Use of second-line biochemical and susceptibility tests for the differential identification of coryneform bacteria

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Objective: To investigate which 'second-line' biochemical and susceptibility tests could be used for the differential identification of coryneform bacteria (*Actinomyces, Arcanobacterium, Brevibacterium, Corynebacterium, Dermabacter, Rothia, Turicella*).

Methods: Tests used for the diagnosis of certain Gram-negative rods and nocardiforms: growth in 10% NaCl; production of β-galactosidase, arginine dihydrolase, ornithine and lysine decarboxylase, γ-glutamylaminopeptidase, and methanethiol; hydrolysis of tributyrin, casein, tyrosine, and starch; assimilation of carbohydrates in the API 20 NE system; gas–liquid chromatography for end products of glucose metabolism, and susceptibility to antimicrobials used for diagnostic purposes.

Results: Some tests proved useful for the differentiation within the genus *Corynebacterium* (e.g. growth in 10% NaCl, tyrosine hydrolysis, furazolidine and O/129 susceptibility), while others differentiated only between genera (e.g. methanethiol formation, bacitracin susceptibility).

Conclusion: Second-line tests can be of use for the differential identification of coryneform bacteria.

Key words: Coryneforms, identification

INTRODUCTION

The identification of coryneform bacteria (i.e. facultatively anaerobic or aerobic, asporogenous, non-partially-acid-fast, irregular Gram-positive rods) is often a difficult task for medical microbiologists [1]. This is due to the enormous heterogeneity within this group of bacteria as well as to the fact that the few identification systems available possess incomplete biochemical panels or databases. In the first systematic approach to identify coryneform bacteria at the species level by means of biochemical reactions, Hollis and Weaver [2] used a broad armamentarium. Their guide for the identification of coryneform bacteria became the basis for the development of commercial identification systems (e.g. the API Coryne systems [3,4]). However, both the Hollis–Weaver system [2] and the two versions of the API Coryne system [3,4] (with identical test panels but an enlarged database) do not provide sufficient discrimination to correctly identify some species [3,4]. In order to supplement these and our recent [5] identification systems, we tried to search for further easy-to-perform and inexpensive biochemical reactions known from the diagnosis of Gram-negative rods and nocardiforms as well as for susceptibility tests which could contribute to the differentiation of coryneform bacteria.

One hundred and ninety-four strains of coryneform bacteria representing 25 different taxa were examined for their reactivity in 11 such biochemical tests and in 12 assimilation reactions, as well as for their end products of glucose metabolism. In addition, patterns of susceptibility of these taxa to five infrequently used antimicrobial agents were determined by disk diffusion. All these tests will henceforth be called 'second-line' tests.

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MATERIALS AND METHODS

Bacterial strains and culture conditions

The 194 strains used in the present study (Table 1) came from the culture collection of the Department of Medical Microbiology, University of Zurich. The majority (178 of 194) were potential human pathogens. Only strains of *Arcanobacterium pyogenes, Corynebacterium kutscheri, C. pseudotuberculosis* and *C. renale* are more frequently encountered in veterinary specimens than in human samples; however, in order to cover a wide range of coryneform bacteria, these four species were also included, albeit with only a few strains. The number of strains tested for each of the human taxa did not reflect their frequency of isolation in clinical specimens; however, we wanted to build a relatively broad database for each taxon.

The strains had been previously identified at the species level using established methods which included both chemotaxonomic and molecular genetic methods [1,3–5]. All strains had initially been cultured at 37°C in a 5% CO_2 atmosphere on Columbia agar (Difco, Detroit, USA) supplemented with 5% sheep blood (SBA). Subculturing was performed as follows: lipophilic *Corynebacterium* species (i.e. *C. afermentans* subsp. *lipophilum, Corynebacterium* CDC group G bacteria, *C. jeikeium,* and *C. urealyticum)* were cultured on SBA supplemented with 1% Tween-80 (Merck, Darmstadt, Germany) at 37°C in ambient air, *Actinomyces* and *Arcanobacterium* strains on SBA plates in a 5% CO_2-enriched atmosphere, and the remaining strains on SBA in ambient air at 37°C. Cells were harvested after 24 h of incubation.

