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Short note

Phylogenetic relationship of equine *Actinobacillus* species and distribution of RTX toxin genes among clusters

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Abstract – Equine *Actinobacillus* species were analysed phylogenetically by 16S rRNA gene (*rrs*) sequencing focusing on the species *Actinobacillus equuli*, which has recently been subdivided into the non-haemolytic *A. equuli* subsp. *equuli* and the haemolytic *A. equuli* subsp. *haemolyticus*. In parallel we determined the profile for RTX toxin genes of the sample of strains by PCR testing for the presence of the *A. equuli* haemolysin gene *aqx*, and the toxin genes *apx*I, *apx*II, *apx*III and *apx*IV, which are known in porcine pathogens such as *Actinobacillus pleuropneumoniae* and *Actinobacillus suis*. The *rrs*-based phylogenetic analysis revealed two distinct subclusters containing both *A. equuli* subsp. *equuli* and *A. equuli* subsp. *haemolyticus* distributed through both subclusters with no correlation to taxonomic classification. Within one of the *rrs*-based subclusters containing the *A. equuli* subsp. *equuli* type strain, clustered as well the porcine *Actinobacillus suis* strains. This latter is known to be also phenotypically closely related to *A. equuli*. The toxin gene analysis revealed that all *A. equuli* subsp. *haemolyticus* strains from both *rrs* subclusters specifically contained the *aqx* gene while the *A. suis* strains harboured the genes *apx*I and *apx*II. The *aqx* gene was found to be specific for *A. equuli* subsp. *haemolyticus*, since *A. equuli* subsp. *equuli* contained no *aqx* nor any of the other RTX genes tested. The specificity of *aqx* for the haemolytic equine *A. equuli* and ApxI and ApxII for the porcine *A. suis* indicates a role of these RTX toxins in host species predilection of the two closely related species of bacterial pathogens and allows PCR based diagnostic differentiation of the two.

***Actinobacillus* / horse / RTX toxin / phylogeny / diagnostic**

1. INTRODUCTION

Equine bacteria of the family of *Pasteurellaceae* are of clinical importance, since they may cause disease in horses and are found in infected wounds of humans bitten by horses [4, 5]. Most of these bacteria fit into the genus *Actinobacillus*, and

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the majority of them belongs to the species *Actinobacillus equuli*. However, phenotypically this species shows a high variability including haemolytic activity. Haemolytic strains of *A. equuli* seem to be part of the normal flora of the equine oral cavity, but are also isolated from tracheal washes, indicating a preference for the respiratory tract [9]. These organisms also seem to represent opportunistic pathogens since they have been found associated with various clinical pictures in horses including respiratory infections, septicaemia, metritis, mastitis, arthritis, endocarditis, meningitis or stillbirth [3, 18]. Non-haemolytic strains of *A. equuli* also seem to belong to the normal flora of the oral cavity and alimentary tract of horses. In addition they are frequently reported as an opportunistic pathogen, mainly in young animals. The most prominent clinical condition associated with these strains is "sleepy foal disease", an acute septicaemia of foals with purulent nephritis which eventually becomes chronic in the form of "joint ill", a polyarticular arthritis. The haemolytic strains of *A. equuli*, previously known as Bisgaard Taxon 11, have only been reported from horses but not specifically associated with "sleepy foal disease". The haemolytic strains of *A. equuli* led to the proposal of two subspecies: *A. equuli* subsp. *haemolyticus* and *A. equuli* subsp. *equuli* [5].

The factor responsible for the haemolytic phenotype of *A. equuli* strains has recently been described to be the Aqx protein, a member of the RTX toxin family [2]. Many representatives of RTX toxins are found in *Pasteurellaceae* [7]. The aims of the present investigation were to examine the phylogenetic relationship of a collection of strains of *A. equuli* and assess the distribution of the RTX toxin genes *aqx* and *apx*IA, *apx*IIA, *apx*IIIA and *apx*IV in phylogenetic clusters established.

