ORIGINAL ARTICLE

Comparison of culture-dependent and -independent bacterial detection results on nasal swabs in dogs with nasal discharge

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Objectives: The role of bacterial communities in the pathophysiology of canine nasal disease is still unclear. How and when to treat dogs with suspected secondary bacterial rhinitis and on which test to rely before making a decision to treat with antimicrobials has not been established. The objective is to compare the results of bacterial identification using agar-plate cultures and 16S rRNA gene amplicon sequencing in dogs with nasal discharge suspected to be of bacterial origin.

Materials and Methods: Twenty-nine client-owned dogs presented for investigation of nasal disease were included in the study. Paired swabs were collected from the same affected nasal cavity. One swab was streaked on 4 agar media (Columbia Blood Agar, MacConkey, Chapman and Edward's). The other swab was stored in a sterile cryotube at −80°. Extracted DNA underwent a polymerase chain reaction targeting the V1-V3 region of the 16S rRNA gene.

Results: At least one of the species detected by amplicon sequencing with a relative abundance of >10% was also identified by culture in 14 cases (48.3%), in association with marked predominance of one taxon (>80% relative abundance) in six of 14 cases. In 12 dogs (41.4%), the cultured isolates were rare or undetected components of the corresponding sequence libraries. A negative culture in the face of bacterial predominance (>50% relative abundance) of a potentially pathogenic bacteria detected by sequencing occurred in 17% (n=5) of cases; however, the use of other agar media may have decreased this percentage.

Clinical Significance: Standard culture does not reliably predict the bacterial profile detected by 16S rRNA gene amplicon sequencing.

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INTRODUCTION

Acute or chronic nasal discharge is a common reason for consultation in canine medicine. Frequent primary nasal diseases diagnosed in dogs include chronic idiopathic rhinitis, fungal rhinitis/rhinosinusitis, nasal/nasopharyngeal neoplasia, nasal/nasopharyngeal foreign body and oronasal defect (Meler et al., 2008; Plickert et al., 2014; Rösch et al., 2019; Tasker et al., 1999; Windsor & Johnson, 2006). In practice, dogs with nasal discharge are commonly treated with antimicrobial treatment (local or systemic) based or not on the results of nasal swab culture. However, the usefulness of this complementary exam as well as the use of systemic antimicrobials in such cases is questionable. Indeed, primary nasal bacterial infections rarely occur in dogs (Hawkins et al., 2008; Lee-Fowler & Reinero, 2012; Meler et al., 2008; Rösch et al., 2019; Tasker et al., 1999; Windsor & Johnson, 2006) although bacterial infections frequently complicate primary conditions (Lee-Fowler & Reinero, 2012; Windsor & Johnson, 2006). The primary cause of nasal discharge has to be determined before considering the use of antimicrobials in dogs (Lee-Fowler & Reinero, 2012). Moreover, culture results may not reflect the actual infection. Indeed, the major infection-causing organism may be difficult to grow on conventional culture media and the microbiota may be of more interest than a single colony type grown in a given culture medium. Furthermore, a cultivable flora also exists in the nose of healthy dogs (Bailie et al., 1978; Clapper & Meade, 1963; Smith, 1961). As a result, the cultured colony type(s) might not be the one(s) responsible for the infection.

Until recently, our understanding of normal nasal microbiota was based on publications of the mid-19 century, in which nasal passages of healthy dogs were cultured (Bailie et al., 1978; Clapper & Meade, 1963; Smith, 1961). The recent contribution of results obtained with next-generation sequencing methods has given a more precise and exhaustive idea of the nasal microbiota in healthy dogs (Banks et al., 2020; Ericsson et al., 2016; Isaiah et al., 2017; Rodriguez et al., 2019; Tress et al., 2017; Vangrinsven et al., 2021). Based on 16S rRNA gene amplicon sequencing, a species-rich bacterial community was detected in the nose of healthy dogs (Banks et al., 2020; Ericsson et al., 2016; Isaiah et al., 2017; Rodriguez et al., 2019; Tress et al., 2017; Vangrinsven et al., 2021) with different alterations of this community in dogs with nasal diseases (Tress et al., 2017; Vangrinsven, 2021).

