Jurnal Medik Veteriner
DOI: 10.20473/jmv.vol7.iss1.2024.88–104
April 2024, Vol.7 No.1, 88–104
online at https://e-journal.unair.ac.id/JMV

Validation of Analytical Method for *Aeromonas hydrophila* Identification using Analytical Profile Index (API) 20E KIT Method

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Abstract

The high demand for fish consumption has an impact on increasing aquaculture productivity and causes the vulnerability of increasing bacterial populations in aquaculture fields, so more rapid handling is needed. The use of phenotypic KIT methods (API 20E) has been applied as a targeted and efficient identification support in targeting better bacterial identification accuracy but often provides unequal results. Method validation is one of the general requirements for the competence of a laboratory evaluation: to provide coherent, interpretable, and accurate results with known uncertainties. The purpose of this study was to validate the API 20E KIT method for the identification of *A. hydrophila*. The conventional method used as a reference is SNI 7303.1:2015. The validation parameters consisted of determining the limit of detection, sensitivity, and specificity tests, as well as the positive predictive value and negative predictive value. The results showed that the limit of the detection value of the API 20E KIT was at a concentration of 100 CFU/mL with an ID of 99.00%. The sensitivity and specificity values in the positive and negative target samples were 100% with a positive predictive value and a negative predictive value of 0%, respectively. In conclusion, the API 20E KIT method as an alternative test method or rapid test was proven valid for identifying *A. hydrophila* by the test results using the reference method.

Keywords: *Aeromonas hydrophila*, API 20E KIT, bacterial identification, method validation

Received: 9 October 2023 **Revised:** 24 January 2024 **Accepted:** 2 March 2024

INTRODUCTION

Aeromonas hydrophila is one of the pathogenic bacteria that cause bacterial diseases in the process of fish farming. *A. hydrophila* were known to infect various types of freshwater fish and king prawns, where the infection is influenced by stress conditions due to overcrowding, malnutrition, poor water quality, and extreme fluctuations in water temperature (Fikri *et al*., 2022). *A. hydrophila* is also known as an opportunistic bacteria, spreading through water, contact with contaminated equipment, or the transfer of fish infected with *A. hydrophila* from one place to another (Darmawan and Rohaendi 2014). *A. hydrophila* has been identified as causing disease-causing septicemia with open skin ulcers, bleeding gastroenteritis, ascites, and cloacal bleeding (El-Son *et al*., 2019). This bacteria was a Gram-negative rod-shaped bacteria, white-beige yellowish in color, circular, convex, slightly jagged at the edges of the colony, and lives in freshwater waters (Ugarte-Torres *et al*., 2018), with high virulence and fish mortality rates reaching 80–100% (Lukistyowati and Kurniasih 2012). Symptoms caused by *A. hydrophila* were all fins damaged and whitish, bleeding in the internal organs (liver, kidney, spleen), flatulence, and damaged eyes (Kusdarwati *et al*., 2017; Saleema *et al*., 2018).

Along with the high demand for animal protein, the productivity of aquaculture is increasing, which has an impact on the vulnerability of increasing bacterial populations in aquaculture fields. Identification of disease agents caused by bacteria is important to prevent

disease in fish. Various fish bacterial identification techniques have been widely used, with varying degrees of accuracy, advantages, and disadvantages depending on the method used. Several studies reported that molecular bacterial identification methods such as real-time PCR, 16S ribosomal RNA (16S rRNA), and next-generation sequencing (NGS) provide more valid results because they provide information on the composition, structure, and genetic diversity of bacteria, but these methods were relatively expensive and inefficient when identifying large numbers of bacterial isolates (Braga *et al*., 2013; Bodor *et al*., 2020). The use of phenotypic-based identification KIT, including Analytical Profile Index (API) 20E and API 20NE KIT, shows a 100% accuracy rate in identifying Gram-negative bacteria (Rakotovao-Ravahatra *et al*., 2021; Balci *et al*., 2023), while reports by Dubey *et al*. (2021); Kazmi *et al*. (2022) showed an identification accuracy rate with API 20E KIT of 90–93%. The API KIT as a replacement for conventional tube-based evaluation for members of the Enterobacteriaceae provides the advantage of being able to reduce by more than half the total cost per identification of an isolate, which is achieved through reduced labor and media. Furthermore, API 20E KIT is a reliable method and provides earlier results when compared to conventional biochemical (Travis, 2019; Al-Howaidi and Al-Hamad, 2020). The interpretation of API 20E identification results was based on the match between the tested bacterial profile and the database, the relative value of the selection, and the number of tests (Popović *et al*., 2022). In the context of clinical laboratory use, the utilization of phenotypic-based KIT has been reported to yield erroneous outcomes in the identification of bacterial species. This can be attributed to a multitude of factors, such as artifactual dissimilarities, verification bias in the visual detection of color changes, variances between the employed test KIT, imperfect reference standards during the interpretation of test results, and inaccuracies within identification databases (Jackson *et al*., 2016; Mok *et al*., 2018; Fernández-Bravo and Figueras, 2020). Vira *et al*. (2016) posited that the efficacy and precision of identification have a substantial impact on the implementation of disease diagnosis caused by bacteria, the determination of pathogenic and non-pathogenic strains of a bacterium, as well as the accuracy of preventing and controlling disease outbreaks. Subsequently, in the scenario of genus or species identification, biochemical tests are imperative to augment the dissimilarities among the tested strains (Christ *et al*., 2017; Ferris *et al*., 2017).

