Development of rapid phenotypic system for the identification of Gram-negative oxidase-positive bacilli in resource-limited settings

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Rapid and accurate identification of bacterial pathogens is a fundamental goal of clinical microbiology. The diagnosis and surveillance of diseases is dependent, to a great extent, on laboratory services, which cannot function without effective reliable reagents and diagnostics. Despite the advancement in microbiology diagnosis globally, resource-limited countries still struggle to provide an acceptable diagnosis quality which helps in clinical disease management and improve their mortality and morbidity data. During this study an indigenous product, Quick Test Strip (QTS) NE, was developed for the rapid identification of biochemically slower group of Gram-negative oxidase-positive bacilli that covers 19 different bacterial genera. Some of the members belonging to these groups are well-established human pathogens, e.g. various species of *Vibrio*, *Pseudomonas*, *Burkholderia*, *Aeromonas*, *Achromobacter* and *Stenotrophomonas*. This study also evaluates the performance of QTS-NE by comparing with genotypic characterization methods. A total of 232 clinical and reference bacterial isolates were tested by three different methods. QTS-NE provides 100% concordant results with other rapid identification and molecular characterization methods and confirms the potential to be used in clinical diagnosis.

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1. Introduction

Infectious diseases are major cause of mortality in developing countries. The statistical data released by the World Health Organization (WHO) in 2008 describe that 8.7 million people die every year because of infectious diseases globally and, of them, 89% belong to lower-middle and lower-income countries (WHO 2008). Gram-negative oxidase-positive non-fermenting bacilli are known for their ability to cause infection in debilitated and immunocompromised individuals (Ribeiro *et al*. 2010). The organisms that belong to genera *Pseudomonas*, *Acinetobacter*, *Ralstonia* or *Burkholdaria*, *Aeromonas*, *Achromobacter* and *Stenotrophomonas* are normally present in air and on moist or dry surfaces (Ali *et al.* 2010), and therefore they have greater chances to infect patients in nosocomial settings. A surveillance study conducted in 10 different developing countries by International Nosocomial Infection Control Consortium (INICC) reported that more than 50% of ventilator-associated pneumonia in neonatal ICUs are caused by *Pseudomonas* and *Acinetobacter* species (Rosenthal *et al.* 2012). Furthermore, they are involved in bacteremia, septicemia, post-surgical, urinary tract and wound infections (Kaleem *et al.* 2010; Rezaei *et al.* 2011; Aguilar-Duran *et al.* 2012; Ramakrishnan *et al.* 2012). The mortality rate is specifically high in the former cases because clinical management only depends on early detection of pathogens.

Modern diagnosis of Gram-negative non-fermenting pathogens depends on bacterial isolation from clinical samples either by automated identification (ID) systems such as VITEK ID cards (Biomerieux Inc.), PHOENIX (BD, Inc.) or MicroScan ID panels (MicroScan USA), etc. (Chatzigeorgiou *et al.* 2011). Also, genotypic and proteomic assays based on the analysis of 16S rRNA gene by PCR and sequencing, fluorescent *in situ* hybridization, restriction enzyme digestion and matrix-assisted laser desorption time-of-flight (MALDI-TOF) and mass spectrometry are on the way to be implemented in

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routine diagnostics in developing countries (Chatzigeorgiou *et al.* 2011; Fernández-Olmos *et al.* 2011; Hagen *et al.* 2011; Oderiz *et al.* 2011; Pinot *et al.* 2011). Molecular techniques for the identification of bacteria are particularly useful for specific cases such as cystic fibrosis in which it is difficult to grow organisms on lab culture media due to their slow metabolic rate (Keating *et al.* 2012). However, these methods are neither efficient in detecting pathogens directly from clinical samples nor they can quantify the accurate bioload of pathogens in a clinical sample. Despite the enormous development in clinical diagnosis, developing countries like Pakistan are still reliant on manual ID methods since above-mentioned systems are costly and require specific skills and expertise. Enzymatic ID test strips like API 20NE (Biomerieux) and others made by well-reputed companies are limited to few reference laboratories due to their high cost and complicated means of transportation from foreign countries. Majority of clinical microbiology labs report genus-level identification that could be misleading due to different virulent potential of Gram-negative non-fermenting bacteria. The situation requires an affordable alternative which provides accurate species level identification and be easily used in resource-limited settings.