To what extent nasal swab culture reflects the bacterial communities in dogs with nasal disease is unknown. The primary aim of this study was to compare the results obtained with agar-plate cultures and 16S rRNA gene-based amplicon sequencing using matched samples of nasal swabs.

MATERIAL AND METHODS

Study population

Dogs presented for investigation of a nasal disease associated with acute or chronic nasal discharge and with a clinical suspicion of bacterial infection were prospectively included. The suspicion of bacterial infection was based on the presence of a continuous or intermittent mucopurulent-like nasal discharge. Pre-treatment with antimicrobials was not an exclusion criterion.

Sampling method

This study was approved by the animal ethical committee (N° 16-1854, October 27, 2016) and all samples were obtained with the consent of the owners.

Clinical data were recorded, general anaesthesia was performed and two consecutive samples were collected in the same nasal cavity before starting rhinoscopic examination. Dogs were premedicated with butorphanol (0.2 mg/kg; Butomidor®, Richter Pharma) intravenously in combination with medetomidine (5 µg/kg; Medetor®, CP-Pharma) followed by propofol (Propovet®, Zoetis) on demand for induction. The dogs were intubated and placed in sternal recumbency. First an eSwab (Copan®, eSwab™, 490 CE.A, Brescia, Italy) was introduced into the distal third of the most affected nasal cavity followed by the introduction of a FLOQswab (Copan®, FLOQSwabs™, 553C, Brescia, Italy) at the same site in the same nasal cavity. The swabs were introduced into the nasal cavity through a sterile speculum which maintained the nostril open and prevailed the swab of being in contact with the skin and nose. Three gentle circular movements were performed with each swab before withdrawal of the swabs through the speculum. The eSwab was immediately re-inserted in his sterile tube and sent for culture. The end of the sew of the FLOQswab was cut with sterile scissors and stored at −80° in a sterile cryotube until batched DNA extraction. Direct and retrograde rhinoscopy was performed in all dogs. Other diagnostic tests (CT of the head, fungal culture, nasal biopsy with histopathologic evaluation, dental probing) were performed at the clinician's discretion depending on the clinical suspicion.

Agar-plate culture method and bacterial identification

The eSwabs were sent to the microbiology laboratory of the same institution site for bacterial culture and isolation, within 6 hours. Specimens were plated and streaked onto MacConkey agar (selective for enterobacteria, Prolabochemical), Mannitol salt agar (selective for staphylococci, VWR), Columbia Blood Agar (non-selective medium, bioMerieux), Edward agar (selective for streptococci and enterococci, Oxoid) and were incubated overnight in aerobic conditions at 37°C. If no growth was observed, the incubation was extended to 48 hours. If no significant growth was observed after 48 hours, culture was reported negative. Growth was categorised based on the number of colonies: 0 colonies=no growth, 1 to 10 colonies=1+, 10 to 50 colonies=2+, 50 to 100 colonies 3+ and >100 colonies=4+. Colonies were counted at first on each selective agar medium and compared to the growth on the Columbia Blood Agar medium. Suspect colonies were pre-identified on the basis of colony shape, Gram staining, bacterial cell shape and catalase and oxidase production (Markey

et al., 2013). Bacterial identification was performed by matrix-assisted laser desorption ionisation/time of flight (MALDI-TOF) mass spectrometry on a Microflex LT platform (Bruker Daltonik, Bremen, Germany) (Descy et al., 2010).

Molecular-based method: extraction, sequencing and data analysis

Total bacterial DNA was extracted from the nasal swabs and two negative control swabs using the DNEasy Blood and Tissue kit (QIAGEN Benelux BV; Antwerp, Belgium) following the manufacturer's recommendations. Spectrophotometry (NanoDrop, Thermo Scientific) was used for total DNA concentration measurement.

