Nasal Bacterial Microbiome: Probing a Healthy Porcine Family

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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

complicated aetiology, which may be associated with higher mortality. Hence, knowledge of aetiology of respiratory disease, as well as host-pathogen interaction, is crucial to understand Upper Respiratory Tract (URT) infection. Defining the normal microbiota of URT is the first step to understand health and disease development.

As the entrance of upper respiratory tract, host nasal cavity inhabits many different microbes, with minority of symbiotic microorganisms, foreign flora and microbes from the environment; including some important associated with URT infection and other related diseases(Pettigrew et al., 2008). All of them coexist on nasal cavity during a certain development stage. In the URT, site-specific microbial flora may play an important role in respiratory disease development as the nasal cavity ecosystem changes from a healthy to a diseased state. Respiratory disease in pig is complex, but vital in population health state, and economic importance. In veterinary clinic, often two or more related etiologies are associated with disease development, indicating the microbe composition and interaction may help to understand respiratory disease development(Moorkamp et al., 2008; Palzer et al., 2008). While in modern industry farming, early piglet colonizer plays an important role in state of health in its life career. As the closest relative, sow may influence early colonized of their offspring. Thus, exploring the bacterial flora of nasal cavities in early-colonized piglet, sow and understanding their relationship is an

111 important to reveal respiratory disease development in herds.

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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.

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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

bacterial colonies were picked up for plasmid DNA purification, and plasmid inserts were sequenced bidirectionally using the M13 primers on an ABI 3730xl sequencer (Applied Biosystems Inc.), including 576 from sow and 615 from two piglets, respectively. Chromatogram data quality and quantity were evaluated using phred Q20 counts and non-vector sequence data remaining after cross_match screening. Sequences were extracted from chromatograms using phred, and bidirectional pairs were assembled using phrap. Vector sequence detected by cross_match was trimmed off. Only assembled sequences about 800 base pairs were studied in further. Assemblies were screened for quality, and all sequences containing >20 consecutive bases of sequence <Q20 were discarded(Grice et al., 2008).

Data analysis

Total valid sequences with chimeric are identified using the Mallard program(Ashelford et al., 2006), leaving 454 from S (sow) group, 479 from PA (piglet A) group and PB (piglet B) group. These 933 sequences were then submitted to RDPII web service(Hamady and Knight, 2009), using the online program Classifier and SeqMatch with a threshold setting for similarity score of 95%, to assign 16S rRNA gene sequences to the new phylogenetically consistent higher-order bacterial taxonomy and assign a genus to each sequence, respectively. To classify sequences based on self-similarities rather than matches to an external database, sequences were grouped into operational taxonomic units (OTUs) with cutoffs of 97%sequence similarity

using the DOTUR software package implemented with the furthest-neighbor option, in which all of the sequences within an OTU are at least 97% similar to all of the other sequences within the OTU(Yu et al., 2006). 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. The representative sequences were incorporated into Green-Gene online tool NAST alignment tool for species assignment. Shannon-Wiener index as diversity indices, Chao1 and rarefaction as richness estimates calculated by DOTUR and FastGroupII were to estimate microbial diversity and richness as described(Yu et al., 2006). 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(Bik et al., 2006). Biohazard level of each species was estimated according to the potential risk of each species according the levels of biohazard definition basing on Risk Group Classification for Infectious Agents available on American Biological Safety Association (http://www.absa.org/riskgroups/index.html). The zoonosis information was determinated according the list of zoonoses available on World Health Organization (WHO) website (http://www.who.int/zoonoses/en/index.html).

Results

Summary the Sequencing Project

Nasal samples were obtained from 3 individuals, consisting of a sow and its two piglets living together. For further extensive analysis bacterial community in a continuous timescale, we focused on a representative family with small individuals. A highly variable portion of the 16S rRNA gene of ~800 base pairs was amplified, cloned and approximately 300 clones were sequenced from each individual; this number of clones is sufficient to capture most of the variation at the level of bacterial genera inferred from the partial 16S rRNA sequences as describe previously(Grice et al., 2008). A total of 1,149 sequences were obtained, which were then analyzed for possible chimeras or other artifacts. We identified 76 potentially chimeric sequences, which represents 6.6% of the sequences. This data was according to the average of 9% potentially chimeric sequences reported previously for full-length 16S rRNA clone libraries. The remaining qualified 933 sequences were analyzed in the followings. The overview information of project was showed in Table 1.