In this study, we indigenously developed a phenotypic characterization system, Quick Test Strip-Non-Enteric (QTS-NE) for the identification of Gram-negative oxidase-positive bacilli. Certain oxidase-negative bacteria such as *Acinetobacter* were also included in the study due to their non-fermentive nature and clinical importance. The locally isolated strains along with standard reference strains were tested. Correlation studies of QTS-12 NE were concluded in 90–100% agreement with genotypic characterization methods.

2. Materials and methods

2.1 *Bacterial strains*

The study was carried out with 13 standard reference strains as listed in table 1 and 219 clinical strains of Gram-negative oxidase-positive bacilli that were collected from several diagnostic microbiology laboratories. Bacterial cultures were maintained on brain–heart infusion (BHI) agar (Oxoid) for 24 h at 37°C. The isolates were preserved in BHI broth containing 50% glycerol at −80°C.

2.2 *Development and description of QTS-NE*

During this study, we developed an indigenous bacterial ID kit, QTS-NE, for rapid identification of metabolically slower group of Gram-negative oxidase-positive bacilli. The kit is based on a total of 14 different enzymatic and assimilation tests, of them dehydrated substrates for 13 reactions are accommodated in one strip whereas one reaction, respiratory enzyme cytochrome oxidase is supplemented with impregnated filter paper strips. As shown in figure 1, strip of twelve micro cupules contain dehydrated substrates for 13 different reactions including indole, glucose, nitrate reduction, urease, asculin, gelatin hydrolysis, ONPG, maltose, mannose and *N*-acetyl glucosamine assimilation. The kit was completely developed inhouse. Finally, selected formulation of indole composed of 1:1 mixture of sterilized 2% tryptone solution (pH 7) and 0.8% trypophane solution. G/NO cupule contained 0.5% peptone, 2% glucose, 0.3% KNO_3 for nitrate reduction, bromothymol blue and cresol red in 0.0075% HCl and 0.2 M phosphate buffer (pH 7.4). The mixture was sterilized by membrane filtration. The production of acetoin, nitrite, nitrate and N_2 gas as well as glucose fermentation can be observed simultaneously in this cupule after the addition of 0.8% sulphanilic acid and 0.5% α-naphthylamine.

In ADH cupule, arginine decarboxylation was observed. The process leads to the production of putrescine, ammonia and carbon dioxide, which bind to hydrogen and gives the alkalinity to the medium. The finally optimized formulation of ADH includes 1.2% thioton, 0.2% dextrose, 0.00012% pyridoxate-5-phosphate, 0.00075% bromothymole blue, 0.00092% cresol red and 2% arginine. The pH of solution was 5.8. The formulation of URE test is composed of solution A and solution B, mixed with 3:1 proportion. Solution A contained 0.25% protease peptone, 0.5% KH_2 PO_4, 0.25% Sodium thioglycolate and 0.00185% solution of cresol red in 50% ethanol (v/v) in basal medium. Solution B was 10% aqueous urea solution. The finally selected formulation of asculin consist of solution A (2% asculin in 0.9% peptone pH 6.6) and solution B (0.6% ferric citrate), mixed in 9:1 ratio and then dried into respective cupules of the strips. For assimilation reactions, the tests were developed on growth or

Table 1. List of reference bacterial strains used in this study

S. No.	Name	Reference No.
1	*Pseudomonas aeruginosa*	ATCC- 27853
2	*Pseudomonas fluorescens*	ATCC -13525
3	*Pseudomonas putida*	ATCC-49128
4	*Pseudomonas stutzeri*	ATCC-17588
5	*Brevundimonas diminuta*	ATCC-19146
6	*Burkholderia cepacia*	ATCC-25416
7	*Stenotrophomonas maltophillia*	ATCC-49130
8	*Plesiomonas shigelloides*	ATCC-51903
9	*Vibrio parahaemolyticus*	ATCC-17802
10	*Acinetobacter lwoffii*	ATCC-15309
11	*Aeromonas hydrophila*	ATCC-35854
12	*Aeromonas hydrophila*	ATCC-35654
13	*Shiwnella putrifaciens*	ATCC-49138

