1 # Nasal Bacterial Microbiome: Probing a Healthy

2 # Porcine Family

3

4 Min Yue* [1] , Weicheng Bei [2,3] , Huanchun Chen [2,3]

5

6 *Department of Pathobiology, University of Pennsylvania, School of Veterinary*

7 *Medicine, Philadelphia, Pennsylvania 19104, United States of America[1]; State*

8 *Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University,*

9 *Wuhan 430070, People's Republic of China[2]; Division of Animal Infectious*

10 *Disease, College of Veterinary Medicine, Huazhong Agricultural University,*

11 *Wuhan 430070, People's Republic of China[3]*

12

13

14

15

16

17

18

19

20

21

22

23

* Corresponding author: Min Yue, University of Pennsylvania School of

Veterinary Medicine, 3800 Spruce Street, Philadelphia, PA 19104-6049, USA

Telephone: 215-573-9596

Fax: 215-898-7887

28

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

Abstract:

Upper respiratory tract (URT) infection caused the leading and devastating diseases in pigs. It was believed that normal microbiome of URT play a vital role in health and disease development. As the entry of URT, little knowledge of bacterial microbiome in porcine nasal was known. A cultivation-independent approach directly to 16s ribosomal RNA genes enabled us to reveal nasal bacterial community, structure and diversity. Here, we found that an unprecedented 207 phylotypes were characterized from 933 qualified clones, indicating the variable, species richness but particular dominant bacterial microbiome. The dominant species were from genus *Comamonas* and *Acinetobacter*, which constitute core normal bacterial microbiome in porcine nasal. Moreover, a set of swine specific pathogens and zoonotic agents were detected in the swine nasal microbiome. Collectively, we provided a snapshot of our current knowledge of the community structure of porcine nasal bacterial ecosystem in a health family that in further enhance our view to understand URT infection and public health.

Keywords:

Porcine Nasal, Bacterial Microbiome, URT infection, Public Health, 16S rDNA

Introduction

The composition of the complex microbial flora inhabiting the human body has a tremendous influence on human health and disease(Stecher and Hardt, 2008). A microbiome is the totality of microbes, their genetic elements, and environmental interaction in a particular niches. Benefiting from the Human Microbiome Project, previous work about vagina, gastrointestinal tract, oral cavity, hands and skin of human bacterial flora largely enhanced our knowledge to understand distinct microbial communities and host-microbe interaction(Bik et al., 2006; Diaz et al., 2006; Grice et al., 2008; Nasidze et al., 2009; Oakley et al., 2008; Pei et al., 2004). However, very little is known about respiratory tract microbiome, particularly host other than human, such as swine.

Respiratory disease has consistently been reported as one of the most important causes of both morbidity and mortality in post-weaning pigs, as one of the limiting factors of production, causing decreased weight gain, and decreased feed efficiency, an overuse of antibiotics and ultimately respiratory distress and death(Moorkamp et al., 2008; Palzer et al., 2008). There are numerous organisms, both bacterial and viral, that can contribute to respiratory distress in pigs, many can act as opportunistic agents on an already compromised immune system, or can themselves become exacerbated by infection with other opportunistic pathogens. This can result in a more

89   complicated aetiology, which may be associated with higher mortality. Hence,

90   knowledge of aetiology of respiratory disease, as well as host-pathogen

91   interaction, is crucial to understand Upper Respiratory Tract (URT) infection.

92   Defining the normal microbiota of URT is the first step to understand health

93   and disease development.

94

95   As the entrance of upper respiratory tract, host nasal cavity inhabits many

96   different microbes, with minority of symbiotic microorganisms, foreign flora and

97   microbes from the environment; including some important associated with

98   URT infection and other related diseases(Pettigrew et al., 2008). All of them

99   coexist on nasal cavity during a certain development stage. In the URT,

100 site-specific microbial flora may play an important role in respiratory disease

101 development as the nasal cavity ecosystem changes from a healthy to a

102 diseased state. Respiratory disease in pig is complex, but vital in population

103 health state, and economic importance. In veterinary clinic, often two or more

104 related etiologies are associated with disease development, indicating the

105 microbe composition and interaction may help to understand respiratory

106 disease development(Moorkamp et al., 2008; Palzer et al., 2008). While in

107 modern industry farming, early piglet colonizer plays an important role in state

108 of health in its life career. As the closest relative, sow may influence early

109 colonized of their offspring. Thus, exploring the bacterial flora of nasal cavities

110 in early-colonized piglet, sow and understanding their relationship is an

111   important to reveal respiratory disease development in herds.

112

113   Until recently, knowledge of the nasal bacteria was limited to

114   culture-dependent assays, and it is estimated that less than 1% of bacterial

115   species can be cultivated(Guclu et al., 2007; Smith-Vaughan et al., 2006). The

116   use of 16S ribosomal RNA (rRNA) gene sequences to study bacterial

117   community is the common and suitable housekeeping genetic marker. The

118   16S small subunit ribosomal genes are universal among prokaryotes and

119   contain species-specific variable regions that are useful for inferring

120   phylogenetic relationships(Janda and Abbott, 2007; Petrosino et al., 2009).

121   Broad-range PCR primers that anneal to highly conserved regions flanking the

122   variable regions of the gene allow amplification from the majority of known

123   bacteria, which has led to the identification of microbial diversity and has

124   provided compelling evidence for the existence of hitherto unknown bacteria.

125   As a result of increasing challenge in modern farm associated with respiratory

126   disease, we tend to understand bacterial microbiota nasal cavity of modern

127   farming sow and its piglets, evaluate their relationship with potential disease

128   development. Summary the previous data of porcine upper respiratory tract as

129   well as our data, we provide a preliminary picture of the community structure of

130   the nasal bacteria ecosystem and discuss the potential pathogens, and their

131   impact on public health and food safety.