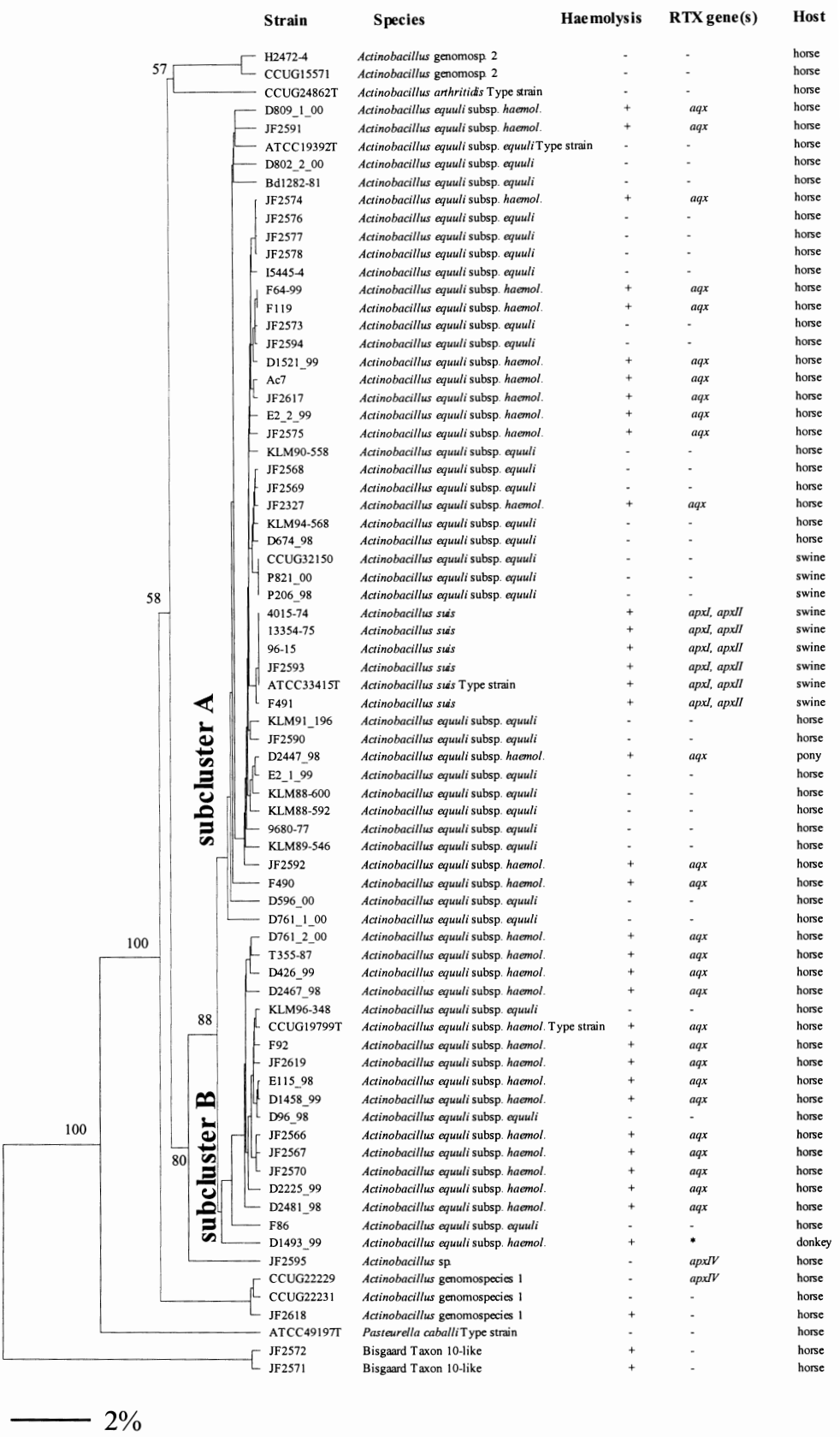
In order to get a broader view on the distribution of *aqx* as well as to consider the recent classification of *A. equuli* into two subspecies we also carried out a thorough study on a variety of equine isolates. These included *Pasteurella caballi*, equine isolates of *Actinobacillus lignieresii* (recently classified as *Actinobacillus* genomospecies 1 [4]) as well as representatives of Bisgaard taxon 9 (recently classified as *Actinobacillus arthritidis* and *Actinobacillus* genomospecies 2 [4]) and Bisgaard taxon 10. In order to compare these findings with the phenotypically and phylogenetically closely related *A. suis* [1, 6], several porcine isolates of this species were included in addition to type strains of taxa investigated.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth

Type and reference strains of the different species were received from the American Type Culture Collection (ATCC), and the Culture Collection at the University of Göteborg (CCUG) and were identified accordingly in Figure 1. Field strains were isolated from equidae and pigs by the diagnostic units of the Institutes of Veterinary Bacteriology of the Universities Bern and Zurich (Switzerland), the Department of Disease Control and Biosecurity, National Veterinary Institute (Sweden), and the Department of Veterinary Microbiology at the Royal Veterinary and Agricultural University (Denmark) (Fig. 1). They were characterized and identified phenotypically by conventional methods in the laboratory where they had been kept. Strains

Figure 1. Phylogeny based on 16S rRNA gene sequence, haemolytic phenotype and RTX toxin gene distribution of equine and porcine isolates of *A. equuli* and related species. Bootstrap values of 500 repeated experiments are indicated as percent at the major branches. The bar represents 2% sequence divergence.
* Strain negative by PCR, but positive by Southern blot.


Strain
Species
Haemolysis
RTX gene(s)
Host
H2472-4 Actinobacillus genomosp. 2 - - horse
CCUG15571 Actinobacillus genomosp. 2 - - horse
CCUG24862T Actinobacillus arthritidis Type strain - - horse
D809_1_00 Actinobacillus equuli subsp. haemol. + aqx horse
JF2591 Actinobacillus equuli subsp. haemol. + aqx horse
ATCC19392T Actinobacillus equuli subsp. equuli Type strain - - horse
D802_2_00 Actinobacillus equuli subsp. equuli - - horse
Bd1282-81 Actinobacillus equuli subsp. equuli - - horse
JF2574 Actinobacillus equuli subsp. haemol. + aqx horse
JF2576 Actinobacillus equuli subsp. equuli - - horse
JF2577 Actinobacillus equuli subsp. equuli - - horse
JF2578 Actinobacillus equuli subsp. equuli - - horse
I5445-4 Actinobacillus equuli subsp. equuli - - horse
F64-99 Actinobacillus equuli subsp. haemol. + aqx horse
F119 Actinobacillus equuli subsp. haemol. + aqx horse
JF2573 Actinobacillus equuli subsp. equuli - - horse
JF2594 Actinobacillus equuli subsp. equuli - - horse
D1521_99 Actinobacillus equuli subsp. haemol. + aqx horse
Ac7 Actinobacillus equuli subsp. haemol. + aqx horse
JF2617 Actinobacillus equuli subsp. haemol. + aqx horse
E2_2_99 Actinobacillus equuli subsp. haemol. + aqx horse
JF2575 Actinobacillus equuli subsp. haemol. + aqx horse
KLM90-558 Actinobacillus equuli subsp. equuli - - horse
JF2568 Actinobacillus equuli subsp. equuli - - horse
JF2569 Actinobacillus equuli subsp. equuli - - horse
JF2327 Actinobacillus equuli subsp. haemol. + aqx horse
KLM94-568 Actinobacillus equuli subsp. equuli - - horse
D674_98 Actinobacillus equuli subsp. equuli - - horse
CCUG32150 Actinobacillus equuli subsp. equuli - - swine
P821_00 Actinobacillus equuli subsp. equuli - - swine
P206_98 Actinobacillus equuli subsp. equuli - - swine
4015-74 Actinobacillus suis + apxI, apxII swine
13354-75 Actinobacillus suis + apxI, apxII swine
96-15 Actinobacillus suis + apxI, apxII swine
JF2593 Actinobacillus suis + apxI, apxII swine
ATCC33415T Actinobacillus suis Type strain + apxI, apxII swine
F491 Actinobacillus suis + apxI, apxII swine
KLM91_196 Actinobacillus equuli subsp. equuli - - horse
JF2590 Actinobacillus equuli subsp. equuli - - horse
D2447_98 Actinobacillus equuli subsp. haemol. + aqx pony
E2_1_99 Actinobacillus equuli subsp. equuli - - horse
KLM88-600 Actinobacillus equuli subsp. equuli - - horse
KLM88-592 Actinobacillus equuli subsp. equuli - - horse
9680-77 Actinobacillus equuli subsp. equuli - - horse
KLM89-546 Actinobacillus equuli subsp. equuli - - horse
JF2592 Actinobacillus equuli subsp. haemol. + aqx horse
F490 Actinobacillus equuli subsp. haemol. + aqx horse
D596_00 Actinobacillus equuli subsp. equuli - - horse
D761_1_00 Actinobacillus equuli subsp. equuli - - horse
D761_2_00 Actinobacillus equuli subsp. haemol. + aqx horse
T355-87 Actinobacillus equuli subsp. haemol. + aqx horse
D426_99 Actinobacillus equuli subsp. haemol. + aqx horse
D2467_98 Actinobacillus equuli subsp. haemol. + aqx horse
KLM96-348 Actinobacillus equuli subsp. equuli - - horse
CCUG19799T Actinobacillus equuli subsp. haemol. Type strain + aqx horse
F92 Actinobacillus equuli subsp. haemol. + aqx horse
JF2619 Actinobacillus equuli subsp. haemol. + aqx horse
E115_98 Actinobacillus equuli subsp. haemol. + aqx horse
D1458_99 Actinobacillus equuli subsp. haemol. + aqx horse
D96_98 Actinobacillus equuli subsp. equuli - - horse
JF2566 Actinobacillus equuli subsp. haemol. + aqx horse
JF2567 Actinobacillus equuli subsp. haemol. + aqx horse
JF2570 Actinobacillus equuli subsp. haemol. + aqx horse
D2225_99 Actinobacillus equuli subsp. haemol. + aqx horse
D2481_98 Actinobacillus equuli subsp. haemol. + aqx horse
F86 Actinobacillus equuli subsp. equuli - - horse
D1493_99 Actinobacillus equuli subsp. haemol. + * donkey
JF2595 Actinobacillus sp. - apxIV horse
CCUG22229 Actinobacillus genomospecies 1 - apxIV horse
CCUG22231 Actinobacillus genomospecies 1 - - horse
JF2618 Actinobacillus genomospecies 1 + - horse
ATCC49197T Pasteurella caballi Type strain - - horse
JF2572 Bisgaard Taxon 10-like + - horse
JF2571 Bisgaard Taxon 10-like + - horse
57
58
100
88
100
80
subcluster A
subcluster B
2%