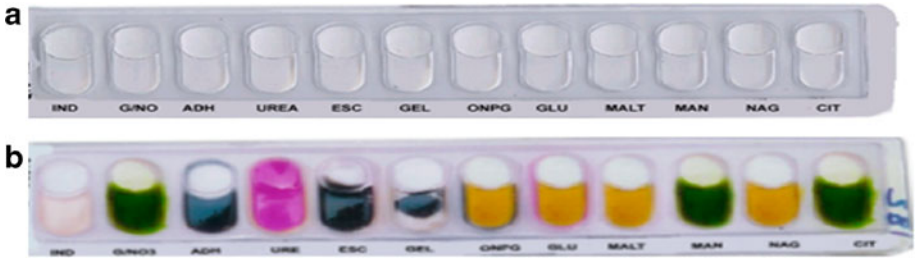

Figure 1. (**a**) The blank and (**b**) inoculated strip of QTS-NE are shown in this figure.

no growth pattern. The basal minimal medium contained 0.1% agar and different substrates were used as a sole source of carbon and nitrogen. pH was kept between 6.7 to 6.8. After filling the cupules, substrates were desiccated to prepare dried strips. Interpretation criteria of each test are illustrated in table 2. The results were found to be stable up to 24 h at room temperature.

To define optimal conditions, the strip was inoculated with *Pseudomonas aeruginosa* ATCC-27853 and incubated at a wide range of temperature and humidity. Different inoculation media such as PBS, 0.85% normal saline and nutrient broth were used to evaluate standard inoculation conditions. Furthermore, moisture content in dehydrated substrates was measured at different points to get reproducible and clear observations. The kit can be stored at room temperature up to one month and at 4°C up to 3 years.

2.3 *Scope of Identification of QTS-NE*

QTS-NE provides the identification of 45 different medically important bacterial species belonging to 19 genera (table 3). Species-level identification is based on a differentiation scheme by applying elimination techniques. The differentiation scheme is shown in figure 2.

2.4 *Evaluation of QTS-NE*

A total of 232 clinical and reference strains were identified using QTS-NE. The suspension of 24-h-old pure bacterial culture was prepared in 0.5 mL phosphate buffered saline (PBS) and turbidity was matched with 0.5 McFarland's nephlometer index. The suspension was appropriately

Table 2. Criteria for the interpretation of QTS-NE strip

Abbreviation	Name of Reaction	Reagents to be added	Interpretation	
			Positive	Negative
IND	Indole	Add 1 drop of Indole reagent	Red	Colourless/yellow brown/ pale green
GLU	a. Acid from glucose	None	Yellow/yellow green/	Green
	b. Nitrate reduction	Add one drop of 0.8% sulphanilic acid and 0.5% α-baphthylamine in 5N acetic acid	Brick red/brown	No change
	c. N_2 gas	If test is negative, add zinc dust	No change/gas bubble	Red
ADH	Arginine dihydrolase	None	Royal blue/deep blue	Yellow/green/greenish grey
URE	Urea hydrolysis	None	Purple	Light orange/light brown
ESC	Asculin hydrolysis	None	Black/brown	No change
GEL	Gelatin hydrolysis	None	Black deposit	No change
ONPG	ONPG	None	Yellow	Colorless/buff
GLU	Glucose assimilation	None	Yellow/yellow green	Green/blue
MALT	Maltose assimilation	None	Yellow/yellow green	Green/blue
MAN	Acid from maltose	None	Yellow/yellow green	Green/blue
NAG	*N*-acetyl glucosamine assimilation	None	Yellow/yellow green	Green/blue
CIT	Sodium citrate	None	Blue/bluish green	Green/dark green