132

Material and Methods

Sample Preparation

A healthy family, sow (about 3 years old) and its piglets (twice week after birth), with no history of clinical medication (mild cold or other common features acceptable) or other chronic medical disorders and with no current infections, were selected and all experiments involving pigs were performed under an NIH/NHGRI ACUC approved protocol. Biological samples were collected from both the left and right nasal cavities with no prior cleaning or preparation of the surface. Swabs were obtained using a sterile cotton pledget soaked in sterile 0.15 M NaCl with 0.1% Tween 20 and wrung of excess solution. All samples were stored at -80°C until further processing.

DNA extraction and purification

All biological specimens were first incubated in a preparation of enzymatic lysis buffer (20 mM Tris at pH 8.0, 2 mM EDTA, 1.2% Triton X-100) and lysozyme (20 mg/mL) for 30 min at 37°C, and samples were incubated overnight at 56°C in Buffer AL and Proteinase K from the DNeasy DNA Extraction Kit (Qiagen). The standard protocol for the genomic DNA isolation was followed for steps provide by QIAamp DNA Stool Mini Kit (Qiagen). The purified genomic DNA was resuspended in 30 µL of Buffer AE and stored at -20°C.

Broad-spectrum amplification

A portion of the bacterial 16S rRNA gene was amplified using forward primer 8F: AGTTTGATC(A/C)TGGCTCAG (Location: 8–27bp) and reverse primer

806R: GGACTAC(C/T/A)AGGGTATCTAAT (Location: 806–787bp) to yield a

PCR product about 800bp (which encompasses the hypervariable V1-V5

region)(Goldenberger et al., 1997; Petrosino et al., 2009). For each 50 µL

reaction, conditions were as follows: 5.0 µL of 10 x buffer with $MgCl_2$ (TaKaRa),

5 µL of dNTP mix (10 mM each; TaKaRa), 1 µL of each primer (20 µM;

Tsingke), 3 µL of DMSO, 5 µL of bacterial genomic DNA, and 0.5 µL of Ex Taq

Polymerase (TaKaRa). For each DNA sample, three replicates were

performed. Thermocycling was as follows: Initial denaturation at 95°C for 5 min,

followed by first round 15 cycles of a 30-sec 95°C denaturation, 30-sec

annealing at 50°C, and 1-min elongation at 72°C, second round 35 cycles of a

30-sec 95°C denaturation, 30-sec annealing at 54°C, and 1-min elongation at

72°C, all followed by a final extension of 10 min at 72°C. PCR products were

then separated on an agarose gel, and bands corresponding to about 0.8-kb

product were extracted with a razor blade. Negative control PCR reactions

were performed with each set of amplifications and in all cases did not produce

an amplification product. PCR products were extracted using the Gel

Extraction kit (Qiagen) and resuspended in 30 µL of Buffer EB and stored at

-20°C.

Plasmid library construction, sequencing amplicons and quality assessment

PCR products were cloned into the pEASY-T1 vector (Transgene Inc.)

according to the manufacturer's protocol. A total of 1191 of the resulting

177   bacterial colonies were picked up for plasmid DNA purification, and plasmid

178   inserts were sequenced bidirectionally using the M13 primers on an ABI

179   3730xl sequencer (Applied Biosystems Inc.), including 576 from sow and 615

180   from two piglets, respectively. Chromatogram data quality and quantity were

181   evaluated using phred Q20 counts and non-vector sequence data remaining

182   after cross_match screening. Sequences were extracted from chromatograms

183   using phred, and bidirectional pairs were assembled using phrap. Vector

184   sequence detected by cross_match was trimmed off. Only assembled

185   sequences about 800 base pairs were studied in further. Assemblies were

186   screened for quality, and all sequences containing >20 consecutive bases of

187   sequence <Q20 were discarded(Grice et al., 2008).

188   **Data analysis**

189   Total valid sequences with chimeric are identified using the Mallard

190   program(Ashelford et al., 2006), leaving 454 from S (sow) group, 479 from PA

191   (piglet A) group and PB (piglet B) group. These 933 sequences were then

192   submitted to RDPII web service(Hamady and Knight, 2009), using the online

193   program Classifier and SeqMatch with a threshold setting for similarity score of

194   95%, to assign 16S rRNA gene sequences to the new phylogenetically

195   consistent higher-order bacterial taxonomy and assign a genus to each

196   sequence, respectively. To classify sequences based on self-similarities rather

197   than matches to an external database, sequences were grouped into

198   operational taxonomic units (OTUs) with cutoffs of 97%sequence similarity

199   using the DOTUR software package implemented with the furthest-neighbor

200   option, in which all of the sequences within an OTU are at least 97% similar to

201   all of the other sequences within the OTU(Yu et al., 2006). The term

202   'phylotype' is used for clusters of clone sequences which differ from known

203   species by more than 3% and are at least 97% similar to members of their

204   cluster. The representative sequences were incorporated into Green-Gene

205   online tool NAST alignment tool for species assignment. Shannon-Wiener

206   index as diversity indices, Chao1 and rarefaction as richness estimates

207   calculated by DOTUR and FastGroupII were to estimate microbial diversity

208   and richness as described(Yu et al., 2006). The estimation of diversity

209   coverage was calculated by Good's method, according to which the

210   percentage of coverage was calculated with the formula (%) = [1-(n/N)] x 100,

211   where n is the number of phylotypes represented by one clone only and N is

212   the total number of sequences(Bik et al., 2006). Biohazard level of each

213   species was estimated according to the potential risk of each species

214   according the levels of biohazard definition basing on Risk Group

215   Classification for Infectious Agents available on American Biological Safety

216   Association (http://www.absa.org/riskgroups/index.html). The zoonosis

217   information was determinated according the list of zoonoses available on

218   World Health Organization (WHO) website

219   (http://www.who.int/zoonoses/en/index.html).