were grown on 5% sheep blood agar plates at 37 °C overnight. Agar plates were prepared by overlaying Blood-Agar-Base (Oxoid, Hampshire, UK) plates with Trypticase-Soy-Agar (BBL, Beckton Dickinson, Cockeyesville, MD, USA) supplemented with 0.1% $CaCl_2$ and 5% sheep blood.

2.2. DNA isolation and PCR analysis

Genomic DNA was extracted using either the QIAamp Tissue kit (Qiagen, Basel, Switzerland) or the Genomic DNA isolation kit PUREGENE® (Gentra Systems, Minneapolis, USA). Specific Aqx PCR, based on the *aqx*A gene, was done as described by Berthoud et al. [2], while specific ApxI, ApxII and ApxIII PCR, amplifying *apx*IA, *apx*IIA, *apx*IIIA plus a small part of the corresponding C genes, was carried out as described by Frey et al. [8]. Specific ApxIV PCR was finally done as reported by Schaller et al. [17].

2.3. 16S rRNA gene sequencing and phylogenetic analysis

Determination of the 16S rRNA gene sequences in both directions was done as described previously [12, 14]. Sequence alignment and editing were done with the software Sequencher™ (Gene Codes Corporation, Ann Arbor, MI, USA). Sequence comparisons, cluster analysis and tree construction using UPGMA were done in BioNumerics Ver. 3.00 (Applied Maths, Kortrijk, Belgium).