Phylotypes, Species Richness and Diversity

The qualified 933 sequences were grouped into operational taxonomic units (OTUs; “Phylotypes”) based on their genetic distance in a neighbor-joining tree with the DOTUR program(Hamady and Knight, 2009). A species-level OTU has historically been defined as containing 16S rDNA sequences that are 97% identical. Using the furthest-neighbor method of calculation and a similarity

threshold of 97%, DOTUR assigned the 933 sequences to 207 OTUs, including 121 singletons. There were 79 swine unique OTUs (120 clones), while 47 (63 clones) and 32 (42 clones) piglet unique OTUs for piglet A and piglet B, respectively. Though only 11 OTUs were commonly shared by such family (As showed in Fig 2a), there were 480 clones belonging to previous 11 OTUs, which constitute of a total 51.4% (As showed in Fig 2b) indicating the general core bacterial flora in the family. The remaining 196 OTUs were from the other half of total clones (453/933, 48.6% in total), which indicated the diversity of bacterial flora in nasal within different individuals. The genus was assigned to each sequence by comparison to the RDPII database revealed 922 classifiable bacterial clones, while the remaining 11 clones cannot be assigned the known genus at a threshold of 95%.

Estimations of species coverage, richness, evenness, and diversity were calculated for the combined data set, as well as for three subsets of nasal samples (As showed in Table 1). The Chao1 estimator of total species richness was 439, which based on the distribution of singletons. Shannon-Wiener index of total species diversity is 3.75, which 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. Good's coverage was 77.8% for the overall sequence set, indicating that twelve additional phylotypes would be expected for every 100 additional sequenced clones. This level of coverage

indicated that the 16S rDNA sequences identified in these samples represent the majority of bacterial sequences present in the porcine nasal samples under study.

Distribution of Clones at Different Levels

Fig.1 showed bacteria distribution in different categories with the phylum identified among the 933 clones in combined nasal sequence data set. The nasal bacterial community was constituted by three phyla: *Proteobacteria* (895 clones), *Firmicutes* (21 clones), *Bacteroidetes* (7 clones), the remaining nasal bacterial sequences were assigned as an uncharacterized composition (12 clones). Among these sequences, *Proteobacteria* was the dominant phylum (95.9% in total) in porcine nasal cavities, which included a wide variety of pathogens, such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, and many other notable genera. As showed in Table 2, current genuses identified in porcine nasal sample were *Empedobacter*, *Myroides*, *Dysgonomonas* in phylum *Bacteroidetes*; *Streptococcus*, *Kurthia*, *Peptostreptococcaceae Incertae Sedis*, *Clostridium* in phylum; *Arcobacter*, *Actinobacillus*, *Pasteurella*, *Proteus*, *Schineria*, *Psychrobacter*, *Acinetobacter*, *Pseudomonas*, *Massilia*, *Naxibacter*, *Comamonas*, *Vitreoscilla* in phylum *Proteobacteria*. Among these genus, *Comamonas* and *Acinetobacter* were the most abundant clones within the porcine nasal libraries. *Comamonas* sequences were found all the three porcine nasal samples (varying from 28.3% to 56.4% of clones per sample library) and constitute of all sequences analyzed (441/933, 47.3% in total).