Table 1 Taxa included in the present study

Species	No. of strains tested
Actinomyces neuii	10
Arcanobacterium haemolyticum	10
Arcanobacterium pyogenes	7[a]
Brevibacterium casei	10
Corynebacterium afermentans subsp. *afermentans*	5
C. afermentans subsp. *lipophilum*	5
C. amycolatum	10
C. auris	8
Corynebacterium CDC group G	10
Corynebacterium CDC group I-1	5
C. diphtheriae	10
C. glucuronolyticum	10
C. jeikeium	10
C. kutscheri	3[a]
C. minutissimum	10
C. propinquum	5
C. pseudodiphtheriticum	10
C. pseudotuberculosis	3[a]
C. renale	3[a]
C. striatum	10
C. ulcerans	3
C. urealyticum	10
Dermabacter hominis	10
Rothia dentocariosa	7
Turicella otitidis	10
Total number of strains tested	194

[a]Mainly isolated from veterinary specimens.

Biochemical tests

Unless otherwise stated, all biochemical reactions were carried out at 35°C in ambient air and read after 24 h of incubation.

Growth (inoculum of approximately 10^6 cells) in 5 mL trypticase soy broth with 10% NaCl (both Becton Dickinson, Cockeysville, USA) was recorded as positive if clouding of the medium occurred within 48 h.

For determination of ornithine decarboxylase (ODC) and lysine decarboxylase (LDC) activities, an ODC or LDC tablet (Rosco, Taastrup, Denmark) was placed into a McFarland 2 bacterial suspension in 0.5 mL 0.9% NaCl which was covered with paraffin oil. A positive reaction was indicated by a blue color.

β-Galactosidase (PNPG), arginine dihydrolase (ADH) and 12 carbohydrate-assimilation reactions (including adipate and malate) were checked in the API 20 NE gallery (bioMérieux, Marcy l'Etoile, France). Assimilation reactions were read up to 1 week, and PNPG and ADH reactions after 48 h, of incubation.

Hydrolysis of tributyrin was observed by preparing a McFarland standard 3 suspension in 0.5 mL 0.9% NaCl to which a diagnostic tablet (Rosco) was added. A yellow color indicated a positive reaction.

Activity of γ-glutamylaminopeptidase (γGT) was assessed by placing a diagnostic tablet (Rosco) into a McFarland 3 suspension covered by paraffin oil. A yellow color indicated a positive reaction.

Production of methanethiol from L-methionine was observed in DTNB medium (skimmed milk powder L31 (Oxoid, Basingstoke, UK), L-methionine, K_2HPO_4, and 5,5-dithio-bis(2-nitrobenzoic acid) (all from Sigma Chemicals, St Louis, USA)) as outlined previously [6]. Appearance of a bright yellow pigment within 24 h was scored as positive (for *Actinomyces* and *Arcanobacterium* spp., the medium had to be incubated in a 5% CO_2 atmosphere).

Casein hydrolysis was observed on 7% skimmed milk (Difco) agar plates, and tyrosine hydrolysis on Nutrient Broth no. 2 (Oxoid) agar plates supplemented with 0.5% tyrosine, both as outlined by Nash and Krenz [7]. For lipophilic bacteria, these plates were supplemented with 1% Tween-80. Plates were incubated for up to 1 week; a clearing of the medium indicated a positive reaction.

Starch hydrolysis was determined on Mueller–Hinton agar plates inoculated with about 10^8 cells per spot (some cells were also stabbed into the agar plate). After 48 h, Lugol's solution [8] was flushed over the agar plate. Zones positive for starch hydrolysis became translucent.

