Acta Veterinaria Hungarica 67 (3), pp. 327–337 (2019)
DOI: 10.1556/004.2019.034

CARBON SOURCE UTILISATION AND EVALUATION OF THE BIOLOG SYSTEM IN THE IDENTIFICATION OF *ACTINOBACILLUS PLEUROPNEUMONIAE*

László MAKRAI*, Rita SÁRKÖZI and László FODOR

Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, H-1581 Budapest, P.O. Box 22, Hungary

(Received 4 March 2019; accepted 31 July 2019)

Sixty-eight *Actinobacillus pleuropneumoniae* strains were isolated from porcine acute pleuropneumonia cases from different parts of Hungary between 2000 and 2014. A total of 41 isolates were identified as *A. pleuropneumoniae* biotype I and 27 strains as biotype II based on cultural, morphological and biochemical characteristics. The aim of this study was to evaluate metabolic fingerprinting in the species-level identification of *A. pleuropneumoniae* isolates. Utilisation of carbon sources by these field isolates and six reference strains was characterised by the Biolog system (GN2 Microplate, MicroLog3 Version 4.20.05 software). Twenty-nine field strains were correctly identified by the Biolog system as *A. pleuropneumoniae*, 36 strains as *A. lignieresii*, two strains as *H. paraphrohaemolyticus* and one strain as *A. equuli* after 24 h of incubation. Among the six *A. pleuropneumoniae* reference strains the Biolog system identified one strain as *A. pleuropneumoniae*, four as *A. lignieresii* and one as *H. paraphrohaemolyticus*. There was no correlation between biotypes and serotypes of *A. pleuropneumoniae* and the carbon source utilisation pattern and species identification by the Biolog system. Our data indicate that the efficacy of the Biolog system used here could be improved by including phenotypes of more *A. pleuropneumoniae* strains representing a wider geographical occurrence into the database.

Key words: *Actinobacillus pleuropneumoniae*, carbon source utilisation, Biolog system, identification

Actinobacillus pleuropneumoniae is one of the most important bacterium species causing respiratory disease in swine all over the world. Acute haemorrhagic-necrotic pneumonia with fibrinous pleuritis can usually be seen among 12- to 16-week-old pigs, but actually the acute or peracute form can be recognised in all ages, while the chronic form generally develops after the disappearance of acute signs. Both forms can cause huge economic losses (Marsteller and Fenwick, 1999; Christensen and Bisgaard, 2004; Gottschalk, 2012).

*Corresponding author; E-mail: makrai.laszlo@univet.hu; Phone: 0036 (1) 251-9900; Fax: 0036 (1) 251-9260

Actinobacillus pleuropneumoniae strains are facultative anaerobic, medium-sized (< 2 μm), non-motile rods covered by a polysaccharide capsule; they can infect only swine (Markey et al., 2013). The bacterium has two biotypes; biotype I strains require nicotinamide adenine dinucleotide (NAD, V-factor) for growth, while biotype II strains are NAD independent. On the basis of surface soluble capsular polysaccharide antigens 18 serovars have been described (Nielsen, 1986a,b; Fodor et al., 1989; Nielsen et al., 1997; Blackall et al., 2002; Sárközi et al., 2015; Bossé et al., 2018).

All 18 serovar reference strains are able to express some of the four different Apx toxins belonging to the pore-forming repeats-in-toxin (RTX) group (Shin et al., 2011; Sárközi et al., 2015; Bossé et al., 2018). Three toxins, ApxI, ApxII and ApxIII are responsible for haemolysis and cytotoxic damage of the lung cells (Sthitmatee et al., 2003), but these toxins are produced by other bacterium species, too, not only by *A. pleuropneumoniae* (Schaller et al., 2001). The *ApxIV* toxin gene is species specific, it can be found only in *A. pleuropneumonae* strains, and ApxIV toxin is produced only in live animals (Schaller et al., 2001). Although *A. lignieresii* is the species most closely related to *A. pleuropneumoniae* as determined by DNA-DNA hybridisation and comparison of the 16S rRNA sequences (Borr et al., 1991), there is no *apxIVA* gene in *A. lignieresii* (Schaller et al., 2001).