Acinetobacter sequences were also found all the three porcine nasal samples (varying from 15.8% to 38.2% of clones per sample library) and constitute of all sequences analyzed (216/933, 23.2% in total). The above abundant genus in porcine nasal samples occupied about 70% clones in species, indicating the specific core species constituted the indigenous bacterial population in porcine nasal cavity. Detail analysis the composition of the genus *Comamonas* revealed the constitution of *Comamonas aquatica*, *Comamonas sp*. and *Comamonas testosteroni*. And *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter lwoffii* and *Acinetobacter sp*. are the representative species of *Acinetobacter* genus. The other remarkable clones in nasal libraries, which ware specific found in sow, were family *Pasteurellaceae* and *Streptococcaceae*. Though the total clone number is from 1 to 3, we cannot draw a conclusion of no significant difference between sow and its piglet because of lack of available data in analysis. The similar results were found in family *Clostridiaceae*, *Campylobacteraceae* which were found specific to sow, while family *Carnobacteriaceae*, *Pseudomonadaceae*, *Xanthomonadaceae* were specific to piglet.

Potential Pathogens and public health

Table 4 summary the most important pathogens frequent found in porcine UTR, which were well recognized in previous knowledge. Many of them were as normal microbiota, but with specific aetiology importance in veterinary clinics. In table 3, we concluded the annotated sequences with aetiology importance

from porcine nasal samples. *Streptococcus suis*, *Pasteurella multocida*, *Actinobacillus suis*, *Haemophilus parasuis* were found according to previous knowledge of aetiology in porcine UTR. We also discovered lots of the previous unknown pathogens in porcine UTR(Brockmeier et al., 2008; Lin et al., 2006; Maes et al., 2008). Some belong to zoonosis such as *Proteus vulgaris*, inhabits the intestinal tracts of humans and animals, known to cause urinary tract infections and wound infections. Large numbers of potential pathogens specific to human were also found. Among the summary in the table 3, most of them were found specific in sow, while relative small number was in piglet. Interesting, we found specie *Bacillus anthracis* from sow sample, which can be classified as biohazard level-3 pathogen. And host specific specie *Shigella flexneri* was also found in nasal sample from sow.

Discussion

Environment samples including seawater, sediments, and soil, about 1% of bacteria can be cultivated; in contrast, cultivability of the human microbiota, such as in the gut, is estimated to be substantially higher (10–50%)(Hamady and Knight, 2009). The molecular approach by 16S rDNA sequencing has significantly enhanced our knowledge in understanding bacteria diversity, though this method has PCR amplification efficacy bias, cloning bias and was dependent largely on updated database. In this study, our data from

sequence-based environmental microbial approach describes a previously unrecognized extent of bacterial diversity in the porcine nasal ecosystem. Analysis of the 933 16S rDNA clones from the nasal samples of a healthy porcine family revealed an unrecognized bacterial species rich microbiota: the 3 phyla and 207 phylotypes represented in the microbiota, with about 75% species coverage estimated.

Though there are 3 phyla and a few unclassified clones in the nasal samples, *Proteobacteria* is, undoubtedly, the dominant phylum. And *Comamonadaceae*, *Moraxellaceae* and *Oxalobacteraceae* are the three dominant families in the porcine nasal samples. All those clones from above three dominant families constitute the major normal bacterial. Among these, Genus *Comamonas* belong to family *Comamonadaceae* covers half of the clones in the total libraries. *Comamonas* species are environmental Gram-negative rods that grow forming pink-pigmented colonies. It is known that *Comamonas* strains are involved in novel degradation capability in environments. Though this genus have potential application in bioremediation and are common in nature, some member, such as *Comamonas testosteroni* is associated implicated as a human pathogen along with rarely case report(Reddy et al., 2009). The only notable infection species is *C. testosterone*, which was found in sow library. Genus *Acinetobacter* from family *Moraxellaceae* is also one of major composition in normal bacterial flora. *Acinetobacter spp*. is widely distributed in

nature. Occasionally, strains are isolated from foodstuffs and some are able to survive on various medical equipments and even on healthy human skin. *Acinetobacter* species are generally considered nonpathogenic to healthy individuals. However, several species persist in hospital environments and cause severe, life-threatening infections in compromised patients, and the strain *A. baumannii* is the second most commonly isolated nonfermenting bacteria in human specimens(Bergogne-Berezin and Towner, 1996; Joly-Guillou, 2005). The bacterial community in major constitution of *Comamonas* and *Acinetobacter* species may play a vital part in balance bacterial flora in porcine nasal. Though sow and piglets have numbers of different phylotypes, the constitution of core genus in bacterial microbiota is the same, indicating the sow play a key role in early colonizers to establish in its piglets.