Laboratory bacterial identification activities require appropriate and validated methods so that the values obtained are valid and correct. Yeung and Thorsen (2016) stated that the process of identifying bacteria requires a high level of accuracy to avoid misidentification. Method validation is the act of assessing certain parameters based on laboratory experiments to prove that the approaches, strategies, experimental procedures, processes, laboratory staff, instrumentation, reagents, and room conditions selected for this method will function properly under a set of predetermined conditions (Gupta, 2014; Peris-Vicente, *et al*., 2015). According to ISO/IEC 17025:2005, laboratories must validate all methods used. The methods will be validated separately for each matrix and working range, even for the same analyte. Full validation is required when applying new methods: self-developed, taken from literature sources, transferred from other laboratories, and reference methods. A new method can be used when it has been validated and conditions are adjusted to the laboratory conditions and equipment available. Jones and Marengo (2016) state that validation is needed to obtain analytical results that are valid, reliable, trustworthy, and can be scientifically accounted for and according to standards based on their intended use.

Currently, conventional method was still practiced in laboratories to ensure the identification of unusual organisms. Even though most other laboratory services already use reliable and effective diagnostic KIT, the analysis of the identification determination of *A. hydrophila* so far refers to the Indonesian National Standard (SNI) 7303.1:2015 as a reference method (standard), which is a conventional biochemical

test. The use of API 20E KIT as a substitute method requires quality assurance evaluating through method validation to prove that the substitute method has the same test results as the reference method. Method validation is one of the general requirements for the competence of a laboratory evaluation, to provide coherent, interpretable, and accurate results with known uncertainties. The goal of any analysis method was to provide consistent, reliable, and accurate data. For this reason, the performance, and limitations of the method, as well as external influences that can modify these features, must be determined before use. In this study, an assessment of the API 20E KIT method was carried out compared to the SNI 7303.1:2015 reference method with an approach consisting of activities to determine the limit of detection, sensitivity, and specificity tests, as well as positive predictive value (PPV) and negative predictive value (NPV).

MATERIALS AND METHODS

Ethical Approval

According to the letter of notification from the secretariat of the research ethics committee, the National Research and Innovation Agency, the experiments were carried out following the guidelines for the care and use of animals for scientific purposes, and this study did not require research ethics approval.

Study Period and Location

This study was conducted for two months, from November to December 2021, at the Microbiology Laboratory at the Research Institute for Fish Breeding, Ministry of Marine Affairs and Fisheries, Subang, West Java, Indonesia.

API 20E KIT Method (BioMérieux, USA)

According to SAC (2019), method validation was divided into primary validation, namely the validation of a completely new test method or modification of a conventional method, and secondary validation, namely the application of a test method that has been recognized in a laboratory.

In this study, primary validation was carried out for the identification of *A. hydrophila* using API 20E KIT as an alternative method to replace the conventional biochemical test SNI 7303.1:2015 to accelerate evaluating, reduce the risk of contamination, and optimize labor. In addition, this validation was also included in the validation of qualitative methods because the analysis method was both a positive and a negative response.

Oxidase Test

The oxidase test must be performed according to the manufacturer's instructions for use. The result should be recorded on the result sheet as it was an integral part of the final profile (21st identification test). Performing of the API 20E KIT method was preceded by an oxidase test using oxidase discs, where if the color turns blue, it showed a positive result. The reference method used in this study was based on (SNI 7303.1 2015) for the identification of *A. hydrophila*. The test samples used include positive bacteria target type *A. hydrophila* isolated from catfish that have been confirmed positive using the API 20E KIT and negative bacteria target type *S. agalactiae*.

Strip Preparation

Strip preparation activities begin with preparing an incubation box (tray and lid) that has been filled with 5 mL of distilled or demineralized water or any water without additives or chemicals that might release gases [(e.g., Cl_2, CO_2, etc.)] into the honey-combed wells of the tray to create a humid atmosphere. Then, the strain reference was recorded on the cover extending from the tray (remove the strip from its packaging and place the strip in the incubation box).

Inoculum Preparation

Inoculum preparation begins with opening a 0.85% NaCl API Medium ampoule (5 mL) or API Suspension Medium ampoule (5 mL) containing 5 mL of sterile saline or sterile distilled water, without additives. Then transfer one well-isolated colony (18–24 hours) from the isolation plate

using a pipette. Next, carefully emulsify to obtain a homogeneous bacterial suspension. After that, the bacterial suspension was distributed to each well on the strip. The filling of the bacterial suspension in the strip wells is divided into two, first for the CIT, VP, and GEL tests (where the volume was filled in full), while for other tests the suspension volume is only partially filled (up to the limit of the good lid). As for the ADH, LDC, ODC, H_2S, and URE tests, mineral oil was added to create anaerobic conditions. After the suspension distribution was completed, the incubation box was closed and then incubated at 36 ± 2°C for 18–24 hours.