There are six members of the *Actinobacillus* genus which are nowadays recognised as significant causes of diseases in animals: *A. pleuropneumoniae*, *A. suis*, *A. lignieresii*, *A. equuli*, *A. seminis*, and *A. capsulatus* (Rycroft and Garside, 2000). Many species of the *Actinobacillus* genus other than *A. pleuropneumoniae* can be found in tonsils of swine, such as *A. minor*, *A. porcinus*, Bisgaard's Taxon 10, *A. rossii*, and *A. porcitonsillarum* (Lowe et al., 2011; Gottschalk, 2012). *Actinobacillus muris*, *A. hominis* and *A. ureae* are species of little veterinary impact (Christensen and Bisgaard, 2004).

Actinobacillus lignieresii can be found on the mucous membranes of cattle and is able to cause wooden tongue or lesions in the oral cavity and the regional lymph nodes (Gottschalk, 2012). Differentiation of *A. pleuropneumoniae* biotype II strains and *A. lignieresii* is difficult because of their close phylogenetic relationship and common characteristics (Rycroft and Garside, 2000).

There are different methods for the identification of bacteria. In addition to the detection of genus-, species- and serotype-specific genes, identification based on phenotypic characteristics is also widely used. Besides the traditional identification using cultural, morphological, biochemical and serological features (Barrow and Feltham, 2003), several identification systems based on the examination of phenotypic characteristics are available on the market. Different manual biochemical identification systems like API (Bio-Mérieux, Lyon, France), RapID System (Thermo Fisher Scientific, Lenexa, KS, USA), BD BBL Identification System (Becton Dickinson, Franklin Lakes, NJ, USA) and automated identifica-

tion systems, like VITEK (Bio-Merieux), BD Phoenix System (Becton Dickinson), Sherlock Microbial ID System (MIDI Inc., Newark, DE, USA) and matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) methods are widely used.

The Biolog Microbial Identification Systems (Biolog Inc., Hayward, CA, USA) are available in manual, semi-automated and fully automated forms; they identify the bacteria on the basis of utilisation of carbon sources (Wong et al., 1992). Their databases include *A. pleuropneumoniae*, *A. lignieresii*, *A. hominis*, *A. equuli* and *A. suis* from the *Actinobacillus* genus.

The aim of this study was to examine metabolic fingerprinting of field isolates and reference *A. pleuropneumoniae* strains based on the utilisation of 95 carbon sources and to evaluate this method in the identification of *A. pleuropneumoniae* strains.

Materials and methods

Bacterium strains

Sixty-eight *A. pleuropneumoniae* field isolates were included in the examination; all were isolated from lung samples collected in slaughterhouses and from postmortem cases of acute porcine pleuropneumonia, submitted to our laboratory from different Hungarian swine farms between 2000 and 2010. One of them was suggested as reference strain of serotype 16 isolated in 2014. Six serotype reference strains of *A. pleuropneumoniae* K17 (serotype 5a), L20 (serotype 5b), CVI13261 (serotype 9), D13039 (serotype 10), 56153 (serotype 11) and N273 (serotype 13) provided by Dr. Ø. Angen (Danish Veterinary Laboratory, Copenhagen) were included in the examinations.

The *A. pleuropneumoniae* strains were isolated on Tryptone Soya Agar (TSA, Biolab Ltd., Hungary) cross-inoculated with *Staphylococcus aureus*, and subcultured on chocolate agar with added 50 µg/ml NAD (Biolab Ltd., Hungary), both containing 10% defibrinated sheep blood. Cultures were incubated at 37 °C for 24 h in aerobic environment with the addition of 5% carbon dioxide. They were identified using standard methods (Barrow and Feltham, 2003), and most strains were serotyped (Sárközi et al., 2018). After identification, the isolated *A. pleuropneumoniae* strains were stored at –80 °C until further examination.