220

221 **Results**

222 **Summary the Sequencing Project**

223 Nasal samples were obtained from 3 individuals, consisting of a sow and its

224 two piglets living together. For further extensive analysis bacterial community

225 in a continuous timescale, we focused on a representative family with small

226 individuals. A highly variable portion of the 16S rRNA gene of ~800 base pairs

227 was amplified, cloned and approximately 300 clones were sequenced from

228 each individual; this number of clones is sufficient to capture most of the

229 variation at the level of bacterial genera inferred from the partial 16S rRNA

230 sequences as describe previously(Grice et al., 2008). A total of 1,149

231 sequences were obtained, which were then analyzed for possible chimeras or

232 other artifacts. We identified 76 potentially chimeric sequences, which

233 represents 6.6% of the sequences. This data was according to the average of

234 9% potentially chimeric sequences reported previously for full-length 16S

235 rRNA clone libraries. The remaining qualified 933 sequences were analyzed in

236 the followings. The overview information of project was showed in Table 1.

237 **Phylotypes, Species Richness and Diversity**

238 The qualified 933 sequences were grouped into operational taxonomic units

239 (OTUs; "Phylotypes") based on their genetic distance in a neighbor-joining tree

240 with the DOTUR program(Hamady and Knight, 2009). A species-level OTU

241 has historically been defined as containing 16S rDNA sequences that are 97%

242 identical. Using the furthest-neighbor method of calculation and a similarity

243   threshold of 97%, DOTUR assigned the 933 sequences to 207 OTUs,

244   including 121 singletons. There were 79 swine unique OTUs (120 clones),

245   while 47 (63 clones) and 32 (42 clones) piglet unique OTUs for piglet A and

246   piglet B, respectively. Though only 11 OTUs were commonly shared by such

247   family (As showed in Fig 2a), there were 480 clones belonging to previous 11

248   OTUs, which constitute of a total 51.4% (As showed in Fig 2b) indicating the

249   general core bacterial flora in the family. The remaining 196 OTUs were from

250   the other half of total clones (453/933, 48.6% in total), which indicated the

251   diversity of bacterial flora in nasal within different individuals. The genus was

252   assigned to each sequence by comparison to the RDPII database revealed

253   922 classifiable bacterial clones, while the remaining 11 clones cannot be

254   assigned the known genus at a threshold of 95%.

255   Estimations of species coverage, richness, evenness, and diversity were

256   calculated for the combined data set, as well as for three subsets of nasal

257   samples (As showed in Table 1). The Chao1 estimator of total species

258   richness was 439, which based on the distribution of singletons.

259   Shannon-Wiener index of total species diversity is 3.75, which is one of several

260   diversity indices used to measure diversity in categorical data. It is simply the

261   Information entropy of the distribution, treating species as symbols and their

262   relative population sizes as the probability. Good's coverage was 77.8% for the

263   overall sequence set, indicating that twelve additional phylotypes would be

264   expected for every 100 additional sequenced clones. This level of coverage

265   indicated that the 16S rDNA sequences identified in these samples represent

266   the majority of bacterial sequences present in the porcine nasal samples under

267   study.

268   **Distribution of Clones at Different Levels**

269   Fig.1 showed bacteria distribution in different categories with the phylum

270   identified among the 933 clones in combined nasal sequence data set. The

271   nasal bacterial community was constituted by three phyla: *Proteobacteria* (895

272   clones), *Firmicutes* (21 clones), *Bacteroidetes* (7 clones), the remaining nasal

273   bacterial sequences were assigned as an uncharacterized composition (12

274   clones). Among these sequences, *Proteobacteria* was the dominant phylum

275   (95.9% in total) in porcine nasal cavities, which included a wide variety of

276   pathogens, such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, and many

277   other notable genera. As showed in Table 2, current genuses identified in

278   porcine nasal sample were *Empedobacter*, *Myroides, Dysgonomonas* in

279   phylum *Bacteroidetes*; *Streptococcus*, *Kurthia*, *Peptostreptococcaceae*

280   *Incertae Sedis, Clostridium* in phylum; *Arcobacter*, *Actinobacillus*, *Pasteurella*,

281   *Proteus*, *Schineria*, *Psychrobacter*, *Acinetobacter*, *Pseudomonas*, *Massilia*,

282   *Naxibacter*, *Comamonas*, *Vitreoscilla* in phylum *Proteobacteria*. Among these

283   genus, *Comamonas* and *Acinetobacter* were the most abundant clones within

284   the porcine nasal libraries. *Comamonas* sequences were found all the three

285   porcine nasal samples (varying from 28.3% to 56.4% of clones per sample

286   library) and constitute of all sequences analyzed (441/933, 47.3% in total).

287   *Acinetobacter* sequences were also found all the three porcine nasal samples

288   (varying from 15.8% to 38.2% of clones per sample library) and constitute of all

289   sequences analyzed (216/933, 23.2% in total). The above abundant genus in

290   porcine nasal samples occupied about 70% clones in species, indicating the

291   specific core species constituted the indigenous bacterial population in porcine

292   nasal cavity. Detail analysis the composition of the genus *Comamonas*

293   revealed the constitution of *Comamonas aquatica*, *Comamonas sp*. and

294   *Comamonas testosteroni*. And *Acinetobacter baumannii*, *Acinetobacter*

295   *calcoaceticus*, *Acinetobacter lwoffii* and *Acinetobacter sp*. are the

296   representative species of *Acinetobacter* genus. The other remarkable clones in

297   nasal libraries, which ware specific found in sow, were family *Pasteurellaceae*

298   and *Streptococcaceae*. Though the total clone number is from 1 to 3, we

299   cannot draw a conclusion of no significant difference between sow and its

300   piglet because of lack of available data in analysis. The similar results were

301   found in family *Clostridiaceae*, *Campylobacteraceae* which were found specific

302   to sow, while family *Carnobacteriaceae*, *Pseudomonadaceae*,

303   *Xanthomonadaceae* were specific to piglet.

304   **Potential Pathogens and public health**

305   Table 4 summary the most important pathogens frequent found in porcine UTR,

306   which were well recognized in previous knowledge. Many of them were as

307   normal microbiota, but with specific aetiology importance in veterinary clinics.