For determination of the end products of glucose metabolism, 6×10^7 cells (cell density determined in an ATB 1550 photometer (bioMérieux)) were transferred into 5 mL of brain–heart infusion broth (Becton Dickinson) supplemented with 1% glucose and incubated for 48 h. The detection method for volatile and non-volatile fatty acids using a gas chromatograph and a 3390A integrator (Hewlett-Packard, Palo Alto, USA) has been outlined previously [9].

Susceptibility tests

Mueller–Hinton agar plates supplemented with 5% sheep blood were inoculated with a bacterial suspension grown in trypticase soy broth to a 0.5 McFarland density. For lipophilic bacteria, plates were supplemented with 1% Tween-80. Susceptibility to bacitracin (0.04 U, Becton Dickinson), colistin (10 μg, Becton Dickinson), furazolidine (50 μg, Rosco), novobiocin (5 μg, Becton Dickinson) and O/129 (150 μg, Oxoid) was determined by placing the disks on the inoculated plates and incubating them for 20 h at 35°C. *Actinomyces, Arcanobacterium* and *Rothia* strains were grown in a 5% CO_2-enriched atmosphere at 37°C. Any inhibition zone around the disks was considered to indicate susceptibility.

RESULTS

Some species known to be inhabitants of the human skin (e.g. *Brevibacterium casei, C. minutissimum, Dermabacter hominis*) [1] were able to grow at a concentration of 10% NaCl, whereas other strains from the same habitat (e.g. *C. amycolatum)* were unable to grow (Table 2). *Actinomyces neuii* [9] and *D. hominis* [10] were the only two taxa expressing ODC activity, and *D. hominis* strains were also consistently positive for LDC [10], while corynebacteria were never positive for either decarboxylase. The same applied to the presence of β-galactosidase, which was detected in all *Actinomyces, Arcanobacterium* and *Dermabacter* species but very rarely in *Corynebacterium*. None of the 194 strains tested exhibited positive ADH or γGT reactions. In contrast, many different species of different genera were able to hydrolyze tributyrin. Production of methanethiol (within 24 h) was an almost exclusive feature of *B. casei* strains. The same bacteria also consistently hydrolyzed casein and tyrosine. All *Arcanobacterium pyogenes* and all *C. renale* strains were also able to hydrolyze casein, while tyrosine was also hydrolyzed by many strains of *Corynebacterium* CDC group I-1, *C. minutissimum, C. propinquum, C. pseudodiphtheriticum,* and *C. striatum*. Starch was hydrolyzed by many different taxa as well. Significantly, *C. auris* and most *C. afermentans* subsp. *afermentans* strains were able to degrade starch, whereas *B. casei* and *Turicella otitidis* were unable to do so. In addition, *C. glucuronolyticum* hydrolyzed starch, whereas the three strains of *C. renale* did not.

Table 2 lists only those assimilation reactions that were positive in ≥90% of all strains tested. Seventy-three of the 194 strains tested, in particular the lipophilic *Corynebacterium* spp., were non-reactive. *B. casei* was the most reactive taxon. *C. amycolatum* and *C. striatum* as well as *Corynebacterium* CDC group I-1 utilized mannose and malate, whereas *C. minutissimum* utilized mannose only. *C. auris* and *T. otitidis* could be clearly separated by the utilization of adipate *(C. auris)*.

The presence of end products of glucose metabolism was found not to be specific for any genus or species, but their absence would at least rule out some species (Table 2). Notable was the production of succinic and lactic acid by *Corynebacterium* CDC group I-1 bacteria, *C. minutissimum,* and *C. striatum*, whereas *C. amycolatum* mainly produced propionic and lactic acid.