Table 3. Scope of identification of QTS-NE

S. No.	Organism	S. No.	Organism
1.	*Pseudomonas aeruginosa*	24.	*Flavo. adoratum*
2.	*Pseudomonas fluorescence/putida*	25.	*Flavo. multivorum*
3.	*Pseudomonas putida*	26.	*Flavo. Spp*
4.	*Pseudomonas pseudomallei*	27.	*Acinetobacter calc. var anitratus*
5.	*Pseudomonas mallei*	28.	*A. Calc. var lowffii*
6.	*Pseudomonas stutzeri*	29.	*A. hemolyticus/alcaligens*
7.	*Pseudomonas mendocina*	30.	*Morexella spp.*
8.	*Ps. vesicularis*	31.	*M. phenylpyroveca*
9.	*Ps. paucimobilis*	32.	*M. lecunata*
10.	*Comamonas acidovorans*	33.	*M. urethreales*
11.	*Comamonas testosteroni*	34.	*Pasteurella multocida*
12.	*Stenotrophomonas maltophilia*	35.	*Pasteurella pneumotropica*
13.	*Brevundimonas diminuta*	36.	*Pst. aerogenes*
14.	*Ralstonia pickettii*	37.	*Pas. heamolytica*
15.	*Burkholdaria cepacia*	38.	*Past. Spp.*
16.	*Shiwnella putrifaciens*	39.	*Chromobacterium violacium*
17.	*Alcaligenes faecalis/odorans*	40.	*Aeromonas hydrophila*
18.	*Alcaligenes denitreficans*	41.	*Plesiomonas shigelloides*
19.	*Achronobacter xylosoxidans*	42.	*Vibrio cholera*
20.	*Achromobacter gpvd*	43.	*Vibrio heamolyticus*
21.	*Agarobacterium Radiobacter*	44.	*Vibrio alginlyt icus*
22.	*Bordetella bronceptica*	45.	*Vibrio vulnificus*
23.	*Flavobacterim menengo cepticum*		

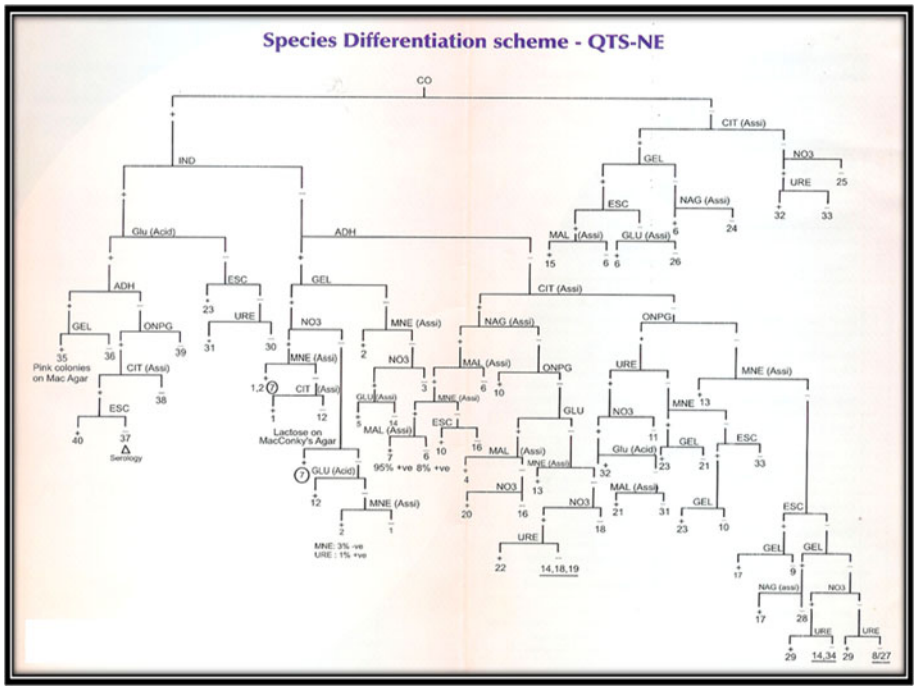

Figure 2. Identification scheme of QTS-NE. Each number represents specific bacterial species, which are further explained in table 3.

diluted with PBS so it finally contained 1–1.5×10^5 CFU bacteria/mL. One mL of diluted suspension was added to QTS-NE and incubated at 37°C aerobically for 8 h.