308   In table 3, we concluded the annotated sequences with aetiology importance

309   from porcine nasal samples. *Streptococcus suis*, *Pasteurella multocida*,

310   *Actinobacillus suis*, *Haemophilus parasuis* were found according to previous

311   knowledge of aetiology in porcine UTR. We also discovered lots of the

312   previous unknown pathogens in porcine UTR(Brockmeier et al., 2008; Lin et al.,

313   2006; Maes et al., 2008). Some belong to zoonosis such as *Proteus vulgaris*,

314   inhabits the intestinal tracts of humans and animals, known to cause urinary

315   tract infections and wound infections. Large numbers of potential pathogens

316   specific to human were also found. Among the summary in the table 3, most of

317   them were found specific in sow, while relative small number was in piglet.

318   Interesting, we found specie *Bacillus anthracis* from sow sample, which can be

319   classified as biohazard level-3 pathogen. And host specific specie *Shigella*

320   *flexneri* was also found in nasal sample from sow.

321

Discussion

323   Environment samples including seawater, sediments, and soil, about 1% of

324   bacteria can be cultivated; in contrast, cultivability of the human microbiota,

325   such as in the gut, is estimated to be substantially higher (10–50%)(Hamady

326   and Knight, 2009). The molecular approach by 16S rDNA sequencing has

327   significantly enhanced our knowledge in understanding bacteria diversity,

328   though this method has PCR amplification efficacy bias, cloning bias and was

329   dependent largely on updated database. In this study, our data from

330 sequence-based environmental microbial approach describes a previously

331 unrecognized extent of bacterial diversity in the porcine nasal ecosystem.

332 Analysis of the 933 16S rDNA clones from the nasal samples of a healthy

333 porcine family revealed an unrecognized bacterial species rich microbiota: the

334 3 phyla and 207 phylotypes represented in the microbiota, with about 75%

335 species coverage estimated.

336

337 Though there are 3 phyla and a few unclassified clones in the nasal samples,

338 *Proteobacteria* is, undoubtedly, the dominant phylum. And *Comamonadaceae*,

339 *Moraxellaceae* and *Oxalobacteraceae* are the three dominant families in the

340 porcine nasal samples. All those clones from above three dominant families

341 constitute the major normal bacterial. Among these, Genus *Comamonas*

342 belong to family *Comamonadaceae* covers half of the clones in the total

343 libraries. *Comamonas* species are environmental Gram-negative rods that

344 grow forming pink-pigmented colonies. It is known that *Comamonas* strains

345 are involved in novel degradation capability in environments. Though this

346 genus have potential application in bioremediation and are common in nature,

347 some member, such as *Comamonas testosteroni* is associated implicated as a

348 human pathogen along with rarely case report(Reddy et al., 2009). The only

349 notable infection species is *C. testosterone*, which was found in sow library.

350 Genus *Acinetobacter* from family *Moraxellaceae* is also one of major

351 composition in normal bacterial flora. *Acinetobacter spp*. is widely distributed in

352   nature. Occasionally, strains are isolated from foodstuffs and some are able to

353   survive on various medical equipments and even on healthy human skin.

354   *Acinetobacter* species are generally considered nonpathogenic to healthy

355   individuals. However, several species persist in hospital environments and

356   cause severe, life-threatening infections in compromised patients, and the

357   strain *A. baumannii* is the second most commonly isolated nonfermenting

358   bacteria in human specimens(Bergogne-Berezin and Towner, 1996;

359   Joly-Guillou, 2005). The bacterial community in major constitution of

360   *Comamonas* and *Acinetobacter* species may play a vital part in balance

361   bacterial flora in porcine nasal. Though sow and piglets have numbers of

362   different phylotypes, the constitution of core genus in bacterial microbiota is

363   the same, indicating the sow play a key role in early colonizers to establish in

364   its piglets.

365

366   There were various swine specific pathogens found in swine nasal microbiome.

367   Organisms from *Pasteurellaceae* family were common agents found in porcine

368   UTRs(Moller and Kilian, 1990). *Haemophilus parasuis* was the most common

369   nasal carriage in pigs, which was found throughout the world and organisms

370   were present even in high health herds(Olvera et al., 2007). *Actinobacillus suis*

371   can persist as a commensal in the upper respiratory tract (URT) with a wide

372   rang of host, under conditions which are poorly understood(MacInnes and

373   Desrosiers, 1999). *Pasteurella multocida* was the causative agent of fowl

374 cholera (FC), hemorrhagic septicemia (HS), and a variety of respiratory

375 syndromes such as atrophic rhinitis of swine (AR) and purulent rhinitis of

376 rabbits ("snuffles"), which were also part of the normal oropharyngeal flora in

377 many species(Frost and Adler, 2000; Hunt et al., 2000). The other important

378 agent belongs to family *Streptococcus*. *Streptococcus suis*, which principally

379 colonized in pig tonsil, is associated with a wide range of clinical syndromes in

380 swine and other domestic animals(Wertheim et al., 2009). The latter three

381 were also notable zoonotic important agents, while zoonotic *S. suis* type 2

382 cause serious public incidence in China in 2005(Segura, 2009). As the healthy

383 carrier, they all were also found in the sow libraries. Though there maybe

384 virulent strains as a result of specific disease outbreaks, the normal bacterial

385 microbiota may balance such virulent strains; result from competitive limiting

386 their growth or other unknown mechanisms. Ecological view may help to

387 understanding the porcine upper respiratory infection and disease

388 development. In such cases, those dominant species in *Comamonas* and

389 *Acinetobacter* may be of vital importance in porcine UTR microbiota

390 interaction.