Bacterial 16S rRNA gene amplicons were generated via amplification of the V1-V3 hypervariable regions of the 16S rRNA gene using the following primers (with Illumina overhand adapters): forward (5′-GAGAGTTTGATYMTGGCTCAG-3′) and reverse (5′-ACCGCGGCTGCTGGCAC-3′) as previously described (Ngo et al., 2018). The DNA was purified with the Agencourt AMPure XP beads kit (Beckman Coulter; Pasadena, CA, USA) and submitted to a second polymerase chain reaction (PCR) round for indexing, using the Nextera XT index primers 1 and 2. A final quantification, by quantitative (q)PCR, of each sample in the library was performed using the KAPA SYBR" FAST qPCR Kit (KapaBiosystems; Wilmington, MA, USA) before normalisation, pooling and sequencing on a MiSeq sequencer using V3 reagents (Illumina; San Diego, CA, USA) (Ravi et al., 2018). Positive control using DNA from 20 defined bacterial species and a negative control (from the PCR step) were included in the sequencing run (*Acinetobacter baumannii*, *Actinomyces odontolyticus*, *Bacillus cereus*, *Bacteroides vulgatus*, *Bifidobacterium adolescentis*, *Clostridium beijerinckii*, *Clostridioides difficile*, *Cutibacterium acnes*, *Deinococcus radiodurans*, *Enterococcus faecalis*, *Escherichia coli*, *Helicobacter pylori*, *Lactobacillus sakei*, *Lactococcus lactis*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Porphyromonas gingivalis*, *Pseudomonas aeruginosa*, *Rhodobacter sphaeroides*, *Staphylococcus aureus* and *Streptococcus agalactiae*).

Alignment and clustering were done with MOTHUR software package (v1.41.0) with an operational taxonomic unit (OTU) clustering distance of 0.03 and based on the SILVA database (V1.32) of full-length 16S rRNA sequences. Vsearch (Rognes et al., 2016) algorithm was used for chimera detection.

After DNA extraction from nasal swab samples, quantification of the total bacterial flora was performed with a quantitative real-time PCR targeting the V2-V3 region of the 16S rRNA gene with the following primers: forward (5′-ACTCCTACGGGAGGCAGCAG-3′) and reverse (5′-ATTACCGCGGCTGCTGG-3′). The standard curve was based upon 10-fold dilution of a quantified PCR product. This PCR product was purified (Wizard® SV Gel and PCR Clean-Up System, Promega, Leiden, The Netherlands), and quantified with PicoGreen targeting double-stranded DNA (Promega).

Data and statistical analyses

Bacterial richness, evenness and alpha-diversity were obtained with MOTHUR (v1.41.0) at species level using the Chao1 index, Simpson index-based measure and the inverse Simpson's index respectively. Beta-diversity was estimated with AMOVA (analysis of molecular variance; 10,000 iterations) and beta-dispersion was assessed with HOMOVA (analysis of molecular variance homogeneity; 10,000 iterations).

The total bacterial flora, intrinsic diversity values, beta-diversity and beta-dispersion were compared between the dogs treated with antimicrobials, ongoing or recent, and untreated dogs using MOTHUR and a Mann–Whitney test (XLstat 2019.3.2, Addinsoft, Paris, France). Differences were considered significant for a P-value <0.05.

Comparison between culture- and molecular-based results

Results were distributed in three tables according to the agreement between the two methods. Good and moderate agreement were attributed when at least one of the most abundant sequenced taxa (all taxa with a relative abundance >10%) was cultured (Table 2). When, among the most abundant sequenced taxa, the one taxon with the highest relative abundance was detected by culture, the agreement was considered good. When, among the most abundant sequenced taxa, the one taxon with the highest relative abundance was not detected by culture, but other lower abundance taxa were detected by culture, the agreement was considered moderate.