Carbon source utilisation

A 96-well automated MicroLog MicroStation System with GN2 Microplates (Biolog Inc., Hayward, CA, USA) was used for the characterisation of carbon source utilisation. Microplates were set up and analysed following the manufacturer's instructions with minor modification. Single colonies of biotype I of *A. pleuropneumoniae* were subcultured three times on Biolog Universal Growth

(BUG) agar plates with NAD, and biotype II strains were cultured on BUG agar containing 10% defibrinated blood. Two pure colonies from the third subculture of each strain were inoculated on two chocolate plates with NAD or blood agar plates evenly covering the whole surface of the plate. Plates were cultured at 37 °C and 5% CO_2. After 24-h incubation, the thin and confluent layer of *A. pleuropneumoniae* was collected with a cotton swab and suspended in 18 ml inoculation fluid (GN/GP IF) to obtain a homogeneous suspension. The turbidity of the bacterium suspensions was set to 20 ± 2% using the Biolog Turbidimeter. GN2 MicroPlates were inoculated with 150 µl bacterium suspension per well and incubated at 37 °C in 5% CO_2 atmosphere. Metabolic activity was determined by visual reading of the plates after 24 h. The results were evaluated and a dendrogram showing the metabolic relationships between the strains was produced by Biolog MicroLog3 software (Biolog Inc., USA, Version 4.20.05).

Results

Identification of bacterium isolates

All 68 field isolates were Gram-negative, < 2 µm, coccoid rods. They produced small grey colonies surrounded by a narrow β-haemolytic zone. They all produced urease, they were oxidase positive but catalase negative. All strains proved to be *A. pleuropneumoniae,* 41 strains needed NAD, and 27 strains were able to grow without NAD.

Carbon source utilisation

The carbon source utilisation of the *A. pleuropneumoniae* strains is presented in Table 1. There were no major differences between biotype I and II strains of *A. pleuropneumoniae* in carbon source utilisation pattern. All strains were able to metabolise 20 carbon sources and 1–99% of the strains could utilise 27 further carbon sources after 24-hour-long incubation. Twenty-nine out of the 68 field isolates were identified by the Biolog system as *A. pleuropneumoniae*, 36 strains as *A. lignieresii*, two strains as *H. paraphrohaemolyticus*, and one strain as *A. equuli.*

The *A. pleuropneumoniae* reference strain 9 was identified as *A. pleuropneumoniae*, type strains 5a, 10, 11 and 13 as *A. lignieresii*, and 5b as *H. paraphrohaemolyticus*.

The dendrograms show the similarity of all the *A. pleuropneumoniae* strains and that of biotype I and II ones (Figs 1, 2 and 3).

There was no connection between biotype and serotype and identification with the Biolog system.

Table 1

Carbon source utilisation of 68 field isolates (biotypes I and II of *A. pleuropneumoniae*) and 6 reference strains

Carbon sources	Total* (74) (68)	Biotype I* (41) (41)	Biotype II* (27) (27)	Reference strains* (6) (6)
Dextrin	100	100	100	100
N-Acetyl-D-Glucosamine	99	98	100	100
L-Arabinose	99	98	100	100
D-Arabitol	4	5	4	0
D-Cellobiose	100	100	100	100
D-Fructose	100	100	100	100
L-Fructose	97	98	96	100
D-Galactose	100	100	100	100
Gentiobiose	10	12	7	0
α-D-Glucose	100	100	100	100
m-Inositol	7	7	7	0
α-D-Lactose	100	100	100	100
Lactulose	100	100	100	100
Maltose	100	100	100	100
D-Mannitol	100	100	100	100
D-Mannose	100	100	100	100
D-Melibiose	3	2	4	0
D-Psicose	100	100	100	100
D-Raffinose	100	100	100	100
L-Rhamnose	94	98	89	100
D-Sorbitol	100	100	100	100
Sucrose	100	100	100	100
D-Trechalose	38	46	26	50
Turanose	97	100	93	100
Xylitol	1	0	4	17
Pyruvic Acid Methyl Ester	94	90	100	100
Succinic Acid Mono-Methyl Ester	56	71	33	83
Acetic Acid	71	71	70	17
Formic Acid	100	100	100	100
D-Galacturonic Acid	54	54	56	33
D-Gluconic Acid	93	90	96	83
D-Glucosaminic Acid	19	17	22	17
D-Glucuronic Acid	50	59	37	33
α-Hydroxybutyric Acid	97	98	96	100
p-Hydroxy-phenylacetic Acid	1	0	4	17
α-Ketobutyric Acid	99	100	96	83
α-Ketoglutaric Acid	40	41	37	50
α-Ketovaleric Acid	1	0	4	0
D,L-Lactic Acid	100	100	100	100
Propionic Acid	1	0	4	0
Quinic Acid	1	0	4	0
Succinic Acid	99	98	100	83
Bromosuccinic Acid	49	51	44	17
Glucuronamide	74	76	70	33
L-Asparagine	68	68	67	67
L-Aspartic Acid	79	78	81	83
Glycyl-L-Aspartic Acid	1	2	0	0
L-Proline	75	71	81	67
L-Threonine	84	83	85	83
Urocanic Acid	68	61	78	50
Inosine	100	100	100	100
Uridine	100	100	100	100
Thymidine	100	100	100	100
Phenylethyl-amine	4	7	0	0
Putrescine	1	0	4	0
D,L,α-Glycerol Phosphate	1	0	4	0
α-D-Glucose-1-Phosphate	57	49	70	83
D-Glucose-6-Phosphate	100	100	100	83