391

392 In addition to above well-recognized agents, we also characterized a group of

393 agents that can be involved in specific human infection. Organisms such as

394 *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter lwoffii*

395 from genus *Acinetobacter*. *Acinetobacter* species are generally considered

396 nonpathogenic to healthy human individuals(Joly-Guillou, 2005). However,

397 several species persist in hospital environments and cause severe,

398 life-threatening infections in compromised patients, and the species *A.*

399 *baumannii* is the second most commonly isolated nonfermenting bacteria in

400 human specimens(Joly-Guillou, 2005). *Arcobacter cryaerophilus*, *Proteus*

401 *vulgaris*, *Pantoea agglomerans* and *Pantoea ananatis* were as opportunistic

402 pathogen, as result of common bacteremic infection and other specific

403 syndrome(Aly et al., 2008; De Baere et al., 2004; Stein and Gechman, 1955;

404 Wesley et al., 1996). Including *Massilia timonae* and *Myroides odoratimimus*,

405 those were all firstly detected in porcine nasal samples. Approximately 80% of

406 the infectious agents that cause disease in humans are shared with various

407 animal hosts. Currently, many newly emerging diseases are caused by

408 zoonotic agents, which focused on wildlife reservoirs that increase human

409 exposure to insect vectors as well as to animal and environmental sources of

410 disease. Importantly, porcine could play an important part in emerging

411 zoonoses, and may be the unrecognized carrier, potential risk to public health

412 and food safety(Tomley and Shirley, 2009). More effects need to discover our

413 close contact animals-pig, by new potential agents explore and routine

414 surveillance. The human microbiome project largely enhances our knowledge

415 to understanding the health and disease. Porcine microbiome will not only aid

416 the above knowledge, but also help to understand swine zoonosis.

417

418 In conclusion, by means of a 16S rRNA gene clone library, we provided a

419 preliminary picture of our current knowledge of the community structure of

420 porcine nasal bacterial ecosystem. Our results indicate that the

421 culture-independent genetic profiling of the 16S rRNA gene is a powerful tool

422 for investigation of the nasal microbiota. Further study is required to determine

423 the occurrence of these organisms in the porcine nasal microbiota, their

424 interaction mechanism and their functional roles in development of health and

425 disease.

426

431

432 **Literature Cited**

433 Aly, N.Y., Salmeen, H.N., Lila, R.A., Nagaraja, P.A., 2008, Pantoea
434   agglomerans bloodstream infection in preterm neonates. Medical
435   principles and practice 17, 500-503.
436 Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., Weightman, A.J.,
437   2006, New screening software shows that most recent large 16S rRNA
438   gene clone libraries contain chimeras. Applied and environmental
439   microbiology 72, 5734-5741.
440 Bergogne-Berezin, E., Towner, K.J., 1996, Acinetobacter spp. as nosocomial
441   pathogens: microbiological, clinical, and epidemiological features.
442   Clinical microbiology reviews 9, 148-165.
443 Bik, E.M., Eckburg, P.B., Gill, S.R., Nelson, K.E., Purdom, E.A., Francois, F.,

444   Perez-Perez, G., Blaser, M.J., Relman, D.A., 2006, Molecular analysis
445   of the bacterial microbiota in the human stomach. Proceedings of the
446   National Academy of Sciences of the United States of America 103,
447   732-737.

448   Brockmeier, S.L., Loving, C.L., Nicholson, T.L., Palmer, M.V., 2008,
449   Coinfection of pigs with porcine respiratory coronavirus and Bordetella
450   bronchiseptica. Veterinary microbiology 128, 36-47.

451   De Baere, T., Verhelst, R., Labit, C., Verschraegen, G., Wauters, G., Claeys,
452   G., Vaneechoutte, M., 2004, Bacteremic infection with Pantoea
453   ananatis. Journal of clinical microbiology 42, 4393-4395.

454   Diaz, P.I., Chalmers, N.I., Rickard, A.H., Kong, C., Milburn, C.L., Palmer, R.J.,
455   Jr., Kolenbrander, P.E., 2006, Molecular characterization of
456   subject-specific oral microflora during initial colonization of enamel.
457   Applied and environmental microbiology 72, 2837-2848.

458   Frost, A.J., Adler, B., 2000, Pasteurella multocida: the elusive determinants of
459   virulence and immunity. Veterinary microbiology 72, 1-2.

460   Goldenberger, D., Kunzli, A., Vogt, P., Zbinden, R., Altwegg, M., 1997,
461   Molecular diagnosis of bacterial endocarditis by broad-range PCR
462   amplification and direct sequencing. Journal of clinical microbiology 35,
463   2733-2739.

464   Grice, E.A., Kong, H.H., Renaud, G., Young, A.C., Bouffard, G.G., Blakesley,
465   R.W., Wolfsberg, T.G., Turner, M.L., Segre, J.A., 2008, A diversity
466   profile of the human skin microbiota. Genome research 18, 1043-1050.

467   Guclu, E., Yavuz, T., Tokmak, A., Behcet, M., Karali, E., Ozturk, O., Egeli, E.,
468   2007, Nasal carriage of pathogenic bacteria in medical students: effects
469   of clinic exposure on prevalence and antibiotic susceptibility. European
470   archives of Oto-Rhino-Laryngology 264, 85-88.

471   Hamady, M., Knight, R., 2009, Microbial community profiling for human
472   microbiome projects: Tools, techniques, and challenges. Genome
473   research.

474   Hunt, M.L., Adler, B., Townsend, K.M., 2000, The molecular biology of
475   pasteurella multocida. Veterinary microbiology 72, 3-25.

476   Janda, J.M., Abbott, S.L., 2007, 16S rRNA gene sequencing for bacterial
477   identification in the diagnostic laboratory: pluses, perils, and pitfalls.
478   Journal of clinical microbiology 45, 2761-2764.

479   Joly-Guillou, M.L., 2005, Clinical impact and pathogenicity of Acinetobacter.
480   Clinical Microbiology Infection 11, 868-873.

481   Lin, J.H., Chen, S.P., Yeh, K.S., Weng, C.N., 2006, Mycoplasma hyorhinis in
482   Taiwan: diagnosis and isolation of swine pneumonia pathogen.
483   Veterinary microbiology 115, 111-116.

484   MacInnes, J.I., Desrosiers, R., 1999, Agents of the "suis-ide diseases" of swine:
485   Actinobacillus suis, Haemophilus parasuis, and Streptococcus suis.
486   Canadian journal of veterinary research = Revue canadienne de
487   recherche veterinaire 63, 83-89.