2.5 *API-NE*

For the comparison of QTS-NE, bacterial strains were identified by compatible foreign product, API-NE (Biomerieux Inc). The kit provides identification of 32 genera and 64 species of non-fastidious Gram-negative rods not belonging to the *Enterobacteriaceae*. Briefly, bacterial suspensions were prepared in 0.85% NaCl to inoculate 8 conventional substrates and in AUX medium to inoculate 12 assimilation cupules included in API-NE strip. The strips were incubated at 37°C aerobically for 8 h. The seven-digit profile number was converted to identification by using the APILAB software, version 3.3.3.

2.6 *PCR*

To validate the performance of the newly developed kit, bacterial strains were simultaneously identified by PCR. DNA extraction was performed using Instagene matrix kit (Bio-Rad, USA) according to the manufacturer's instructions. DNA preparations were stored at −20°C. PCR assays were performed with 1 µg template DNA using different sets of primers including DG74, 65d (Klausegger *et al.* 1999), rtxA rtxC (Chow *et al.* 2001), PS, PAL (De Vos *et al.* 1997), RAPDPCR (Krzewinski *et al.* 2001), lipase (lip) (Cascón *et al.* 1996), VA, VP and VC (Di Pinto *et al.* 2005) as per previously described protocols. Universal and Gram-specific PCR were performed for each strains while genus- and species-specific PCR assays were performed for strains belonging to respective genera. Further details about primer sequences and PCR conditions are given in table 4. DNA from standard strains, listed in table 1, served as positive control and PBS served as negative control. The amplified products were analysed in 2% agarose gel containing 0.05% ethidium bromide by using gel documentation system (Bio-Rad, USA).

3. Results

During this study, we were able to develop a low-cost kit for phenotypic identification of clinically relevant Gram-negative oxidase-positive bacilli. The kit costs only 2 US dollars per sample, which is 5 times lower than a compatible foreign brand such as API-NE; whereas in automated identification systems such as Phoenix and VITEK ID, only identification cards or panels cost around 25 US dollars per sample. In addition, they require sophisticated and costly automated machines.

Table 4. Primers and PCR conditions used for the identification of bacteria

Primers	Targets	Sequences	References
02–F 02-R	Universal bacterial	5′AACTGGAGGAAGGTGGGGAT3′ 5′AGGAGGTGATCCAACCGCA3′	
DG74 65d	Gram-type-specific (Gram-negative)	5′AACTGGAGGAAGGTGGGG AY3′ 5′AYGACGTCAAGTCMTCAT GG3′	Hussain *et al.* 2009
rtxA-F rtxA-R	*Vibrio cholerae*	5′CTGAATAGTGGGTGACTTACG3′ 5′GTGTATTGTTCGATATCCGCTAG3′	Kaleem *et al.* 2010
rtxC-F	*Vibrio cholerae* O-395	5′CGACGAAGATCATTGACGAC3′	
rtxC-R		5′CATCGTCGTTATGTGGTTGC3′	
RAPDPCR-F RAPDPCR-F	*S. maltophilia*	5′TGC GCG CGG G3′ 5′AGC GGG CCA A3′	Khan *et al.* 2003
PS-1 PS-2	*Pseudomonas aeruginosa*	5′ATGAACAACGTTCTGAAATTCTCTGCT3′ 5′CTTGCGGCTGGCTTTTTCCAG3′	Keating *et al.* 2012
PAL-1		5′ATGGAAATGCTGAAATTCGGC3′	
PAL-2		5′ CTTCTTCAGCTCGACGCGACG3′	
Lip-F Lip-R	*Aeromonas hydrophila*	5′AACCTGGTTCCGCTCAAGCCGTTG 5′TTGCTCGCCTCGGCCCAGCAGCT3′	Klausegger *et al.* 1999
VA-F VA-R	*Vibrio alginolyticus*	5′CGAGTACAGTCACTTGAAAGCC 3′ 5′CACAACAGAACTCGCGTTACC3′	Krzewinski *et al.* 2001
VP-F VP-R	*Vibrio parahaemolyticus*	5′GAAAGTTGAACATCATCAGCACGA3′ 5′GGTCAGAATCAAACGCCG3′	Krzewinski *et al.* 2001
VC-F VC-R	*Vibrio cholerae*	5′CGGCGTGGCTGGATACATTG3′ 5′GTCACACTTAAATAGTAGCGTCC3′	Krzewinski *et al.* 2001