*proportion of the strains utilising carbon sources (%)

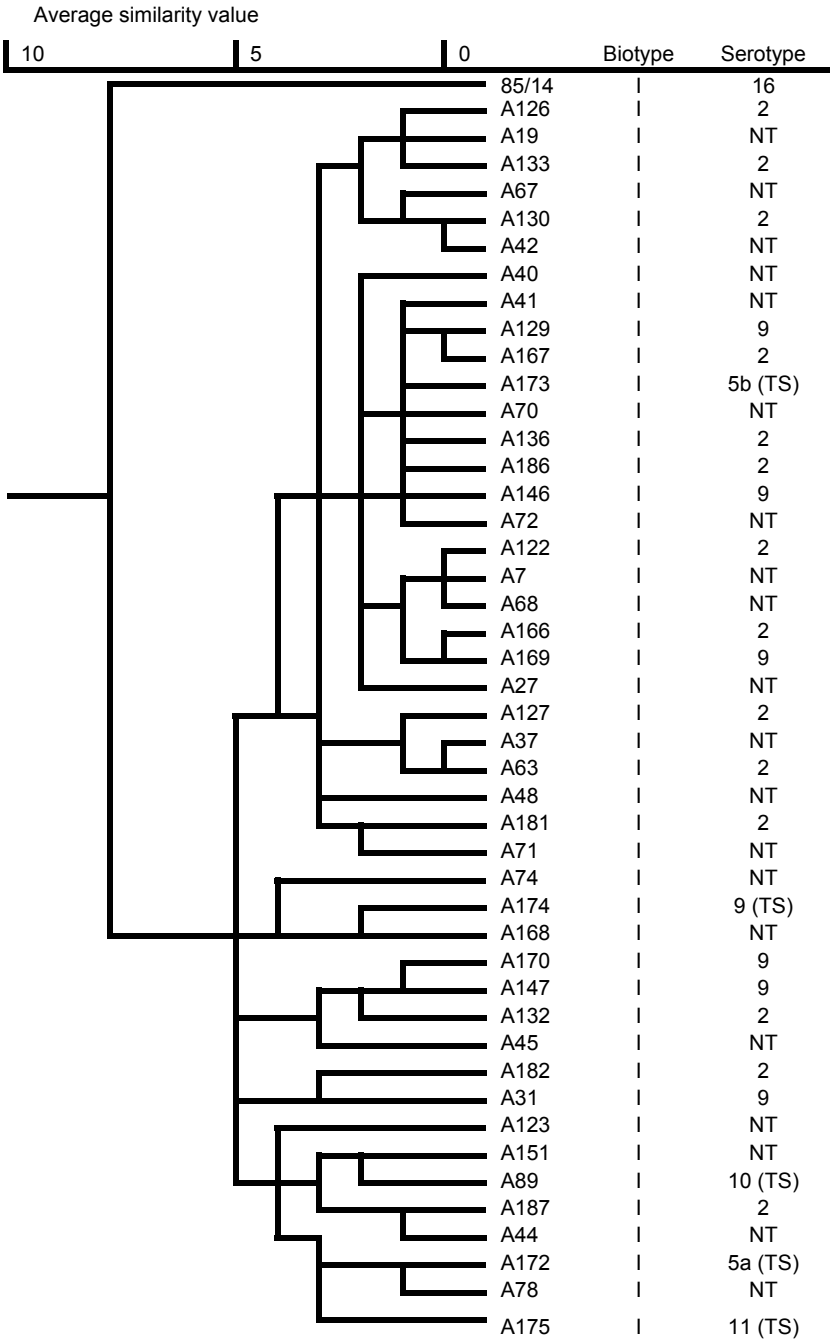

Fig. 1. Dendrogram based on the metabolic fingerprint of biotype I strains (n = 46). NT: non-typeable, TS: type strain