2.4. Southern-blot analysis

Genomic DNA of bacterial strains was digested by *Eco*RI and size separated on a 0.7% agarose gel. Southern-blotting was done by alkaline transfer of DNA onto positively charged nylon membranes using an LCB 2016 Vacu Gene vacuum blotting pump (Pharmacia LKB biotechnology AB, Bromma, Sweden). After hybridization with *aqx*-specific probes [2], blots were washed at room-temperature two times, 5 min each, with 2× SSC-0.1% SDS (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate), followed by two times washes at room-temperature, 15 min each, with 0.2× SSC-0.1% SDS. Signals were detected by chemiluminescence using the CDP-Star substrate (Roche Applied Science, Rotkreuz, Switzerland) followed by exposition to X-ray films.

3. RESULTS

Figure 1 summarizes the results of our analyses. Strains of the species *A. equuli* and *A. suis* formed a mono-phyletic group. This cluster can be divided into two subclusters matching the *rrs* sequences of the type strains of *A. equuli* subsp. *equuli* (ATCC19392) and *A. equuli* subsp. *haemolyticus* (CCUG19799), respectively. The *aqx* gene was found in both subclusters A and B and specifies the haemolytic strains. No correlation between haemolysis and phylogenetic grouping was observed, even though within subcluster A out of 37 equine isolates 14 were haemolytic (38%) whereas in subcluster B, 15 of 18 equine strains were haemolytic (83%). The strain D1493_99 isolated from a donkey was negative for *aqx* by PCR, but showed a strong hybridisation signal at high stringency in a Southern-hybridisation with an *aqx*CA specific probe. The *Eco*RI DNA fragment which reacted with this probe was larger than the *Eco*RI fragment reacting with the same probe in equine strains of *A. equuli* subsp. *haemolyticus*, indicating the presence of a variant of the *aqx* operon (data not shown). Within subcluster A clustered also 3 non-haemolytic porcine *A. equuli* as well as the haemolytic *A. suis* strains. The equine isolates *A. equuli* subsp. *haemolyticus* always contained the *aqx* gene and neither *apx*I nor *apx*II genes, while haemolytic swine isolates contained always *apx*I and *apx*II but never *aqx*. No *aqx* genes could be detected in species other than *A. equuli*.

Isolate JF2618 clustered together with the strains CCUG22231 and CCUG22229, belonging to *Actinobacillus* genomospecies 1. Strain CCUG22229 as well as JF2595 showed a positive PCR reaction for the *apx*IV. Strain JF2595 forms a branch of its own, most closely related to the *A. equuli* cluster showing in absolute numbers 98% similarity of its *rrs* sequence to its nearest neighbour. It shares still 97% *rrs* sequence similarity with the other *apx*IV containing strain CCUG22229.

Actinobacillus arthritidis and genomospecies 2 as well as *Pasteurella caballi* clustered separately but showed neither haemolysis nor the presence of any of the investigated RTX toxin genes. Similar observations were made for the haemolytic Bisgaard taxon 10-like isolates which form an outgroup in the tree.

None of the equine nor porcine strains analysed showed up positive for the *apx*III genes.

4. DISCUSSION

To investigate the relation between phylogeny and haemolytic phenotype we analysed the 16S rRNA gene sequences (*rrs*) of field and reference strains of *A. equuli* and related species isolated mainly from horses and assessed haemolysis on sheep blood agar. In parallel, we defined the RTX toxin gene profile of these strains by PCR analysis. We limited our investigation to the *aqx* gene described for *A. equuli* subsp. *haemolyticus*, and *apx*I, *apx*II, *apx*III and *apx*IV which are present in *A. pleuropneumoniae* and other members of the *Pasteurellaceae* family, including *A. suis* [16].