Strip Interpretation

After the incubation period, evaluated the strip by referring to the Reading Table on the API 20E KIT. If 3 or more tests (GLU test + or -) were positive, record all the spontaneous reactions on the result sheet, and then reveal the tests that require the addition of following reagents i.e. (a) TDA Test: add 1 drop of TDA reagent. A reddish-brown color indicates a positive reaction to be recorded on the result sheet; (b) IND Test: add 1 drop of JAMES reagent. A pink color developed in the whole cupule indicates a positive reaction to be recorded on the result sheet; (c) VP Test: add 1 drop each of VP 1 and VP 2 reagents. Wait at least 10 minutes. A pink or red color indicates a positive reaction to be recorded on the result sheet. If a slightly pink color appears after 10 minutes, the reaction should be considered negative.

If the number of positive tests (including GLU tests) before adding reagents was less than 3, then re-incubate the strip for a further 24 hours (± 2 hours) without adding any reagents.

The identification process was based on the numerical profile. The numerical profile values on the result sheet were divided into 3 groups of values, namely 1, 2, and 4, for each test type, which will indicate a specific result. By adding together the values corresponding to positive reactions within each group, a 7-digit profile number was obtained for the 20 tests of the API 20E strip. The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive. Isolate identification was performed using the WEB Biomérieux Analytical Profile Index software (https://apiweb.biomerieux.com/). Strain identification (ID) at the species level was divided into four subgroups: (i) ID ≥ 99.9% (excellent); (ii) ID ≥ 99.0% (very good); (iii) ID ≥ 90.0% (good); and (iv) ID < 90% (acceptable).

Limit of Detection (LoD)

Validation of the API 20E KIT method was carried out based on the guidelines published by ISO 16140-2:2016.

The limit of detection (LoD) was defined as the minimum amount of analyte concentration in a sample that can still be detected under experimental conditions in units of CFU/mL or CFU/g (the samples used in the study were microbial analytes). LoD measurements were performed to establish a baseline detection value under optimal conditions and to detect a single target organism in a large sample. If no organism was detected, the result was reported as <1 target organism per sample volume or mass (The FEM Microbiology Action Team, 2009; Pum, 2019).

In this study, the LoD value was determined using *A. hydrophila* suspensions with concentrations of 10 CFU/mL and 100 CFU/mL. The number of replicates was 6 replications. The LoD value was indicated by the lowest concentration value in the sample, which was able to show positive results during evaluation.

Sensitivity and Specificity Test

The sensitivity test was performed to measure how well a test identifies true positive results, indicating the ratio that compares the number of correct results to the total number of tests performed. The specificity test was performed to quantify how well a test identifies true negatives, based on the ability of the method to measure only certain substances carefully and thoroughly in the presence of other components that may appear in the sample matrix. Sensitivity and specificity determinations were made from 6 replicate tests on each test medium contaminated with *A. hydrophila* based on the lowest limit value produced. The sensitivity and specificity tests of the method were obtained from the results of

calculations that refer to Boyce (2017) and Pum (2019):

$$Sensitivity = \frac{TP}{TP+FN} x\ 100$$

$$Specificity = \frac{TN}{FP+TN} x\ 100$$

Positive Predictive Value (PPV) and Negative Predictive Value (NPV)

The positive predictive value test describes the proportion of samples with a positive test result, where the desired condition was known in advance, while the negative predictive value test describes the proportion of samples with a negative test result, where the desired condition is not present. Determination of PPV and NPV was performed from 6 replicate tests on each test medium contaminated with *A. hydrophila* based on the lowest cut-off value generated. The PPV and NPV test methods were obtained from the calculation results, according to the formula by Monaghan *et al*. (2021):

$$\text{Positive Predictive Value (PPV)} = \frac{TP}{TP+FP} x\ 100$$

$$\text{Negative Predictive Value (NPV)} = \frac{TN}{FN+TN} x\ 100$$

Description:
TP (True Positive), FN (False Negative), TN (True Negative), FP (False Positive).

Identification of *A. hydrophila* (SNI 7303.1: 2015)

Bacterial identification activities consist of isolating and purifying bacteria on common media, followed by physical, morphological, and biochemical identification. Bacterial isolation was collected from the kidney, liver, and lymph of catfish, then grown on blood agar media and incubated for 18–24 hours at 25–28ºC. The purification process of β-hemolytic colonies was carried out on TSA media and incubated for 18–24 hours at 25–28°C. The reference method of identification of *A. hydrophila* consists of a hemolytic properties test, tolerance to incubation temperature at 4ºC, 37ºC, and 50ºC, tolerance to media pH at 3, 5, 9, and 11, tolerance to NaCl concentration in media (0.5%; 3%; and 5%), Gram staining and morphology test, Gram test (KOH 3%), motility test, oxidase test, oxidative-fermentative (O/F) test, and RS test.

Data Analysis

Calculation of the LoD, sensitivity, specificity, PPV, and NPV rate parameters was performed by comparing the positive and negative results obtained from the two test methods (API 20E KIT and SNI 7303.1: 2015) (SAC, 2019). Data analysis was performed with descriptive analysis.