Bacitracin (0.04 U) and colistin caused zones for many *Corynebacterium* spp., whereas only few *Actinomyces* and *Arcanobacterium* strains were inhibited (Table 3). Interestingly, however, only one strain of the *C. diphtheriae* group *(C. diphtheriae, C. ulcerans, C. pseudotuberculosis)* showed an inhibition zone around the colistin disk, whereas all the other strains showed none. Furazolidine exhibited limited activity: nine of 10 *C. minutissimum* strains were found to have inhibition zones, whereas none of the *C. amycolatum* strains yielded a zone and all *Arcanobacterium pyogenes* and *C. renale* strains were inhibited. In contrast, all strains except two *Corynebacterium* CDC group G strains and one *C. urealyticum* strain showed inhibition zones around a 5-μg novobiocin disk. Finally, none of the *C. striatum, Corynebacterium* CDC group I-1 bacteria and *C. minutissimum* strains were resistant to O/129, whereas all *C. amycolatum* strains were. Eight of 10 *C. pseudodiphtheriticum* as well as all *D. hominis* and *T. otitidis* strains were also resistant to O/129.

DISCUSSION

We do not know why the biochemical reactions and susceptibility patterns reported in the present study have not been systematically examined before. One of the reasons might be that extensive biochemical profiling has, in the past, been directed more towards

Table 2 Biochemical tests for species identification of coryneform bacteria

Species	Biochemical features (% of positive reactions)												
	10% NaCl	ODC	LDC	PNPG	ARG	TRI	γGT	Methane-thiol	Casein	Tyrosine	Starch	*Assimilations* (≥90% of strains)	*End* products
Actinomyces neuii	0	100	70	100	0	100	0	0	0	0	40		A, L, S
Arcanobacterium haemolyticum	0	0	0	100	0	80	0	0	0	0	50		L, S
Arcanobacterium pyogenes	0	0	0	100	0	0	0	0	100	0	14		A, L, S
B. casei	90	0	0	0	0	60	0	90	100	100	0	NAG, GNT, MLT, CIT, PAC	NU
C. afermentans subsp. *afermentans*	0	0	0	0	0	20	0	20	0	20	67		NU
C. afermentans subsp. *lipophilum*	0	0	0	0	0	60	0	0	0	0	0		NU
C. amycolatum	0	0	0	10	0	100	0	0	0	0	100	MNE, MLT	L, P
C. auris	0	0	0	0	0	100	0	0	0	0	100	ADI, MLT	NU
Corynebacterium CDC group G	40	0	0	0	0	50	0	0	0	0	50		A, L, S
Corynebacterium CDC group I-1	80	0	0	0	0	80	0	0	0	80	40	MNE, MLT	A, L, S
C. diphtheriae	0	0	0	0	0	40	0	0	0	0	20	MNE, MLT	A, L, P, S
C. glucuronolyticum	0	0	0	10	0	100	0	0	0	0	100		A, L, P, S
C. jeikeium	30	0	0	0	0	90	0	0	0	0	40		NU
C. kutscheri[a]	0	0	0	0	0	67	0	0	0	0	0		A, L, P, S
C. minutissimum	90	0	0	0	0	20	0	0	0	90	90	MNE	A, L, S
C. propinquum	60	0	0	0	0	0	0	0	0	80	0		NU
C. pseudodiphtheriticum	40	0	0	0	0	0	0	0	0	50	0		NU
C. pseudotuberculosis[a]	0	0	0	33	0	0	0	0	33	0	100		A, L, S
C. renale[a]	67	0	0	0	0	0	0	0	100	0	0		A, L, S
C. striatum	10	0	0	0	0	100	0	0	0	100	90	MNE, MLT	L, S
C. ulcerans[a]	0	0	0	0	0	100	0	0	0	0	100		A, L, P, S
C. urealyticum	20	0	0	0	0	100	0	0	0	0	0		NU
D. hominis	60	100	100	100	0	100	0	0	0	0	80		A, L
R. dentocariosa	14	0	0	14	0	57	0	0	0	0	29		A, L, S
T. otitidis	40	0	0	0	0	100	0	0	0	0	0	MLT	NU