A total of 232 different clinical and reference strains representing 11 different genera and 16 different species were identified by QTS-NE. All strains were subjected to the identification by API-NE, bacterial universal PCR targeting 16SrRNA gene and Gram-type-specific PCR to compare the efficiency of QTS-NE. Out of them, a total of 152 strains representing 7 different species from genera *Vibrio*, *Aeromonas*, *Pseudomonas* and *Stenotrophomonas* were subjected to species-specific PCR. As shown in table 5, QTS-NE yielded clear identification of all genera and species. The results were comparable with API-NE, universal, Gram-type-specific and species-specific PCR assays. Table 6 summarizes the concordance of QTS-NE with API-NE and molecular identification at species level. Out of 50, 48 (96%) strains of *Pseudomonas aeruginosa* were identified by QTS-NE, while API-NE identified 42 (85%) strains and conventional specie-specific PCR gave 100% positive identification. QTS-NE provided 100% identification for *P. florescence*, *S. maltophilia*, *Burkholderia cepacia*, *Aeromonas hydrophila* and different species of *Vibrio*, which is better than API-NE. Eighty percent of the *P.putida* strains were identified by QTS-NE, while sensitivity of API-NE was only 70%. *P. putida* and *P. stutzeri*, each 8 out of 10, (96%) strains corresponded well with PCR. *Acinetobacter lwoffii* gave 75% and *Acinetobacter baumannii* gave 78% positive results by QTS-NE, which are comparable with API-NE; however, both species were 100% identified by PCR. *Ralstonia pickettii* gave 93% positive in QTS-12NE and 100% positive results were obtained by API-NE and PCR. Gram-type-specific primer did not amplify standard strain of *Brevundimonas diminuta* ATCC-19146, probably because additional optimization procedure was required. However, this strain was identified by universal bacterial PCR, QTS-NE and API-NE.

Sensitivity and specificity of QTS-NE were 95% and 99%, respectively, using the strains (n=232) included in this study. In case of API-NE, sensitivity and specificity were 93% and 96% respectively, while PCR was found to be more sensitive method with 99% sensitivity. QTS-NE and API-NE had comparable turnaround times (TAT), i.e. 8 h, whereas TAT for automated systems such as VITEK-2 and PHOENIX was 4–5 h.

Table 5. Comparison of identification at taxonomic level

Identification vehicle	No. of identified/No. of total tested (%)	
	Genus	Species
QTS-NE	11/11 (100%)	16/16 (100%)
API-NE	11/11 (100%)	16/16 (100%)
Universal bacterial PCR	11/11 (100%)	16/16 (100%)
Gram-type-specific PCR	10/11 (90%)	15/16 (94%)
Species-specific PCR	4/4 (100%)	7/7 (100%)

4. Discussion

In Pakistan, standard microbiology diagnosis is only offered by a few public and private sector hospitals which are within the reach of less than 10% of country's population. In general, clinical diagnosis is the most deprived sector of healthcare settings. Special diagnosis areas such as clinical microbiology labs are usually not the part of small-scale setup. While most of medium- to large-scale laboratories operate without properly trained and educated personnel, and in very limited resources, the labs find difficulty in accurate and timely diagnosis which directly affects on clinical management of infectious diseases and sometimes lead to loss of precious lives even in treatable cases (Filmer *et al.* 2000; Khan *et al.* 2003; Ahmed *et al.* 2006; Hussain *et al.* 2009; Farooqui *et al.* 2009). The purpose of this study was to develop a rapid, accurate and affordable bacterial ID system which can be specifically used in resource-limited settings.