RESULTS AND DISCUSSION

Validation was confirmation through evaluation and the provision of objective evidence that certain requirements for a specific purpose were met. In this study, validation was carried out on an alternative method of identifying *A. hydrophila* using the API 20E KIT. The API 20E assay is a conventional biochemical assay in which the substrate was dehydrated in a microtube. After inoculation with the bacterial suspension, the substrate was dissolved, and the reaction that occurred was usually identical to the reaction using the macro method. The API 20E KIT is a test strip consisting of 20 microtubes or capsules. These 20 microtubes contain conventional biochemical test materials in the form of dried pH-based substrates. After inoculation with a bacterial suspension in saline solution, the bacteria metabolize the substrate during overnight culture and produce distinct color complexes with other chemicals in the tubes. The reactions that occur in these test strips are usually identical to conventional macromethods.

The web API provides species identification probability and typicality index, and metabolic tests can be effectively used to identify specific bacterial isolates. According to Awong-Taylor *et al*. (2008); Tomas *et al*. (2018), biochemical methods are cheaper, easier to use, give rapid results, and do not require the use of specialized equipment and procedures required for molecular methods. This is especially important when trying to isolate and identify many bacterial isolates in a short period. Biochemical methods also make it possible to build a database of the characteristics and metabolic profiles of isolates. The API 20E KIT is a bacterial identification system, where the

identification process to recognize and determine the species of an unknown bacterium within a species is based on the choice of a series of biochemical tests performed by specific software (Rakotovao-Ravahatra *et al*., 2021).

Limit of Detection

According to ISO 16140-2:2016, the lowest limit of the count range was the lowest concentration of a microorganism in a sample that can be detected but not counted as a true value under agreed test conditions. To determine the limit of detection of this method, inoculation was carried out with low inoculum concentrations of 10 and 100 CFU/mL. This detection limit determination was carried out six times. Based on Table 1, the test results of the detection limit of *A. hydrophila* using the API 20E KIT at a concentration of 100 CFU/mL and 10 CFU/mL show differences in reading results in the IND, VP, GLU, MAN, SAC, and AMY tests. The IND test at a concentration of 100 CFU/mL was still able to show positive results in all replicates, according to the characteristics of *A. hydrophila*. As for the sample with a concentration of 10 CFU/mL, there were only 3 replicates with positive results, and the other replicates showed negative results. Evaluating of parameters VP, GLU, MAN, SAC, and AMY samples with a concentration of 10 CFU/mL began to show the inability of the method to read test results in accordance with the characteristics of *A. hydrophila* in each replicate. This result was different from the concentration of 100 CFU/mL, where the test results showed the suitability of the characteristics of the target-positive bacteria (*A. hydrophila*). Santos *et al*. (1993) stated that fish isolates show false negative reactions for sugar fermentation, gelatinase, and VP tests that occur on substrates that have delayed conventional test reactions and/or weakly positive conventional test reactions. Conventional phenotypic methods are still commonly used for bacteria, although they have problems such as inaccuracy in bacterial identification due to differences in incubation temperature (Balcı *et al*., 2023; Rasmussen-Ivey *et al*., 2016).

The results of the software reading on bacterial identification using API 20E are presented in Table 2. The ID value at a concentration of 100 CFU/mL shows that the type of bacteria identified from 5 replicates is *A. hydrophila* with an ID of 99.00%. As for the concentration of 10 CFU/mL, there was only 1 replicate identified as *A. hydrophila* with an ID of 99.80%. Table 3 showed the calculation value of recovery (% recovery) at a concentration of 100 CFU/mL of 5/6 × 100% = 83.33% and a concentration of 10 CFU/mL of 1/6 × 100% = 16.66%. Based on these results, it is known that the concentration of 100 CFU/mL has a % recovery that meets the prerequisites for the acceptability of the detection limit parameters, this is under the % recovery requirement value for the detection limit according to ISO 16140-2:2016, which is more than 50%.

According to some previous study results, it was known that API 20E KIT can indicate false positive and false negative results. The API 20E KIT has the drawback of relying on a limited number of samples and is subjectively based on the visual detection of color changes when reporting results. Thus, individual color interpretation may affect the reported profile and species identification (Iversen *et al*., 2004; Jackson and Forsythe, 2016). It was also reported that the accuracy of identification databases can be problematic due to bacterial taxonomic changes that are not reflected immediately, where several taxonomic changes have occurred very rapidly and some species are very closely related (Brady *et al*., 2014; Stephan *et al*., 2014). The case of misidentification in API was caused by the indicators used in its identification system, derived from the ability of bacteria to utilize carbohydrates in their biological system. If two bacterial species have similar carbohydrate utilization characteristics, the API will most likely misidentify them as the same species. This error will be a problem if this KIT was used exclusively for identification, especially if the results provide a high identification percentage value. In this study, the API KIT are considered accurate if the identification percentage value exceeds 80% (Desem *et al*., 2023).

