of API 20E for fish bacteria

In its current identification database, API 20 E lists the following bacteria that are potential bacterial pathogens, according to Austin and Austin (1999): *Acinetobacter* spp., *Aeromonas hydrophila*, *A. salmonicida* subsp. *salmonicida*, *Citrobacter freundii*, *Edwardsiella tarda*, *Escherichia vulneris*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Moraxella* spp., *Pantoea* spp., *Photobacterium damsela*, *Plesiomonas shigelloides*, *Providencia rettgeri*, *Pseudomonas aeruginosa*, *P. fluorescens*/*putida*, *Salmonella arizonae*, *Serratia liquefaciens*, *Serratia plymuthica*, *Shewanella putrefaciens*, *Vibrio alginolyticus*, *V. cholerae*, *V. vulnificus*. Moreover, the same authors suggested diagnostic schemes based on API 20E system readings which need to be updated. The biochemical data, along with numerical profiles obtained from the strips can be used for further analyses and comparison with classical test reactions using standard plates and tube media until the final identification of the strain is done.

According to Santos et al. (1993) some motile *Aeromonas* strains in the API 20E system gave false positive or negative reactions for lysine decarboxylase (LDC), Voges Proskauer (VP), gelatinase (GEL) and fermentation of arabinose (ARA), sorbitol (SOR) and rhamnose (RHA). Regardless of these false reactions, the API system identified 65% of motile *Aeromonas* isolates. The strains identified as *A. hydrophila* were divided into 26 profile numbers with four of them being predominant (3047125, 3247125, 3247127, and 3247137). Israil et al. (2003) demonstrated that API 20E had some limits in the diagnostics of the bacterial genera of aquatic origin *Vibrio* and *Aeromonas*, when comparatively tested with classical biochemical reactions, mainly being discordant for GEL, LDC, arginine dihydrolase (ADH), saccharose (SAC), mannitol (MAN), and inositol (INO) reactions. Important biochemical reactions in conventional tests were compared

with counterpart reactions in the API 20E system to evaluate its accuracy for the identification of motile *Aeromonas* spp. isolated from fish (Toranzo et al., 1986). False negative or false positive reactions were detected in VP, indole (IND), GEL, ARA, LDC reactions, while good correlations, as opposed to Israil et al., (2003), were found for MAN, INO, ADH and ornithine decarboxylase (ODC) reactions. When identifying phenotypic characteristics and pathogenicity of *Aeromonas* genomospecies isolated from common carp (*Cyprinus carpio* L.) Kozinska et al. (2002) found that all evaluated strains were positive in API 20E for ortho-nitrophenyl-galactopyranosidase (ONPG), GEL and glucose (GLU) reactions. Moreover, MAN and ADH showed positive for each strain in API 20E as well as in conventional tube tests. Invariably, negative results were observed for H_2S production, urease (URE) and INO reactions when tested by API 20E. *A. salmonicida* strains confirmed serologically by FAT and by the standard biochemical tests gave 2006104 and 0006104 API profile and were correctly identified as *A. salmonicida* ssp. *salmonicida* (McCasland and True, 2001) with a note that *A. salmonicida* generally fails to produce positive reactions for ONPG, ADH and LDC when incubated at room temperature (22–25°C). For these tests, longer incubation times are required.