The correlation between phenotypic and genotypic characterization of Gram-negative oxidase-positive bacteria was established by comparing the data obtained from conventional PCR assays, API-NE and QTS-NE ID systems. Specific PCR assays were chosen for correlation studies since they were considered as reliable comparable methods in previous studies (Matsuki *et al.* 1999; Degand *et al.* 2008; Carmody *et al.* 2011). Gram-specific PCR was used as an additional assay to universal bacterial PCR assay. The results obtained from QTS-NE confirms that the kit accurately identifies most important clinical strains including *Pseudomonas*, *Aeromonas*, *Plesisomonas*, *Vibrio*, *Burkholderia cepacia* and *Stenotrophomons maltophilia* with 100% identification rate. However, the identification rate of *Acinetobacter* species ranged 75–78%. The data is based on 232 clinically relevant bacteria which circulate in local clinical environment. The sensitivity and specificity of QTS-NE was found to be superior to API_NE.

The scope of QTS-NE that identifies 19 different genera and 45 bacterial species can be considered limited compared to databases of phenotypic ID systems such as API-NE, VITEK-2 and PHOENIX cards. However, QTS-NE covers the majority of clinically important oxidase-positive non-fermenting bacteria, provides simpler interpretation method and does not require expertise to handle automated systems. Furthermore, it provides better coverage than the battery of 5–6 biochemical reactions traditionally and unanimously used by clinical microbiology labs in Pakistan. The old ID system is based on the manual preparation of individual biochemical reactions such as oxidase, growth at 42°C, pigmentation, triple sugar iron (TSI) that requires more elaborated lab setup, is loaded with numerous preparation

Table 6. Concordance of QTS-NE with PCR-based identification at species level

Organism	Total number of strains tested	QTS-NE			API-NE			PCR		
		No. of identified strains	% sensitivity	% specificity	No. of identified strains	% sensitivity	% specificity	No. of identified strains	% sensitivity	% specificity
Pseudomonas aeruginosa	50	48	96	100	42	84	98	50	100	100
Pseudomonas fluorescens	20	20	100	100	19	95	96	20	100	100
Pseudomonas putida	10	08	80	99	07	70	98	10	100	100
Pseudomonas stutzeri	10	08	80	100	08	80	93	10	100	100
Stenotrophomonas maltophilia	20	20	100	100	20	100	100	20	100	100
Acinetobacter baumannii	09	07	78	100	07	78	93	09	100	100
Acinetobacter lwoffii	08	06	75	99	06	75	94	08	100	100
Ralstonia pickettii	14	13	93	100	14	100	100	14	100	100
Burkholderia cepacia	03	03	100	100	03	100	97	03	100	100
Shewanella putrefaciens	05	05	100	100	05	100	97	05	100	100
Aeromonas hydrophila	05	05	100	100	05	100	98	05	100	100
Vibrio cholerae	50	50	100	100	48	96	98	50	100	100
Vibrio parahaemolyticus	20	20	100	100	20	100	100	20	100	100
Vibrio alginolyticus	07	07	100	100	06	86	96	05	71	99
Plesiomonas shigelloides	05	05	100	100	05	100	98	05	100	100
Brevundimonas diminuta	01	01	100	100	01	100	100	0	0	0
Total	232	221	95	99	216	93	96	229	99	99

steps, interpretation errors and identifies only 6 or 7 bacterial species.

In conclusion, this study presents the development and evaluation of rapid bacterial identification system for Gram-negative oxidase-positive non-fermenting bacteria which can be useful for clinical diagnosis in resource-limited countries.

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