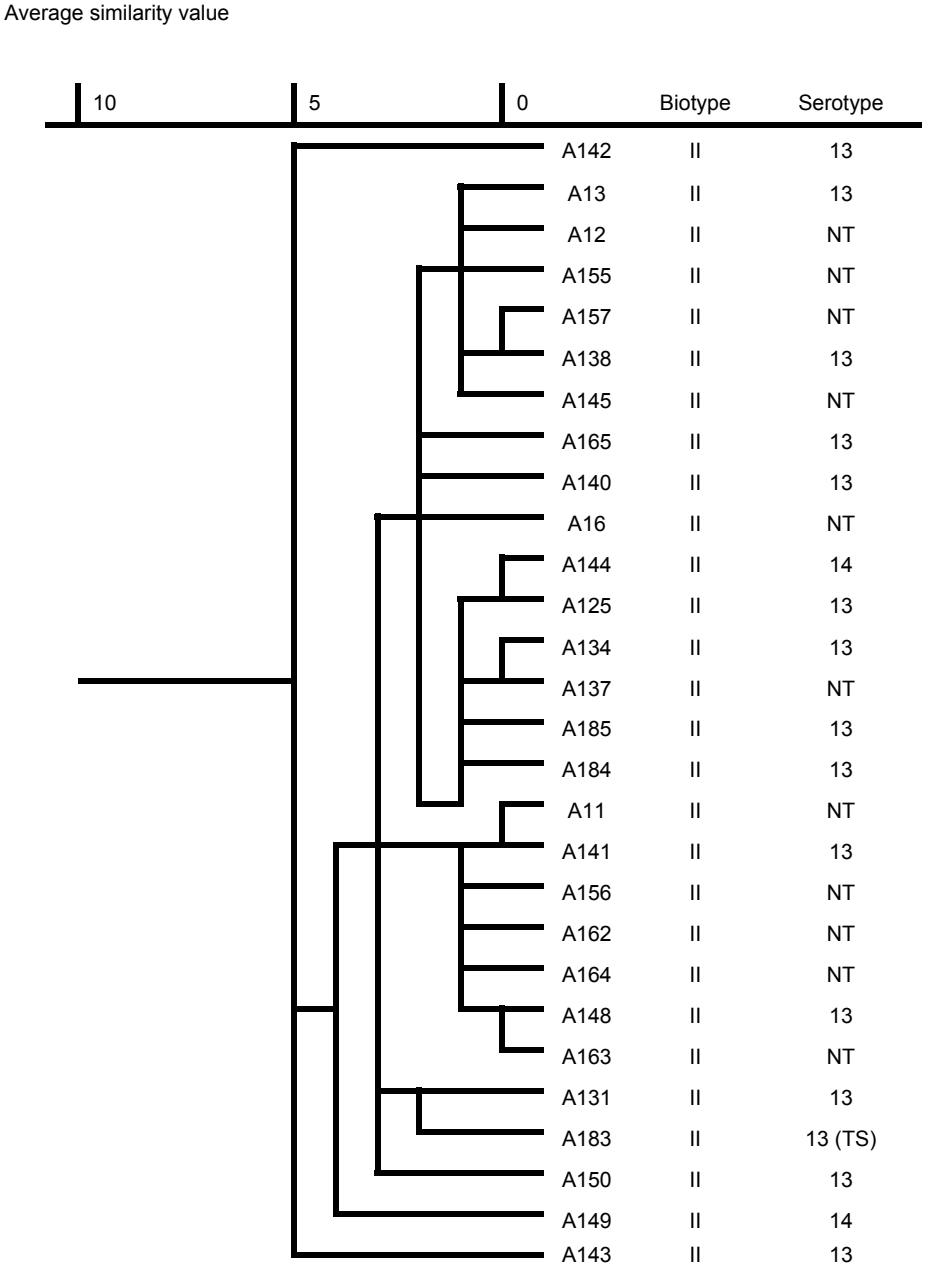

Fig. 2. Dendrogram based on the metabolic fingerprint of biotype II strains (n = 28).
NT: non-typeable, TS: type strain