Although API 20E was originally designed for the identification of members of the family Enterobacteriaceae, some members of Vibrionaceae have also been included in its database. However, the number of listed species affecting fish health is very incomplete. *Vibrio vulnificus* is a pathogenic species comprising two biotypes that can be distinguished by some phenotypic traits and host range. Biotype 1 is associated with human infections and can also be recovered from water and shellfish, whereas biotype 2 comprises only strains pathogenic to eels (Biosca et al., 1993). When they tested *V. vulnificus* strains by the API 20E system, Biosca et al. (1993) noticed some false negative reactions, mainly in citrate (CIT) utilization. *V. vulnificus* generated the following profiles for biotype 1: 5146105, 5246105, 5346005, 5346105, which were all correctly identified as *V. vulnificus*, and for biotype 2: 4206005, 5006004, 5006005, 5106005, 5206005, 5306005, which were recognized as *V. vulnificus*, but more often as *Burkholderia cepacia*. The authors suggest an oxidative-fermentative (O/F) test to ensure rapid discrimination between *B. cepacia* and *V. vulnificus*. Heterogeneity was recorded in phenotypic characterization by the API 20E identification system when identifying *Photobacterium damselae, V. ordalii* and *V. salmonicida* (Austin et al., 1997). A large variability occurred for the CIT reaction when tested for *V. ordalii* and *V. anguillarum*, therefore the CIT reaction was discarded when reading the strips (Grisez et al., 1991). The authors confirmed API 20E to be a good tool for the characterization of fish pathogenic vibrios and that the variability observed within the different phena of *V. anguillarum* was mainly the result of their ability to ferment the sugars amygdalin (AMY) and ARA. While performing the biotyping of *V. anguillarum* by API 20E, Kuhn et al. (1996) demonstrated lower diversity among isolates from fish than from the environment, rotifers or *Artemia* and concluded that the strains with specific characteristics were associated with certain geographic areas, and also certain fish species. That needs to be kept in mind while reading the API 20E strips. *V. anguillarum* is one of the most important agents causing epizootic outbreaks in fish cultured in sea waters, but it is not considered in the API 20E system, however revealing helpful profiles for identification. Up to 66% of *V. anguillarum* isolates were identified as *A. hydrophila,* exhibiting false negative or positive reactions for CIT, GEL, SOR, RHA and AMY in the work of Santos et al. (1993).

Yersinia ruckeri is another important fish disease agent not included in the API 20E database, but its use along with standard biochemical tests helps make the right diagnosis (Oraic et al., 2002). When Austin et al. (2003) examined reference strains of *Y. ruckeri* in API 20E, they were identified as *Hafnia alvei*, albeit with a possibility of *Y. ruckeri*. The fresh isolates were generally equated with *H. alvei*, but with doubtful profiles, and with a possibility of *Y. ruckeri*. Typical profiles attained for reference cultures of *Y. ruckeri* were 5305112, 5315113, 5104100, 5305100 and 5107100. Santos et al. (1993) also found profile number 514100, along with 1104100 and 5105100 for reference strains of *Y. ruckeri*, which all indicated to *H. alvei* with a possibility of *Y. ruckeri*.

The taxonomic position of *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish pasteurellosis, is controversial as this organism has also been described as *Pasteurella piscicida.* API 20E, along with other tests, was used for its phenotypic characterization (Thyssen et al., 1998). API 20E was also used to confirm the first observed pasteurellosis outbreak at a low sea water tempera-

ture in cultured sea bass (*Dicentrarchus labrax*) in Turkey (Korun and Timur, 2005). All the *P. damsela* subsp. *piscicida* strains Zorrilla et al. (1999) isolated from different sources gave the same code profile when tested by API 20E – 2005004, which in a V4.0 apiweb database identifies it as *Pseudomonas fluorescens/putida* with 79.6% and as *Photobacterium damsela* with a 13.9% possibility. API 20E has thus been proved useful in the rapid identification of *P. damsela* subsp. *piscicida*.

4. Conclusions

It seems that the biochemical protocols proposed for the API 20E strips are of limited importance for identification and differentiation of ichthyopathological bacterial species. For some species, their identification must be regarded as only presumptive. However, being already introduced into fish diagnostic laboratories since it is rapid and simple to use, API 20E does have a role in the diagnosis of bacterial fish pathogens. Nevertheless, for comparable results, it has to be adapted for bacterial fish isolates, and there should be a consensus among the fish health professionals regarding the incubation temperature and the duration of the incubation time before performing readings. In our fish health laboratory, we attained the best results when incubating at 22°C and reading the strips after 48–72 hours. Also, we recommend inoculation in a suspension of 1.5% saline for sea fish bacteria. Sealing the cups of carbohydrate tests with mineral oil may or may not enhance the accuracy of these reactions. Our experiences with the system are that in order to confirm the diagnosis and/or exclude an erroneous API diagnosis, API 20E reactions should be compared with the diagnostic schemes based on reactions in conventional phenotypic tests, therefore, if considering only biochemical results, misidentifications could be ignored and only reaction profiles accepted. Also, some strains are wrongly identified because they are not included in the database of API 20E system. However, these profiles could be very useful in future identifications of similar strains. It is important to note that wrong identifications because of low discrimination profiles should be clarified by performing the supplementary tests proposed. The proposed profiles already obtained by other researchers on the reference strains of respective bacteria should be consulted. Care should be advocated when experiencing delayed positive tube tests, especially fermentation of carbohydrates, which could respond to delayed or weakly positive conventional test reactions. And finally, due to their significance for fish health and impact on the aquaculture, and because of the need for their rapid identification, some important fish bacteria should be included in the API 20E system, such as *Y. ruckeri, E. ictaluri, V. anguillarum*.