Table 1. *A. hydrophila* detection limit test results at concentrations of 100 CFU/mL and 10 CFU/mL (API 20E KIT Method)

Parameters	Concentration											
	100 CFU/mL						10 CFU/mL					
	1	2	3	4	5	6	1	2	3	4	5	6
Ortho nitrophenyl-ßD-galactopyranosidase (ONPG)	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase (ADH)	+	+	+	+	+	+	+	+	+	+	+	+
Lysine decarboxylase (LDC)	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase (ODC)	-	-	-	-	-	-	-	-	+	-	-	-
Cirate utilization (CIT)	+	+	+	+	+	+	+	+	+	+	+	+
Sodium thiosulfate (H_2S)	-	-	-	-	-	-	-	-	-	-	-	-
Urea (URE)	-	-	-	-	-	-	-	-	-	-	-	-
Tryptophane deaminase (TDA)	-	-	-	-	-	-	-	-	-	-	-	-
Indole (IND)	+	+	+	+	+	+	-	+	+	-	-	+
Voges Proskauer (VP)	+	-	+	+	+	+	-	-	-	+	-	-
Gelatinase (GEL)	+	+	+	+	+	+	+	+	+	+	+	+
Glucose (GLU)	+	+	+	+	+	+	-	+	-	+	+	+
Mannitol (MAN)	+	+	+	+	+	+	-	+	-	+	+	-
Inositol (INO)	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol (SOR)	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose (RHA)	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose (SAC)	+	+	+	+	+	+	-	+	+	-	+	+
Melibiose (MEL)	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin (AMY)	+	+	+	+	+	+	-	+	+	+	+	+
Arabinose (ARA)	-	-	-	-	-	-	-	-	-	-	-	-

(+) Positive response; (-) Negative response.

Table 2. Limit of detection (LoD) test at concentrations of 100 CFU/mL and 10 CFU/mL (API 20E KIT Method)

Concentration	n	Significant Taxa	ID	Category
100 CFU/mL	1	*A. hydrophila*/caviae/sobria 2	99.00	Very good
	2	*A. hydrophila*/caviae/sobria 2	89.80	Excellent to the genus
	3	*A. hydrophila*/caviae/sobria 2	90.00	Very good
	4	*A. hydrophila*/caviae/sobria 2	99.00	Very good
	5	*A. hydrophila*/caviae/sobria 2	99.00	Very good
	6	*A. hydrophila*/caviae/sobria 2	99.00	Very good
10 CFU/mL	1	*Burkholderia cepacia*	63.70	Doubtful profile
	2	*A. hydrophila*/caviae/sobria 2	89.80	Excellent to the genus
	3	*A. hydrophila*/caviae/sobria 2	-	Unacceptable profile
	4	*A. hydrophila*/caviae/sobria 2	99.80	Very good
	5	*A. hydrophila*/caviae/sobria 2	89.80	Very good to the genus
	6	*A. hydrophila*/caviae/sobria 2	89.80	Very good to the genus

Table 3. Calculation value of LoD test at concentration of 100 CFU/mL and 10 CFU/mL (API 20E KIT Method)

Concentration	Results	Replication					
		1	2	3	4	5	6
100 CFU/mL	Presumptive	+	+	+	+	+	+
	Confirmed	+	-	+	+	+	+
10 CFU/mL	Presumptive	+	+	+	+	+	+
	Confirmed	-	-	-	+	-	-

Table 4. Results of sensitivity, specificity test, PPV, and NPV in target samples (API 20E KIT Method)

Parameters	Code											
	Sample Target (+)						Sample Target (-)					
	1	2	3	4	5	6	1	2	3	4	5	6
Ortho nitrophenyl-ßD-galactopyranosidase (ONPG)	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase (ADH)	+	+	+	+	+	+	-	+	-	-	-	-
Lysine decarboxylase (LDC)	+	+	+	+	+	+	-	-	-	-	-	-
Ornithine decarboxylase (ODC)	-	-	-	-	-	-	-	-	-	-	-	-
Cirate utilization (CIT)	+	+	+	+	+	+	-	-	-	-	-	-
Sodium thiosulfate (H_2S)	-	-	-	-	-	-	-	-	-	-	-	-
Urea (URE)	-	-	-	-	-	-	-	-	-	-	-	-
Tryptophane deaminase (TDA)	-	-	-	-	-	-	-	-	-	-	-	-
Indole (IND)	+	+	+	+	+	+	-	+	+	+	-	-
Voges Proskauer (VP)	+	+	+	+	+	+	-	+	+	+	+	+
Gelatinase (GEL)	+	+	+	+	+	+	-	-	-	-	-	-
Glucose (GLU)	+	+	+	+	+	+	+	-	-	-	-	-
Mannitol (MAN)	+	+	+	+	+	+	+	+	+	+	+	+
Inositol (INO)	-	-	-	-	-	-	-	+	+	+	+	+
Sorbitol (SOR)	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose (RHA)	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose (SAC)	+	+	+	+	+	+	-	-	-	-	-	-
Melibiose (MEL)	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin (AMY)	+	+	+	+	+	+	-	-	-	-	-	-
Arabinose (ARA)	-	-	-	-	-	-	-	-	-	-	-	-

(+) Positive response; (-) Negative response.