488 Maes, D., Segales, J., Meyns, T., Sibila, M., Pieters, M., Haesebrouck, F.,
489   2008, Control of Mycoplasma hyopneumoniae infections in pigs.
490   Veterinary microbiology 126, 297-309.
491 Moller, K., Kilian, M., 1990, V factor-dependent members of the family
492   Pasteurellaceae in the porcine upper respiratory tract. Journal of clinical
493   microbiology 28, 2711-2716.
494 Moorkamp, L., Nathues, H., Spergser, J., Tegeler, R., Beilage, E.G., 2008,
495   Detection of respiratory pathogens in porcine lung tissue and lavage
496   fluid. The veterinary journal 175, 273-275.
497 Nasidze, I., Li, J., Quinque, D., Tang, K., Stoneking, M., 2009, Global diversity
498   in the human salivary microbiome. Genome research 19, 636-643.
499 Oakley, B.B., Fiedler, T.L., Marrazzo, J.M., Fredricks, D.N., 2008, Diversity of
500   human vaginal bacterial communities and associations with clinically
501   defined bacterial vaginosis. Applied and environmental microbiology 74,
502   4898-4909.
503 Olvera, A., Cerda`-Cue´llar, M., Mentaberre, G., Casas-Diaz, E., Lavin, S.,
504   Marco, I., Aragon, V., 2007, First isolation of Haemophilus parasuis and
505   other NAD-dependent Pasteurellaceae of swine from European wild
506   boars. Veterinary Microbiology. 125, 182-186.
507 Palzer, A., Ritzmann, M., Wolf, G., Heinritzi, K., 2008, Associations between
508   pathogens in healthy pigs and pigs with pneumonia. The Veterinary
509   record 162, 267-271.
510 Pei, Z., Bini, E.J., Yang, L., Zhou, M., Francois, F., Blaser, M.J., 2004,
511   Bacterial biota in the human distal esophagus. Proceedings of the
512   National Academy of Sciences of the United States of America 101,
513   4250-4255.
514 Petrosino, J.F., Highlander, S., Luna, R.A., Gibbs, R.A., Versalovic, J., 2009,
515   Metagenomic pyrosequencing and microbial identification. Clinical
516   chemistry 55, 856-866.
517 Pettigrew, M.M., Gent, J.F., Revai, K., Patel, J.A., Chonmaitree, T., 2008,
518   Microbial interactions during upper respiratory tract infections.
519   Emerging infectious diseases 14, 1584-1591.
520 Reddy, A.K., Murthy, S.I., Jalali, S., Gopinathan, U., 2009, Post-operative
521   endophthalmitis due to an unusual pathogen, Comamonas testosteroni.
522   Journal of medical microbiology 58, 374-375.
523 Segura, M., 2009, Streptococcus suis: an emerging human threat. The Journal
524   of infectious diseases 199, 4-6.
525 Smith-Vaughan, H., Byun, R., Nadkarni, M., Jacques, N.A., Hunter, N., Halpin,
526   S., Morris, P.S., Leach, A.J., 2006, Measuring nasal bacterial load and
527   its association with otitis media. BMC ear, nose, and throat disorders 6,
528   10.
529 Smith, M.D., Gradon, J.D., 2003, Bacteremia due to Comamonas species
530   possibly associated with exposure to tropical fish. Southern medical
531   journal 96, 815-817.

532 Stecher, B., Hardt, W.D., 2008, The role of microbiota in infectious disease.
533   Trends in microbiology 16, 107-114.
534 Stein, M.H., Gechman, E., 1955, Blood-stream infection due to Proteus
535   vulgaris and causing thrombocytopenic purpura; recovery with the use
536   of chloramphenicol. The New England journal of medicine 252,
537   906-908.
538 Tomley, F.M., Shirley, M.W., 2009, Livestock infectious diseases and
539   zoonoses. Philosophical transactions of the Royal Society of London
540   364, 2637-2642.
541 Wertheim, H.F., Nghia, H.D., Taylor, W., Schultsz, C., 2009, Streptococcus
542   suis: an emerging human pathogen. Clinical Infection Disease 48,
543   617-625.
544 Wesley, I.V., Baetz, A.L., Larson, D.J., 1996, Infection of cesarean-derived
545   colostrum-deprived 1-day-old piglets with Arcobacter butzleri,
546   Arcobacter cryaerophilus, and Arcobacter skirrowii. Infection and
547   immunity 64, 2295-2299.
548 Yu, Y., Breitbart, M., McNairnie, P., Rohwer, F., 2006, FastGroupII: a
549   web-based bioinformatics platform for analyses of large 16S rDNA
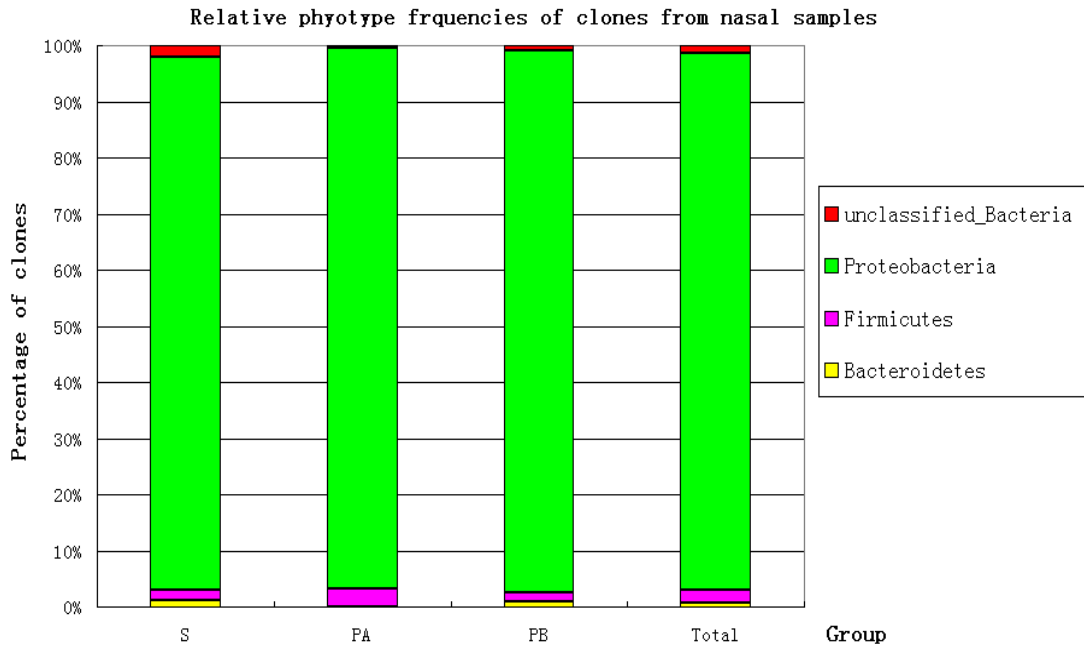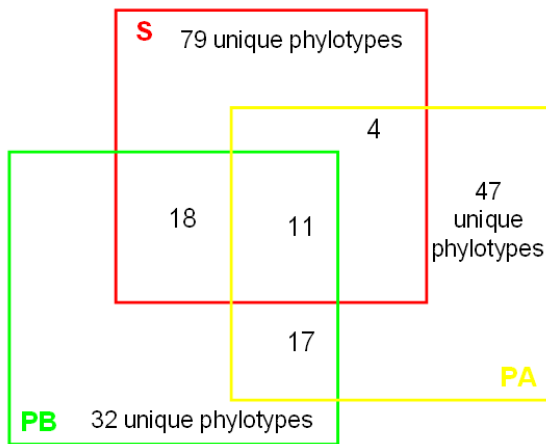550   libraries. BMC bioinformatics 7, 57.
551
552