5. REFERENCES

Arias C.R., Shoemaker C.A., Evans J.J., Klesius P.H. (2003): A comparative study of *Edwardsiella ictaluri* parent (EILO) and *E. ictaluri* rifampicin-mutant (RE-33) isolates using lipopolysaccharides, outer membrane proteins, fatty acids, Biolog, API 20E and genomic analyses. Journal of Fish Diseases, 26, 415–421.

Athanassopoulou F., Prapas T., Rodger H. (1999): Diseases of *Puntazzo puntazzo* Cuvier in marine aquaculture systems in Greece. Journal of Fish Diseases, 22, 215–218.

Austin B. Austin D.A. (eds.) (1999): Bacterial Fish Pathogens: Disease of Farmed and Wild Fish. Springer-Praxis, Chichester, UK.

Austin B., Austin D.A., Blanch A.R., Cerda M., Grimont F., Grimont P.A.D., Jofre J., Swings J. (1997): A comparison of methods for the typing of fish-pathogenic *Vibrio* spp. Systematic and Applied Microbiology, 20, 1, 89–101.

Austin D.A., Robertson P.A.W., Austin B. (2003): Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum). Systematic and Applied Microbiology, 26, 127–131.

Bertone S., Gill C., Moizo A., Calegari L. (1996): *Vibrio carchariae* associated with a chronic skin ulcer on a shark, *Carcharhinus plumbeus* (Nardo). Journal of Fish Diseases 19, 429–434.

Biosca E.G., Esteve C., Gray E., Amaro C. (1993): Evaluation of the API 20E system for identification and discrimination of *Vibrio vulnificus* biotypes 1 and 2. Journal of Fish Diseases, 16, 79–82.

Brown B.J., Leff L.G. (1996): Comparison of fatty acid methyl ester analysis with the use of API 20E and NFT strips for identification of aquatic bacteria. Applied and Environmental Microbiology, 62, 2183–2185.

Coz-Rakovac R., Strunjak-Perovic I., Topic Popovic N., Hacmanjek M., Simpraga B., Teskeredzic E. (2002): Health status of wild and cultured sea bass in the northern Adriatic Sea. Veterinarni Medicina, 47, 222–226.

Doukas V., Athanassopoulou F., Karagouni E., Dotsika E. (1998): Aeromonas hydrophila infection in cultured sea bass, *Dicentrarchus labrax* L., and *Puntazzo puntazzo* Cuvier from the Aegean Sea. Journal of Fish Diseases, 21, 317–320.

Grisez L., Ceusters R., Ollevier F. (1991): The use of API 20E for the identification of *Vibrio anguillarum* and *V. ordalii*. Journal of Fish Diseases, 14, 359–365.

Israil A.M., Balotescu M.C., Alexandru M.C., Dobre G. (2003): Discordances between classical and API 20E microtest biochemical identification of *Vibrio* and *Aeromonas* strains. Bacteriology, Virusology, Parasitology and Epidemiology, 48, 141–143.

Jaksic S., Uhitil S., Petrak T., Bazulic D., Gumhalter Karolyi L. (2002): Occurrence of *Vibrio* spp. in sea fish, shrimps and bivalve molluscs harvested from Adriatic Sea. Food Control, 12, 491–493.

Korun J., Timur G. (2005): The first pasteurellosis case in cultured sea bass (*Dicentrarchus labrax* L.) at low marine water temperatures in Turkey. Israeli Journal of Aquaculture-Bamidgeh, 57, 3, 197–206.