Table 5. Results of sensitivity, specificity test, PPV, and NPV of *A. hydrophila* in target samples (API 20E KIT Method)

Code	n	Significant Taxa	ID	Category
Target sample (+)	1	*A. hydrophila/caviae/sobria* 2	99	Very good
	2	*A. hydrophila/caviae/sobria* 2	99	Very good
	3	*A. hydrophila/caviae/sobria* 2	99	Very good
	4	*A. hydrophila/caviae/sobria* 2	99	Very good
	5	*A. hydrophila/caviae/sobria* 2	99	Very good
	6	*A. hydrophila/caviae/sobria* 2	99	Very good
Target sample (-)	1	*Yersinia pestis*	83.9	Presumptive
	2	*Escherichia coli* 2	55.3	Doubtful Profile
	3	*Escherichia coli* 2	84.6	Acceptable
	4	*Escherichia coli* 2	84.6	Acceptable
	5	*Yersinia pestis*	83.9	Presumptive
	6	*Yersinia pestis*	83.9	Presumptive

Table 6. Presumptive test results of *A. hydrophila* identification in target samples (API 20E KIT Method)

Code	Results	Number of Presumptive Test Results		Total
		Positive	Negative	
Target sample (+)	Confirmed Positive	(TP) 6	(FN) 0	6
Target sample (-)	Confirmed Negative	(FP) 0	(TN) 6	6
Total		6	6	12

Table 7. Calculated values of sensitivity, specificity test, PPV, and NPV of *A. hydrophila* identification in target samples (API 20E KIT Method)

Parameters	Yield (%)	Requirements
Sensitivity	100	≥ 70%
Specificity	100	≥ 70%
Positive predictive value (PPV)	0	≤ 30%
Negative predictive value (NPV)	0	≤ 30%

Table 8. LoD test of *A. hydrophila* results at concentrations of 10^6 CFU/mL and 10^5 CFU/mL (SNI Method 7303.1: 2015)

Parameters	Concentration											
	10^6 CFU/mL						10^5 CFU/mL					
	1	2	3	4	5	6	1	2	3	4	5	6
Hemolytic properties	β	β	β	β	β	β	β	β	β	β	β	β
Incubation at 4^{oC}	+	+	+	+	+	+	+	+	+	+	+	+
Incubation at 37^{oC}	+	+	+	+	+	+	+	+	+	+	+	+
Incubation at 50^{oC}	-	-	-	-	-	-	-	-	-	-	-	-
Media pH 3	-	-	-	-	-	-	-	-	-	-	-	-
Media pH 5	+	+	+	+	+	+	+	+	+	+	+	+
Media pH 9	+	+	+	+	+	+	+	+	+	+	+	+
Media pH 11	-	-	-	-	-	-	-	-	-	-	-	-
Media NaCl 0.5%	+	+	+	+	+	+	+	+	+	+	+	+
Media NaCl 3%	+	+	+	+	+	+	+	+	+	+	+	+
Media NaCl 5%	-	-	-	-	-	-	-	-	-	-	-	-
Gram Stain and morphology test	- BP	- BP	- BP	- BP	- BP	- BP	- BP	- BP	- BP	- BP	- BP	- BP
Gram test (3% KOH)	-	-	-	-	-	-	+	+	+	+	+	+
Motility test	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+	+	+	+	+
Oxidative-Fermentative Test	F	F	F	F	F	F	+	+	+	+	+	+
RS Test	+	+	+	+	+	+	+	+	+	+	+	+

(+) positive; (-) negative; (BP) short stem; (F) fermentative; (β) beta hemolytic.

Table 9. Calculation value of LoD test at concentrations of 10^6 CFU/mL and 10^5 CFU/mL (SNI Method 7303.1: 2015)

Concentration	Results	Replication					
		1	2	3	4	5	6
10^6 (CFU/mL)	Presumptive	+	+	+	+	+	+
	Confirmed	+	+	+	+	+	+
10^5 (CFU/mL)	Presumptive	+	+	+	+	+	+
	Confirmed	-	-	-	-	-	-

Table 10. Results of sensitivity, specificity test, PPV, and NPV on target samples (Method SNI 7303.1: 2015)

Parameters	Code											
	Target Sample (+)						Target Sample (-)					
	1	2	3	4	5	6	1	2	3	4	5	6
Hemolytic properties	β	β	β	β	β	β	γ	γ	γ	γ	γ	γ
Incubation at 4^{oC}	+	+	+	+	+	+	-	-	-	-	-	-
Incubation at 37^{oC}	+	+	+	+	+	+	+	+	+	+	+	+
Incubation at 50^{oC}	-	-	-	-	-	-	-	-	-	-	-	-
Media pH 3	-	-	-	-	-	-	+	+	+	+	+	+
Media pH 5	+	+	+	+	+	+	+	+	+	+	+	+
Media pH 9	+	+	+	+	+	+	+	+	+	+	+	+
Media pH 11	-	-	-	-	-	-	+	+	+	+	+	+
Media NaCl 0.5%	+	+	+	+	+	+	+	+	+	+	+	+
Media NaCl 3%	+	+	+	+	+	+	+	+	+	+	+	+
Media NaCl 5%	-	-	-	-	-	-	+	+	+	+	+	+
Gram Stain and morphology test	- BP	-BP	-BP	-BP	-BP	-BP	+ Blt	+ Blt	+ Blt	+ Blt	+ Blt	+ Blt
Gram test (3% KOH)	-	-	-	-	-	-	+	+	+	+	+	+
Motility test	+	+	+	+	+	+	-	-	-	-	-	-
Oxidase test	+	+	+	+	+	+	-	-	-	-	-	-
Oxidative-Fermentative Test	F	F	F	F	F	F	F	F	F	F	F	F
RS Test	+	+	+	+	+	+	-	-	-	-	-	-

(+) positive; (-) negative; (BP) short stem; (Blt) round; (F) fermentative; (β) beta hemolytic; (γ) gamma hemolytic.