There were various swine specific pathogens found in swine nasal microbiome. Organisms from *Pasteurellaceae* family were common agents found in porcine UTRs(Moller and Kilian, 1990). *Haemophilus parasuis* was the most common nasal carriage in pigs, which was found throughout the world and organisms were present even in high health herds(Olvera et al., 2007). *Actinobacillus suis* can persist as a commensal in the upper respiratory tract (URT) with a wide rang of host, under conditions which are poorly understood(MacInnes and Desrosiers, 1999). *Pasteurella multocida* was the causative agent of fowl

cholera (FC), hemorrhagic septicemia (HS), and a variety of respiratory syndromes such as atrophic rhinitis of swine (AR) and purulent rhinitis of rabbits (“snuffles”), which were also part of the normal oropharyngeal flora in many species(Frost and Adler, 2000; Hunt et al., 2000). The other important agent belongs to family *Streptococcus*. *Streptococcus suis*, which principally colonized in pig tonsil, is associated with a wide range of clinical syndromes in swine and other domestic animals(Wertheim et al., 2009). The latter three were also notable zoonotic important agents, while zoonotic *S. suis* type 2 cause serious public incidence in China in 2005(Segura, 2009). As the healthy carrier, they all were also found in the sow libraries. Though there maybe virulent strains as a result of specific disease outbreaks, the normal bacterial microbiota may balance such virulent strains; result from competitive limiting their growth or other unknown mechanisms. Ecological view may help to understanding the porcine upper respiratory infection and disease development. In such cases, those dominant species in *Comamonas* and *Acinetobacter* may be of vital importance in porcine UTR microbiota interaction.

In addition to above well-recognized agents, we also characterized a group of agents that can be involved in specific human infection. Organisms such as *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter lwoffii* from genus *Acinetobacter*. *Acinetobacter* species are generally considered

nonpathogenic to healthy human individuals(Joly-Guillou, 2005). However, several species persist in hospital environments and cause severe, life-threatening infections in compromised patients, and the species *A. baumannii* is the second most commonly isolated nonfermenting bacteria in human specimens(Joly-Guillou, 2005). *Arcobacter cryaerophilus*, *Proteus vulgaris*, *Pantoea agglomerans* and *Pantoea ananatis* were as opportunistic pathogen, as result of common bacteremic infection and other specific syndrome(Aly et al., 2008; De Baere et al., 2004; Stein and Gechman, 1955; Wesley et al., 1996). Including *Massilia timonae* and *Myroides odoratimimus*, those were all firstly detected in porcine nasal samples. Approximately 80% of the infectious agents that cause disease in humans are shared with various animal hosts. Currently, many newly emerging diseases are caused by zoonotic agents, which focused on wildlife reservoirs that increase human exposure to insect vectors as well as to animal and environmental sources of disease. Importantly, porcine could play an important part in emerging zoonoses, and may be the unrecognized carrier, potential risk to public health and food safety(Tomley and Shirley, 2009). More effects need to discover our close contact animals-pig, by new potential agents explore and routine surveillance. The human microbiome project largely enhances our knowledge to understanding the health and disease. Porcine microbiome will not only aid the above knowledge, but also help to understand swine zoonosis.

In conclusion, by means of a 16S rRNA gene clone library, we provided a preliminary picture of our current knowledge of the community structure of porcine nasal bacterial ecosystem. Our results indicate that the culture-independent genetic profiling of the 16S rRNA gene is a powerful tool for investigation of the nasal microbiota. Further study is required to determine the occurrence of these organisms in the porcine nasal microbiota, their interaction mechanism and their functional roles in development of health and disease.