10% NaCl, growth in 10% NaCl trypticase soy broth; ODC, ornithine decarboxylase; LDC, lysine decarboxylase; PNPG, *p*-nitrophenyl-β-D-galactopyranoside (β-galactosidase activity); ARG, arginine dihydrolase; TRI, tributyrin; γGT, glutamylaminopeptidase; *methanethiol, production of methanethiol from L-methionine; Casein, casein hydrolysis; Tyrosine, tyrosine hydrolysis;* Starch, starch hydrolysis; NAG, *N*-acetylglucosamine; GNT, gluconate; MLT, malate, CIT, citrate; PAC, phenylacetate; MNE, mannose; ADI, adipate; S, succinic acid; L, lactic acid; A, acetic acid; P, propionic acid; NU, Reactions were very weak and could not be used for diagnostic purposes.

[a]The small number of strains tested makes it difficult to draw valid conclusions from the data.

Table 3 Susceptibility tests for species identification of coryneform bacteria

Species	Any inhibition zone around the following disks (% of positive strains)				
	Bacitracin (0.04 IU)	Colistin (10 μg)	Furazolidine (50 μg)	Novobiocin (5 μg)	O/129 (150 μg)
Actinomyces neuii	20	0	30	100	100
Arcanobacterium haemolyticum	10	0	40	100	0
Arcanobacterium pyogenes	0	0	100	100	0
B. casei	60	70	10	100	0
C. afermentas subsp. *afermentans*	100	100	0	100	60
C. afermentans subsp. *lipophilum*	100	80	20	100	80
C. amycolatum	100	60	0	100	0
C. auris	100	100	0	100	75
Corynebacterium CDC group G	90	80	30	80	80
Corynebacterium CDC group I-1	100	100	0	100	100
C. diphtheriae	90	10	0	100	90
C. glucuronolyticum	100	100	10	100	100
C. jeikeium	90	80	30	100	100
C. kutscheri	100	67	0	100	100
C. minutissimum	100	80	90	100	100
C. propinquum	100	100	0	100	40
C. pseudodiphtheriticum	100	100	0	100	20
C. pseudotuberculosis	67	0	0	100	0
C. renale	100	0	100	100	100
C. striatum	100	50	10	100	100
C. ulcerans	100	0	0	100	67
C. urealyticum	100	50	0	90	20
D. hominis	100	0	0	100	0
R. dentocariosa[a]	83	17	50	100	83
T. otitidis	100	100	0	100	0

[a]Only six strains tested.

Table 4 Second-line tests useful in the differential identification of biochemically or morphologically close coryneforms

Feature	Use in differential diagnosis of:
Growth in 10% NaCl	*C. minutissimum* (90% +) versus *C. amycolatum* (–)
Ornithine decarboxylase	*Actinomyces neuii* and *D. hominis* (+) versus other fermentative coryneforms (–)
Lysine decarboxylase	*Actinomyces neuii* (70% +) and *D. hominis* (+) versus other fermentative coryneforms (–)
β-Galactosidase	*Actinomyces neuii, Arcanobacterium haemolyticum, Arcanobacterium pyogenes, D. hominis* versus other fermentative coryneforms (mostly –)
Tributyrin hydrolysis	*C. glucuronolyticum* (+) versus *C. renale* (–)
Methanethiol formation	*B. casei* (90% +) versus other non-fermentative coryneforms (–)
Casein hydrolysis	*C. glucuronolyticum* (–) versus *C. renale* (+)
	B. casei versus other non-fermentative coryneforms (–)
Tyrosine hydrolysis	*C. striatum* (+) versus *C. amycolatum* (–)
	C. minutissimum (90% +) versus *C. amycolatum* (–)
Starch hydrolysis	*C. auris* (+) versus *T. otitidis* (–)
Adipate assimilation	*C. auris* (+) versus *T. otitidis* (–)
Malate assimilation	*C. amycolatum* (+) versus *C. minutissimum* (–)
Bacitracin (0.04 U)	*Actinomyces* and *Arcanobacterium* spp. tested (NZ) versus *Corynebacterium* spp. (Z)
Furazolidine	*C. minutissimum* (90% Z) versus *C. amycolatum* (NZ)
O/129	*C. minutissimum* (Z) versus *C. amycolatum* (NZ)

NZ, no zone; Z, zone.

non-fastidious Gram-negative rods, which generally exhibit faster and stronger reactions than coryneform bacteria.