Table 11. Presumptive test results of *A. hydrophila* identification on target samples (SNI Method 7303.1: 2015)

Code	Results	Number of Presumptive Test Results		
		Positive	Negative	Total
Target Sample (+)	Confirmed Positive	(TP) 6	(FN) 0	6
Target Sample (-)	Confirmed Negative	(FP) 0	(TN) 6	6
Total		6	6	12

Table 12. Calculation values of sensitivity, specificity test, PPV, and NPV identification of *A. hydrophila* in target samples (SNI Method 7303.1: 2015)

Parameters	Yield (%)	Requirements
Sensitivity	100	≥ 70%
Specificity	100	≥ 70%
Positive predictive value (PPV)	0	≤ 30%
Negative predictive value (NPV)	0	≤ 30%

Sensitivity, Specificity Test, PPV, and NPV (API 20E KIT Method)

Sensitivity in this study describes the number of colonies of *A. hydrophila* that are confirmed positive from suspected colonies and correctly identified by the rapid test. Specificity is the ability of the method to detect the analyte carefully and thoroughly in the presence of *A. hydrophila* compared to other bacteria. Specificity can be calculated using the number of positive samples that show positive test results divided by the positive test results compared to the positive control multiplied by 100%. According to Leeflang *et al*. (2013), test accuracy can be expressed as sensitivity and specificity, as positive and negative predictive values, or as positive and negative likelihood ratios. The sensitivity value describes the proportion of positive samples of the target bacteria that are correctly identified by the rapid test, while the specificity value indicates the proportion of negative samples of the target bacteria that are correctly identified by the rapid test. The presumptive false positive value represents the proportion of samples negative for the target bacteria that are falsely identified as positive by the rapid test, while the presumptive false negative value represents the proportion of samples positive for the target bacteria that are falsely identified as negative by the rapid test. In primary validation, all presumptive positive and presumptive negative cultures should be verified. Validation should include natural samples studied over time. Meanwhile, in secondary validation, only presumptive positive colonies are isolated and verified (MMC, 2011; SAC, 2019).

The sensitivity and specificity values of *A. hydrophila* identification using the API 20E KIT method were obtained from the test results of the target (+) sample and target (-) sample with the appropriate concentration based on the detection limit test results. Based on Table 4, it is known that the test results of the target (+) sample on 20 test parameters show the suitability of the confirmed characteristics of *A. hydrophila* according to the API 20E software reading (Table 5), where the results of the API 20E software reading on 6 positive target (+) samples show an ID value of 99.00% with the Very Good identification category. This is different for the negative target (-) samples. From 6 replicate samples, it is known that there are three different reading categories consisting of the acceptable identification category (samples 2 and 3 as *E. coli* 2) with ID 84.60%, the presumptive identification category (samples 1,5 and 6 as Y. pestis) with ID 83.90%, and the doubtful profile category (sample 2 as *E. coli* 2) with ID 55.30%. Based on these readings, it can be concluded that API 20E KIT has a high selectivity value because it can distinguish different types of bacteria at the species level. The high sensitivity and specificity values indicate that the API 20E KIT test alternative method has high sensitivity and is very

specific in diagnosing *A. hydrophila*. The calculation results in this study showed the recovery value from the method validation was by reference, whereas in microbiological analysis, ideally, the lowest recovery value reaches 80%, although the analytical method is still considered convincing if the % recovery value ranges from 50 to 95% (AOAC, 2002).

The results from 6 replicates of positive (+) target samples showed true positive results for 6 samples and false negative results for 0 samples. As for the negative (-) target sample, the false positive value was 0 samples, and the true negative value was 6 samples (Table 6). True positivity refers to individuals who have a positive outcome and were accurately assigned a positive assignment. On the other hand, true negative refers to individuals who have a negative outcome and were correctly assigned a negative assignment. Conversely, false positive refers to individuals who have a negative outcome but were mistakenly assigned a positive assignment. Lastly, false negative indicates individuals who have a positive outcome but were erroneously assigned a negative assignment (Safira *et al*., 2023). The results in this study indicate that the API 20E KIT method has the maximum calculation value for sensitivity and specificity of identification of *A. hydrophila*, which reaches 100% (Table 7). Based on Table 7, Based on this value, the positive predictive value and negative predictive value were 0%. This value reflects the proportion of positive and negative test results, respectively, that are truly positive and truly negative. High sensitivity and specificity values will reduce the number of false positives and false negatives. Vetter *et al*. (2018) stated that higher sensitivity values occur at relatively lower cut points, and higher specificity is achieved at higher cut points. In addition, this value is also determined by the number of false positives and false negatives.