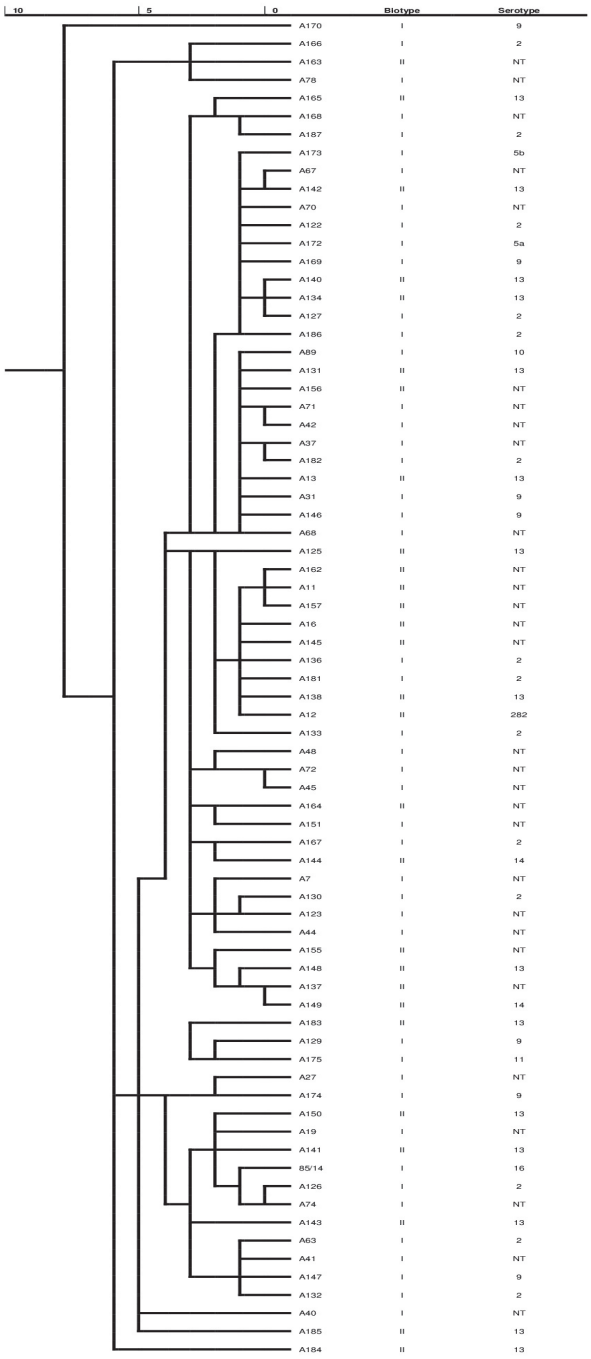

Fig. 3. Dendrogram based on the metabolic fingerprint of 74 *Actinobacillus pleuropneumoniae* strains. NT: non-typeable, TS: type strain (A89, 172–175, 183)

Discussion

In addition to widely used nucleic acid typing methods, identification systems based on the detection of various phenotypic characteristics are also available on the market and used both in human and veterinary medicine. Biolog Microbial Identification Systems are used successfully for the identification of Gram-positive and Gram-negative bacteria (Gyuranecz et al., 2010; Zasada and Mosiej, 2018).

Our results show that identification of the primary pig pathogen *A. pleuropneumoniae* based on carbon source utilisation using the Biolog system has only limited value due to the high similarity of *A. pleuropneumoniae* and *A. lignieresii*. If the metabolic fingerprint shows questionable results, these two bacterium species can be appropriately differentiated by taking into consideration the pathological origin of the bacterial isolate (*A. pleuropneumoniae*: haemorrhagic, necrotic fibrinous pleuropneumonia of swine, *A. lignieresii*: granulomatous mastitis of pigs, or tongue, lymph node, ruminal wall or skin lesions of ruminants) and some cultural (growth on MacConkey agar of *A. lignieresii*) and haemolytic features [haemolysis on blood agar and positive CAMP test with *Staphylococcus aureus (A. pp.)*] of the isolates.