Kozinska A., Figueras M.J., Chacon M.R., Soler L. (2002): Phenotypic characteristics and pathogenicity of *Aeromonas* genomospecies isolated from common carp (*Cyprinus carpio* L.). Journal of Applied Microbiology, 93, 1034–1041.

Kuhn I., Austin B., Austin D.A., Blanch A.R., Grimont P.A.D., Jofre J., Koblavi S., Swings J. (1996): Diversity of *Vibrio anguillarum* isolates from different geographical and biological habitats, determined by the use of a combination of eight different typing methods. Systematic and Applied Microbiology, 19, 442–450.

Li J., Yie J., Foo R.W.T., Ling J.M.L., Xu H., Woo N.Y.S. (1999): Antibiotic resistance and plasmid profiles of *Vibrio* isolates from cultured silver sea bream, *Sparus sarba*. Marine Pollution Bulletin, 39, 245–249.

MacDonnel M.T., Singleton F.L., Hood M.A. (1982): Diluent composition for use of API 20E in characterizing marine and estuarine bacteria. Applied and Environmental Microbiology, 44, 423–427.

McCasland B., True K. (2001): Bacteriology. Chapter 5. In: True K. (ed.): National Wild Fish Health Survey, Laboratory Procedures Manual. USFWS, Washington, D.C. 1–28.

OIE (Office International des Epizooties) (2003): Manual of Diagnostic Tests for Aquatic Animals, available at: http://www.oie.int/eng/normes/fmanual/A_00002.htm

Oraic D., Zrncic S., Sostaric B. (2002): Occurrence of enteric redmouth disease in rainbow trout (*Oncorhynchus mykisss*) on farms in Croatia. Acta Veterinaria Hungarica, 50, 283–291.

Padros F., Zarza C., Dopazo L., Cuadrado M., Crespo S. (2006): Pathology of *Edwardsiella tarda* infection in turbot, *Scophthalmus maximus* (L.). Journal of Fish Diseases, 29, 87–94.

Santos Y. Romalde J.L., Bandin I., Magarinos B., Nunez S., Barja J.L., Toranzo A.E. (1993): Usefulness of the API-20E system for the identification of bacterial fish pathogens. Aquaculture, 116, 111–120.

Thyssen A., Grisez L, Van Houdt R., Ollevier F. (1998): Phenotypic characterization of the marine pathogen *Photobacterium damselae* subsp. *piscicida*. International Journal of Systematic Bacteriology, 48, 1145–1151.

Topic Popovic N., Benussi Skukan A., Strunjak-Perovic I., Coz-Rakovac R., Hacmanjek M., Hunjak B. (2004): Comparison of the API 20E and BBL Crystal E/NF identification systems for differentiating bacterial isolates from apparently healthy reared sea bass (*Dicentrarchus labrax*). Veterinary Research Communications, 28, 93–101.

Toranzo A.E., Santos Y., Nieto T.P., Barja J.L. (1986): Evaluation of different systems for identification of environmental *Aeromonas* strains. Applied and Environmental Microbiology, 51, 652–656.

Villamil L, Figueras A., Toranzo A.E., Planas M., Novoa B. (2003): Isolation of a highly pathogenic *Vibrio pelagius* strain associated with mass mortalities of turbot, *Scophthalmus maximus* (L.) larvae. Journal of Fish Diseases, 26, 293–303.

Zorilla I., Balebona M.C., Morinigo M.A., Sarasquete C., Borrego J.J. (1999): Isolation and characterization of the causative agent of pasteurellosis, *Photobacterium damsela* ssp. *piscicida*, from sole, *Solea senegalensis* (Kaup). Journal of Fish Diseases, 22, 167–172.

Received: 2006–09–11
Accepted: 2007–01–17

Corresponding Author:

N. Topic Popovic, Rudjer Boskovic Institute, Division of Materials Chemistry, Ichthyopathology Group – Biological Materials, Bijenicka 54, 10002 Zagreb, Croatia
Tel./fax +385 1 457 1232; e-mail: ntopic@irb.hr