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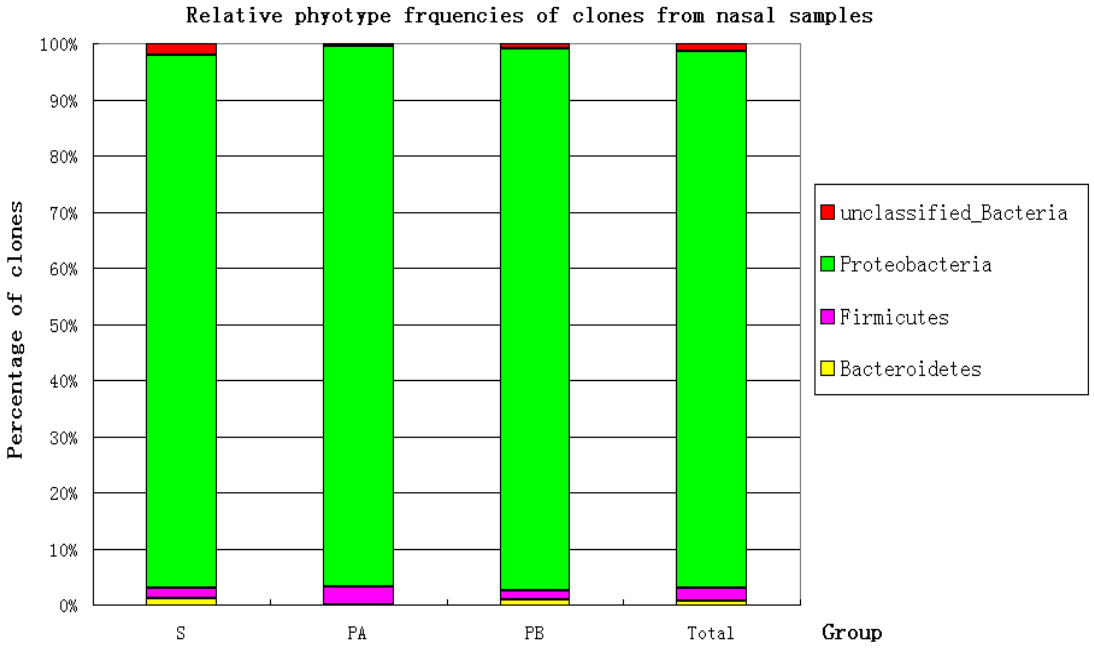
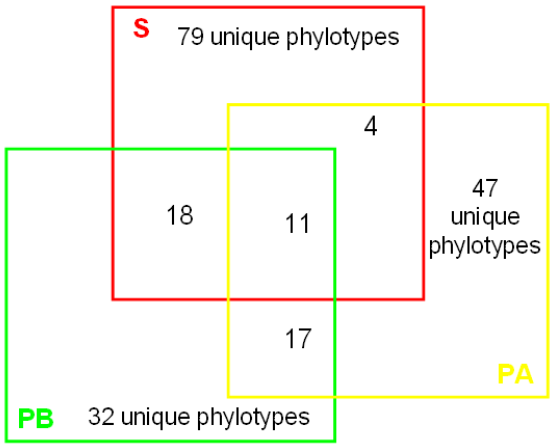


Fig 1. Relative genus frequencies of clones determined from porcine nasal cavity.

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



b

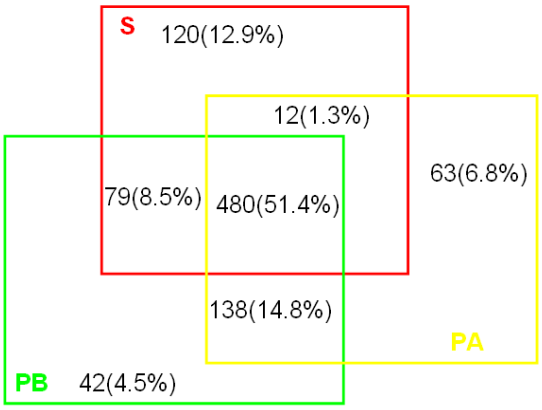


Fig 2. A Venn diagram illustrates observed overlap of OTUs among different individuals within the family. S represent Sow, PA and PB represent Piglet A and Piglet B, respectively. Fig 2a shows the distribution of OUTs in S, PA and PB. Fig 2b shows the distribution of clones in S, PA and PB.

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).