Besides the main cluster formed by the *A. equuli* isolates other phylogenetic groups can be discerned. Strains of *Actinobacillus* genomospecies 1 [4] clustering together have formerly been described as equine *Actinobacillus lignieresii*, a species which is phenotypically closely related to *A. pleuropneumoniae*. This close phenotypic relationship is supported by the earlier description that strain CCUG22229 was typed as *A. pleuropneumoniae* serotype 3 [4]. Surprisingly this strain as well as JF2595 showed a positive PCR reaction for the *apx*IV gene which is considered as specific marker for *A. pleuropneumoniae* [17], indicating a potential horizontal gene transfer of *apx*IV or a similar gene among certain rare species of *Pasteurellaceae*. The haemolytic determinant of JF2618 however, could not be attributed to any of the investigated RTX genes. The same holds true for the Bisgaard Taxon 10-like strains. They both showed haemolysis on sheep blood agar plates but none of the 5 RTX toxin genes we looked for was detected in these strains. Therefore, the haemolytic determinant of these strains remains unknown.

In the phylogenetic group containing *A. arthritidis* and *Actinobacillus* genomospecies 2 the absence of the analysed RTX genes corresponds with the non-haemolytic phenotype of the strains.

No *apx*IIIA genes were found in the strains analysed. The ApxIII determinant as well as the very similar Pax RTX toxin have so far only been found associated with porcine isolates like *Actinobacillus pleuropneumoniae*, *Actinobacillus rossii* or *Pasteurella aerogenes* [13, 16].

The main focus of our study was on the *A. equuli* isolates which formed two phylogenetic clusters, one of them including also the *A. suis* isolates. These two subclusters, characterized by a major branching point with a boots-trap value of 88% (Fig. 1), correspond to previously reported phylogenetic subcluster around the *A. equuli* type strain ATCC19392 and the subcluster characterized by strain CCUG19799 tentatively named *A. equuli*-like [2]. Three non-haemolytic porcine isolates clustered within subcluster A and probably represent *A. equuli* subsp. *equuli* previously reported from pigs [3]. Genuine porcine isolates of *A. suis* also clustered within subcluster

A confirming previous observations [6], but formed a small group of their own within the tree, phenotypically corresponding to haemolysis, unique negative reactions in D(-) mannitol and formation of a yellowish pigment on blood agar. No correlation could be found between the two clusters and the recent classification of *A. equuli* into the two subspecies *A. equuli* subsp. *equuli* and *A. equuli* subsp. *haemolyticus*. However, all *A. equuli* subsp. *haemolyticus* contained the recently described haemolysin gene *aqx*, which confers the haemolytic phenotype of this subspecies [2]. No *apx* genes were detected in any of the *A. equuli* isolates. On the other side the haemolytic species *A. suis* always contained the *apx*I and *apx*II genes but no *aqx*, which was only found in strains of equine origin. Therefore, the haemolytic phenotype common to *A. suis* and *A. equuli* subsp. *haemolyticus* is clearly mediated by different haemolysins, and toxin gene detection by PCR provides a good method to differentiate these phenotypically as well as phylogenetically so closely related species. Furthermore, the different toxin gene profiles of *A. suis* and *A. equuli* subsp. *haemolyticus* could be an indication that RTX toxins contribute to the host specificity of the two bacterial species *A. suis* and *A. equuli* subsp. *haemolyticus* containing the *apx* and the *aqx* genes, respectively. We could recently show, that the RTX toxins ApxI, ApxII and Aqx have host-cell specific cytotoxic activities [15]. In these experiments ApxI and ApxII showed a higher toxic activity towards swine lymphocytes compared to equine lymphocytes, and on the other side Aqx was more toxic for horse than for pig lymphocytes. Differences in the toxic activities are most probably due to specific binding of the toxin to receptors on the target cells and/or differences in the signaling cascade after toxin binding. This was shown for other RTX toxins like the leukotoxin Lkt of *Mannheimia haemolytica* which after binding to the LFA-1 receptor is toxic for bovine leukocytes but has no effect on porcine or equine lymphocytes [10, 11, 19].

Our investigations clearly show, that the haemolytic phenotype common to *A. suis* and *A. equuli* subsp. *haemolyticus* is due to different toxin genotypes which is conserved and associated with the host origin of the isolates. This fact indicates that previous reports on isolation of *A. suis* from horses represent misidentifications or at least misnaming, which can easily be avoided by specific PCR for toxin gene detection. This study therefore shows that RTX toxin analysis provides a good tool for identification of animal pathogens and the assessment of their virulence.

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