Fig 1. Relative genus frequencies of clones determined from porcine nasal cavity.

Sequences were assigned to a bacterial phylum according to their position in the phylogenetic level in TABLE 2. Total combines libraries from previous three subjects, including swine and piglets.

a

S 79 unique phylotypes

4

18   11

47 unique phylotypes

17

PA

PB 32 unique phylotypes

b

S 120(12.9%)

12(1.3%)

63(6.8%)

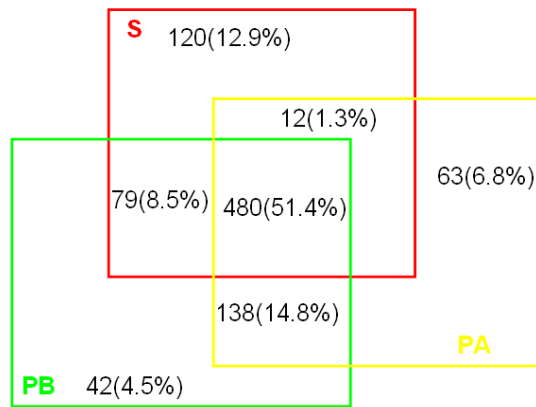79(8.5%) 480(51.4%)

138(14.8%)

PA

PB 42(4.5%)

Fig 2. A Venn diagram illustrates observed overlap of OTUs among different individuals within the family. S represent Sow, PA and PB represent Piglet A and Piglet B, respectively. Fig 2a shows the distribution of OUTs in S, PA and PB. Fig 2b shows the distribution of clones in S, PA and PB.

Measurement	Sow (S)	Piglet A (PA)	Piglet B (PB)	Total
No. of clones sequenced	540	323	286	1149
No. of chemic clones [a]	37	21	18	76
No. of clones for further study	454	251	228	933
Average length of sequence reads (bp)	782	770	762	774
No. of classifiable bacterial sequences [b]	446	250	226	922
Phylotypes [c]	117	77	87	207
Singletons	64	49	49	121
Sow unique phylotypes	79	/	/	/
Piglet A unique phylotypes	/	47	/	/
Piglet B unique phylotypes	/	/	32	/
Common phylotypes	/	/	/	11
Chao 1 (ribotypes) [d]	222±5	197±2	142±2	439±8
Shannon-Wiener index (nats) [e]	3.42±0.19	3.44±0.10	3.64±0.12	3.75±0.22
Coverage [f] (%)	74.2	69.3	61.8	77.8

TABLE 1. Summary description of projects and bacterial community

a Chimeric sequences were determined by using the Mallard program; b Total No. of classifiable bacterial sequences of each libraries were determined by screening the RDPII database at a threshold of 95%; c The term 'phylotype' is used for clusters of clone sequences which differ from known species by more than 3% and are at least 97% similar to members of their cluster; d 'Richness' calculates Chao1 richness index; Chao1 values, a nonparametric estimate of species richness; e 'Diversity' calculates Shannon-Wiener diversity index; The index is one of several diversity indices used to measure diversity in categorical data. It is simply the Information entropy of the distribution, treating species as symbols and their relative population sizes as the probability; f The estimation of diversity coverage was calculated by Good's method, according to which

the percentage of coverage was calculated with the formula (%) = [1-(n/N)] x 100, where n is the number of phylotypes represented by one clone only and N is the total number of sequences.

	S	PA	PB
phylum *Bacteroidetes*			
class *Flavobacteria*			
order *Flavobacteriales*			
family *Flavobacteriaceae*			
genus *Empedobacter*	1		
genus *Myroides*	2		2
class *Bacteroidetes*			
order *Bacteroidales*			
family *Porphyromonadaceae*			
genus *Dysgonomonas*	1		
unclassified *Bacteroidetes*	2		
phylum *Firmicutes*			

class "*Bacilli*"

    order "*Lactobacillales*"

        family *Streptococcaceae*

            genus *Streptococcus*   1

        family "*Carnobacteriaceae*"

            unclassified "*Carnobacteriaceae*"   3   1

    order *Bacillales*

        family *Planococcaceae*

            genus *Kurthia*   6   3   1

    unclassified_"*Bacilli*"   1

class "*Clostridia*"

    order *Clostridiales*

        family "*Peptostreptococcaceae*"