Table 4 summarizes the features which can be used with confidence in the differential diagnosis of certain coryneform bacteria. The difficulties in the differential diagnosis of *C. auris* and *T. otitidis*, both of which are isolated from patients with ear infections, have been outlined previously [11,12]. The same is true for the differentiation between *C. amycolatum* and *C. minutissimum* [13,14], both of which are relatively frequently encountered *Corynebacterium* species in clinical specimens. *C. glucuronolyticum* [15] and *Actinomyces neuii* (G. Funke, unpublished observations) are both frequently isolated from genitourinary specimens and might be confused with each other, as both exhibit a strongly positive CAMP reaction [1]. Besides β-glucuronidase, ODC and/or LDC reactions may assist in the differentiation of these two taxa. All true *Corynebacterium* spp. tested were negative for both ornithine and lysine decarboxylase activity, which differentiates them from *D. hominis* [10] and *Actinomyces neuii* [1].

Similar to fosfomycin, which can be used in a selective medium for the isolation of *Corynebacterium* spp. [16], a medium containing bacitracin and/or colistin could be used for the selective isolation of the *Actinomyces* and *Arcanobacterium* species included in the present study. Whether such a medium would allow the isolation of other aerobically growing *Actinomyces* spp. remains to be determined. Furazolidine has been used in selective media for the recovery of *Corynebacterium* spp. [17].

It is evident that testing for the enzymes ADH and γGT is of no value for the differentiation of coryneform species. The same is true, in our view, for the determination of the end products of glucose metabolism with our system. Although brain–heart infusion broth allows aerobic incubation (rather than anaerobically incubated chopped meat broth), our data on *Arcanobacterium* spp. are in contrast to published data listing acetic, lactic and succinic acid as the major end products of glucose metabolism [18]. The non-fermenting coryneforms accumulated end products too weakly for them to be of use for differentiation. The entire set of API 20 NE assimilation reactions seems much too expensive (25 strips = sFr 235) for the identification of coryneform bacteria because of the limited conclusions (e.g. malate and adipate assimilation) that can be drawn from the resulting pattern. An exception would be species identification within the genus *Brevibacterium* [19].

Single reactions which have not been reported before include the ability of some *C. pseudodiphtheriticum* strains to hydrolyze tyrosine, which makes this test unsuitable for separation from *C. propinquum* (reported as consistently positive for tyrosine hydrolysis [20]), the closest phylogenetic neighbor [1]. The usefulness of the O/129 susceptibility test has been demonstrated before in the identification of *C. amycolatum* [21]. Interestingly, many *C. pseudodiphtheriticum* strains were also resistant to this compound. However, the fermenting *C. amycolatum* and the non-fermenting *C. pseudodiphtheriticum*, both of which may present with dry colonies, will not easily be confused in the routine laboratory. Tyrosine degradation observed in *C. minutissimum* (in contrast to *C. amycolatum*) confirms the previous data of Wauters et al [13]. Reactions for *C. striatum* and *Corynebacterium* CDC group I-1 were almost identical, thereby reinforcing the close affinity between these two taxa which has also been demonstrated by mycolic acid analysis [22]. Although it is most likely that *Corynebacterium* CDC group I-1 strains represent sucrose-negative *C. striatum* strains, we have kept them as two different entities, since final confirmation of this hypothesis by means of DNA–DNA hybridization is still lacking.

In summary, we conclude that easy-to-perform and inexpensive biochemical tests like growth in 10% NaCl, ornithine and lysine decarboxylase, β-galactosidase, hydrolysis of tributyrin, casein, tyrosine and starch, as well as methanethiol production, are suitable complementary tests for the differential identification of coryneform bacteria.

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