There was no correlation between the biotypes and serotypes of *A. pleuropneumoniae* and carbon source utilisation pattern and species identification by the Biolog system.

Comparing our results with the Biolog standard of *A. pleuropneumoniae* and *A. lignieresii*, it is evident that some patterns of *A. pleuropneumoniae* strains included in the Biolog database have not been represented in our study, as certain carbon sources were not utilised at all by our isolates. No major difference could be seen between the carbon source utilisation of biotype I and II strains of the field isolates.

The dendrograms based on carbon source utilisation show a high level of similarity, especially in the case of biotype II strains of *A. pleuropneumoniae*, where the difference was below 5%. A higher variability was seen in the case of biotype I strains, but the difference was below 7.5% in this case as well. Our data confirm the results of other authors on the low variability of *A. pleuropneumoniae* strains (Fussing et al., 1998; Kokotovic and Angen, 2007; Sassu et al., 2018; Ito et al., 2018).

Actinobacillus pleuropneumoniae strains show a high level of antigenic variability in different geographical locations (Gottschalk, 2012; Perry et al., 2012), and a similar metabolic variability could be expected in the utilisation of carbon sources, too. The efficacy of the Biolog system could be improved by including phenotypes of more *A. pleuropneumoniae* strains representing a wider geographical occurrence into the database.

Acknowledgement

This work was supported by the Hungarian Scientific Research Fund (OTKA 84220 and OTKA 112826).

References

Barrow, G. I. and Feltham, R. K. A. (eds) (2003): Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd edition. Cambridge University Press, Cambridge. 331 pp.

Blackall, P. J., Klaasen, H. L. B. M., Bosch, H. V. D., Kuhnert, P. and Frey, J. (2002): Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet. Microbiol. **84,** 47–52.

Borr, J. D., Ryan, D. A. and MacInnes, J. I. (1991): Analysis of *Actinobacillus pleuropneumoniae* and related organisms by DNA-DNA hybridization and restriction endonuclease fingerprinting. Int. J. Syst. Bacteriol. **41,** 121–129.

Bossé, J. T., Li, Y., Sárközi, R., Fodor, L., Lacouture, S., Gottschalk, M., Casas Amoribieta, M., Angen, Ø., Nedbalcova, K., Holden, M. T. G., Maskell, D. J., Tucker, A. W., Wren, B. W., Rycroft, A. N. and Langford, P. R. (2018): Proposal of serovars 17 and 18 of *Actinobacillus pleuropneumoniae* based on serological and genotypic analysis. Vet. Microbiol. **217,** 1–6.

Christensen, H. and Bisgaard, M. (2004): Revised definition of *Actinobacillus* sensu stricto isolated from animals. A review with special emphasis on diagnosis. Vet. Microbiol. **99,** 13–30.

Fodor, L., Varga, J., Molnár, É. and Hajtós, I. (1989): Biochemical and serological properties of *Actinobacillus pleuropneumoniae* biotype 2 strains isolated from swine. Vet. Microbiol. **20,** 173–180.

Fussing, V., Barfod, K., Nielsen, R., Møller, K., Nielsen, J. P., Wegener, H. and Bisgaard, M. (1998): Evaluation and application of ribotyping for epidemiological studies of *Actinobacillus pleuropneumoniae* in Denmark. Vet. Microbiol. **62,** 145–162.

Gottschalk, M. (2012): Actinobacillosis. In: Zimmerman, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. (eds): Diseases of Swine. Wiley-Blackwell, Chichester. pp. 653–669.

Gyuranecz, M., Erdélyi, K., Fodor, L., Jánosi, K., Szépe, B., Füleki, M., Szőke, I., Dénes, B. and Makrai, L. (2010): Characterisation of *Francisella tularensis* strains, comparing their carbon source utilization. Zoonoses Public Hlth **57,** 417–422.