The value in this study was in line with the report of Wahjuningrum *et al*. (2018) that the results of isolate verification using several API KIT, namely the API 20E KIT, which shows 99.00% similarity to *A. hydrophila*, API 20 Strep KIT, which shows 99.30% similarity to *S. agalactiae* API Listeria KIT shows 93.40% similarity to *L. grayi*. Similarly, Christ *et al*. (2017) found that the API® 20 Strep KIT showed a 92.00% identification success rate, identifying 111 strain isolates at the Enterococcus genus level. A lower value was reported by Maina *et al*. (2014); the use of API 20E KIT in the identification of *A. hydrophila* showed a profile (17.2%) of definite identity and 24 (82.8%) of closest identity. O'Hara *et al*. (1993) reported that the results of API 20E KIT identification in the Enterobacteriaceae family at the genus and species level showed results of 77%, while higher results were reported by Robinson *et al*. (1995), with an additional biochemical test of API 20E KIT showed an identification result of the Enterobacteriaceae family of 90.2%. The API 20E KIT test results on Gram-negative bacteria showed an identification conformity rate at the genus and species level of 98% (Al-Howaidi and Al-Hamad, 2020; Fikri *et al*., 2023).

In other species, it has been reported that biochemical tests using API 20E were insufficient to identify Cronobacter isolates at the species level, and reliance on this method will result in false positive and false negative identifications. Only about 80% of Cronobacter strains were correctly identified to genus level with the current version of the database associated with AP20E (Jackson and Forsythe, 2016). Similar results were reported by Ferris *et al*. (2017), who found that the API® 20E™ identification system could only correctly identify 38% of the isolates tested, with the best identification for P. aeruginosa and K. pneumoniae isolates (60% and 56%, respectively). As for Desem *et al*. (2023), another API method using the API 20E KIT successfully identified a bacterial isolate as *P. multocida* with an ID of 96%.

A. hydrophila Identification (SNI 7303.1 Method: 2015)

The identification results of positive (+) target samples on 17 test parameters showed results that were based on the characteristics of *A. hydrophila* (Table 8). Evaluating the detection limit of *A. hydrophila* using the SNI 7303.1 reference method: 2015 obtained the results of the

detection limit value at a concentration of 10^6 CFU/mL because the recovery value (% recovery) ≥ 50% was obtained, namely 6/6 × 100% = 100% (Table 9). This value is a reference for the lowest concentration in samples that are still able to be identified using this method.

Sensitivity values and specificity of identification of *A. hydrophila* using the SNI 7303.1 reference method: 2015 based on the test results of the target (+) sample on 17 test parameters showed the suitability of the characteristics of *A. hydrophila* (Table 10), where the test results of 6 replicates of positive (+) target samples showed true positive results as many as 6 samples and false negative results as many as 0 samples. As for the negative target sample, the false positive was 0 samples, and the true negative was 6 samples (Table 11). Based on these data, the calculation of sensitivity and specificity values reached 100% with a positive probable value and a negative probable value of 0% (Table 12).

Based on the test results in this study, it was known that the use of the API 20E KIT method as an alternative test method or rapid test is proven valid for identifying *A. hydrophila* by the test results using the SNI 7303.1 reference method: 2015, both showed the same results, namely the suitability of the characteristics of the test bacteria. In its application, the API 20E KIT identification method has advantages over the SNI 7303.1 method: 2015, as shown by evaluating at lower sample concentrations, API 20E KIT still has high sensitivity in identifying test samples. The API 20E system still maintains an important role in bacterial identification in settings where automatic identification instruments are out of reach. Even in modern laboratories, the API system is still useful for identifying certain organisms that may not have panels in automatic systems. Rapid and accurate identification of bacterial pathogens is a fundamental goal of clinical microbiology (Maina *et al*., 2014; Safira *et al*., 2022). Therefore, to identify effectively, it was important to follow the manufacturer's standard operating procedure instructions to minimize misidentification and replication. Ayunina (2015) stated that in its application, the rapid test method has advantages over conventional methods because it is more economical, saves time, and is more efficient in the use of equipment and storage. stated that the rapid test method was more practical, did not require special skills, was efficient in storage, produced little waste, did not require a lot of space during incubation, and had a longer shelf life compared to agar culture media. Bottini *et al*. (2011) proved that commercial rapid tests have a test sensitivity of up to 100%, but this can increase the number of false positives, so to ensure that the results obtained are truly positive for *A. hydrophila*, it was still necessary to carry out conventional biochemical tests. Different results can be caused by false positives during biochemical tests, thus in clinical evaluation, false positive results are treated as positive until bacterial identification (Wulandari *et al*., 2019; Amano *et al*., 2022).

CONCLUSION

Validation of the API 20E KIT rapid method has consistent results with the conventional test of SNI 7303.1:2015, which is indicated by a low limit of detection values, high sensitivity, and specificity values, as well as low positive predictive and negative predictive values. Thus, this method can be used for the identification of *A. hydrophila*.

ACKNOWLEDGEMENTS

Thanks to Asep Sopian, S.Si, M.Si and Dr. Ikhsan Khasani, M.Si who have provided advice and input in the implementation of this study.

AUTHORS' CONTRIBUTIONS

DA: Conceptualization and drafted the manuscript. DA and AR: Validation, supervision, and formal analysis. DA, MO, S, DS, and AR: Performed sample evaluation. DA, MO, S, DS, and AR: Performed the statistical analysis and the preparation of tables. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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