genus "*Peptostreptococcaceae Incertae Sedis*"	1	2
family *Clostridiaceae*		
subfamily "*Clostridiaceae 1*"		
genus *Clostridium*	2	
unclassified *Firmicutes*		1
phylum *Proteobacteria*		
class *Epsilonproteobacteria*		
order *Campylobacterales*		
family *Campylobacteraceae*		
genus *Arcobacter*	3	
class *Gammaproteobacteria*		
order *Pasteurellales*		
family *Pasteurellaceae*		

genus *Actinobacillus*	1		
genus *Pasteurella*	1		
unclassified *Pasteurellaceae*	3		
order *Enterobacteriales*			
family *Enterobacteriaceae*			
genus *Proteus*			1
unclassified *Enterobacteriaceae*	2	3	1
order *Xanthomonadales*			
family *Xanthomonadaceae*			
genus *Schineria*		2	
order *Pseudomonadales*			
family *Moraxellaceae*			
genus *Psychrobacter*	1	1	1

genus *Acinetobacter*	84	96	36
unclassified *Moraxellaceae*	4	3	1
family *Pseudomonadaceae*			
genus *Pseudomonas*		1	1
unclassified *Pseudomonadaceae*		4	1
unclassified *Pseudomonadales*	4		2
unclassified *Gammaproteobacteria*	2	2	3
class *Betaproteobacteria*			
order *Burkholderiales*			
family *Oxalobacteraceae*			
genus *Massilia*	1	3	6
genus *Naxibacter*		2	3
unclassified *Oxalobacteraceae*	3	27	12

family *Comamonadaceae*			
genus *Comamonas*	256	71	114
unclassified *Comamonadaceae*	18	7	6
unclassified *Burkholderiales*		6	13
order *Neisseriales*			
family *Neisseriaceae*			
genus *Vitreoscilla*		2	
unclassified *Burkholderiales*	18		
unclassified *Betaproteobacteria*	1		2
unclassified *Proteobacteria*	29	14	17
unclassified *Bacteria*	8	1	2

TABLE 2. Hierarchy view of genus distribution in different samples by Naive Bayesian rRNA Classifier

Sequence Name	Annotated Species [a]	Identity [b]	Genebank	Library	Bioharzard Level [c]	Zoonoses [d]
S080;S304;PA077;PA004;PA;PB021	*Acinetobacter baumannii* AIIMS 11	0.96-0.98	EU883589	S;PA	2	No
S070;	*Acinetobacter calcoaceticus* A2	0.95	AF159045	S	2	No
S386;S526;S534;S210;S091	*Acinetobacter lwoffii* CMG 851	0.95-0.98	EU697389	S	2	No
S456	*Actinobacillus suis* ATCC 33415	0.99	AY362899	S	2	Yes
S071;S129;S259	*Arcobacter cryaerophilus* ATCC 49615	0.98	U25805	S	2	Yes
S406;S549;PB262	*Bacillus anthracis* HDDMM10	0.97	EU723830	S;PB	3	Yes
S393;S307	*Comamonas testosteroni* WAB1945	0.95-0.98	AM184284	S	2	No
PA196	*Enterobacter hormaechei* TMPSB-T10	0.95	EU047556	PA	2	No
PB105	*Escherichia coli* O157:H7	0.95	CP001368	PB	3	Yes
S141;S150;S181	*Haemophilus parasuis* SW114	0.99	AB004039	S	2	No
S179;PB177	*Massilia cf. timonae* 96A14209	0.98	AY157762	S	2	No
PA157;PB125;PB234	*Massilia timonae* UR/MT95	0.95-0.99	NR_026014	PA;PB	2	No
PA245;PA158;PA283	*Moraxella catarrhalis* ATCC 25238	0.96	U10876	PA	2	No
S048	*Myroides odoratimimus* CM9	0.9	EU660317	S	2	Yes
S299;PB038;PB041	*Myroides odoratimimus* GJ1-8	0.9-0.91	EU331413	S;PB	2	Yes
PA219	*Pantoea agglomerans* WAB1872	0.95	AM184214	PA	2	No
PA161	*Pantoea ananatis* BD 543	0.99	DQ133545	PA	2	No
S187	*Pasteurella multocida* Tabriz98	0.98	AE004439	S	2	Yes
PB188	*Proteus vulgaris* IFAM 1731	0.99	X07652	PB	2	Yes
PA132	*Pseudomonas mendocina* NCIB 10541	0.96	D84016	PA	2	No
S276	*Shigella flexneri* FBD002	0.95	EU009187	S	2	No
S293	*Streptococcus suis* NCTC1046	0.99	AF009490	S	2	Yes

TABLE 3 Summary the potential pathogens associated with porcine and public health. a, the annotated sequence information was according to the seqmatch method described previously; b, the querying sample sequence identity is according to the N-BLAST identity to the preference sequence; c, the bioharzard level of each species was estimated as described in the methods; d, Zoonoses information of each species was determinated in the methods.

Porcine UTR bacterial agents	Host	Normal habitat	Primary disease in pig	Preference
Streptococcus suis	swine	palatine tonsil	meningoencephalitis;septicemia;arthritis	Wertheim, et al. 2009
Haemophilus parasuis	swine	nose	polyserositis (Glässer's disease) respiratory disease;septicemia;arthritis	Olvera, et al. 2007
Pasteurella multocida	birds, mammals	oropharyngeal;UTR	atrophic rhinitis(synergistically with Bordetella bronchiseptica)	Frost and Adler. 2000
Actinobacillus pleuropneumoniae	swine	tonsils; nose	pleuropneumonia	Moller, et al. 1990
Actinobacillus suis	birds, mammals	tonsils;UTR	septicemia;pneumonia;arthritis;enteritis	MacInnes, et al. 1999
Bordetella bronchiseptica	warm-blooded animals	nose	atrophic rhinitis(synergistically with toxic Pasteurella multocida)	Brockmeier, et al. 2008
Mycoplasma hyopneumoniae	swine	lung	pneumonia	Maes, et al. 2008
Mycoplasma hyorhinis	swine	tonsils;UTR	polyserositis;arthritis	Lin, et al. 2006

TABLE 4. Summary the most important bacterial pathogens in porcine upper respiratory tract (UTR).