Ito, H., Takahashi, S., Asai, T., Tamura, Y. and Yamamoto, K. (2018): Isolation and molecular characterization of a urease-negative *Actinobacillus pleuropneumoniae* mutant. J. Vet. Diagn. Invest. **30,** 172–174.

Kokotovic, B. and Angen, Ø. (2007): Genetic diversity of Actinobacillus pleuropneumoniae assessed by amplified fragment length polymorphism analysis. J. Clin. Microbiol. **45,** 3921–3929.

Lowe, B. A., Marsch, T. L., Isaacs-Cosgrove, N., Kirkwood, R. N., Kiupel, M. and Mulks, M. H. (2011): Microbial communities in the tonsils of healthy pigs. Vet. Microbiol. **147,** 346–357.

Markey, B. K., Leonard, F. C., Archambault, M., Cullinane, A. and Maguire, D. (2013): Actinobacillus species. In: Clinical Veterinary Microbiology. Mosby Elsevier, Edinburgh. pp. 297–305.

Marsteller, T. A. and Fenwick, B. (1999): *Actinobacillus pleuropneumoniae* disease and serology. Swine Health Prod. **7,** 161–165.

Nielsen, R. (1986*a*): Serology of *Haemophilus (Actinobacillus) pleuropneumoniae* serotype 5 strains: establishment of subtypes A and B. Acta Vet. Scand. **27,** 49–58.

Nielsen, R. (1986*b*): Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of a new serotype: serotype 12. Acta Vet. Scand. **27,** 453–455.

Nielsen, R., Andresen, L. O., Plambeck, T., Nielsen, J. P., Krarup, L. T. and Jorsal, S. E. (1997): Serological characterization of *Actinobacillus pleuropneumoniae* biotype 2 strains isolated from pigs in two Danish herds. Vet. Microbiol. **54,** 35–46.

Perry, M. B., Angen, Ø., MacLean, L. L., Lacouture, S., Kokotovic, B. and Gottschalk, M. (2012): An atypical biotype I *Actinobacillus pleuropneumoniae* serotype 13 is present in North America. Vet. Microbiol. **156,** 403–410.

Rycroft, A. N. and Garside, L. H. (2000): *Actinobacillus* species and their role in animal disease. Vet. J. **159,** 18–36.

Sárközi, R., Makrai, L. and Fodor, L. (2015): Identification of a proposed new serovar of *Actinobacillus pleuropneumoniae*: serovar 16. Acta Vet. Hung. **63,** 444–450.

Sárközi, R., Makrai, L. and Fodor, L. (2018): *Actinobacillus pleuropneumoniae* serotypes in Hungary. Acta Vet. Hung. **66,** 343–349.

Sassu, E. L., Bossé, J. T., Tobias, T. J., Gottschalk, M., Langford, P. R. and Hennig-Pauka, I. (2018): Update on *Actinobacillus pleuropneumoniae* – knowledge, gaps and challenges. Transbound. Emerg. Dis. **65 (Suppl. 1),** 72–90.

Schaller, A., Djordjevic, S. P., Eamens, G. J., Forbes, W. A., Kuhn, R., Kuhnert, P., Gottschalk, M., Nicolet, J. and Frey, J. (2001): Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene *apx*IVA. Vet. Microbiol. **79,** 47–62.

Shin, M. K., Cha, S. B., Lee, W. J. and Yoo, H. S. (2011): Predicting genetic traits and epitope analysis of apxIVA in *Actinobacillus pleuropneumoniae*. J. Microbiol. **49,** 462–468.

Sthitmatee, N., Sirinarumitr, T., Makonkewkeyoon, L., Sakpuaram, T. and Tesaprateep, T. (2003): Identification of the *Actinobacillus pleuropneumoniae* serotype using PCR based-Apx genes. Mol. Cell. Probes **17,** 301–305.

Wong, J. D., Janda, J. M. and Duffey, P. S. (1992): Preliminary studies on the use of carbon substrate utilization patterns for identification of *Brucella* species. Diagn. Microbiol. Infect. Dis. **15,** 109–113.

Zasada, A. A. and Mosiej, E. (2018): Contemporary microbiology and identification of *Corynebacteria* spp. causing infections in human. Lett. Appl. Microbiol. **66,** 472–483.