Poor agreement was attributed when none of the most abundant sequenced taxa were identified by culture while at least one of them was expected to grow on the selected media (cf. material and methods) (Table 3). A third table (Table 4) regroups the results of samples for which the selected growth conditions did not allow the growth of the most abundant species detected by sequencing. An agreement category could therefore not be established for these samples.

RESULTS

Study population

Twenty-nine client-owned dogs were included between December 2019 and January 2021. All dogs were presented for the investigation of a nasal disease. Clinically all dogs had continuous or intermittent muco-purulent-like nasal discharge, which was sometimes associated with other clinical signs of nasal disease such as sneezing, reverse sneezing, hyperkeratosis, ulceration, depigmentation, pain and decreased or increased permeability of the nasal passages (Table 1).

Four dogs were treated with systemic antimicrobials at the time of sampling while in five other dogs, the treatment had been interrupted within the 15 days before sampling. Three dogs were under non-steroidal anti-inflammatory therapy at the time of sampling and in another dog this treatment was stopped 5 days before sampling. Two dogs were receiving an oral antifungal treatment at the time of sampling. Sinonasal aspergillosis was diagnosed in 11 dogs, idiopathic lymphoplasmacytic rhinitis in eight dogs and nasal neoplasia in three dogs. Other primary nasal problems were the presence of nasal foreign body (among which

Table 1. Clinical data

	Breed	Age (years)	Sex	Nasal discharge (day of sampling)	Diagnosis	Treatment (day of sampling)
1	Border Collie	5	M	Seromucous	Sinonasal aspergillosis	Ophthalmic terramycin
6	Standard poodle	4.7	M	Mucopurulent	Sinonasal aspergillosis	None
7	Rottweiler	4.1	M	Purulent/ haemorrhagic	Sinonasal aspergillosis	Non-steroidal anti-inflammatory drug
10	Rottweiler	3.9	F	Mucopurulent	Sinonasal aspergillosis	Itraconazole
11	Rottweiler	4.2	M	Mucohaemorrhagic	Sinonasal aspergillosis	Amoxicillin clavulanic acid
12	Golden retriever	5.1	M	Serohaemorrhagic	Sinonasal aspergillosis	Itraconazole
20	Border Collie	5	F	Seromucous	Sinonasal aspergillosis	None
22	Newfoundland	7.2	M	Serohaemorrhagic	Sinonasal aspergillosis	Doxycycline
25	Mixed-breed	11	F	Mucopurulent/ haemorrhagic	Sinonasal aspergillosis	None
3	Dachshund	0.7	M	Mucopurulent/ haemorrhagic	Sinonasal aspergillosis with nasal foreign body	None
23	Italian pointing dog	1.4	M	Mucopurulent/ haemorrhagic	Sinonasal aspergillosis with nasal foreign body	None
21	Rhodesian ridgeback	10.2	F	Mucopurulent	Lymphoplasmacytic rhinitis (sequel sinonasal aspergillosis)	None
2	Akita Inu	5.9	M	Purulent	Lymphoplasmacytic rhinitis	None
5	Parson jack russel	11.9	M	Mucopurulent	Lymphoplasmacytic rhinitis	Meloxicam
17	Husky	7.1	M	Seromucous	Lymphoplasmacytic rhinitis	None
18	Jack Russel	9.9	M	Purulent	Lymphoplasmacytic rhinitis	None
26	Mixed-breed	8.7	M	Mucopurulent	Lymphoplasmacytic rhinitis	None
27	Münsterländer	4.6	M	Seromucous	Lymphoplasmacytic rhinitis	None
29	Jack Russel	10.9	F	Mucopurulent	Lymphoplasmacytic rhinitis	Amoxicillin clavulanic acid until 2 days ago
8	Weimaraner	6.9	F	Mucopurulent/ haemorrhagic	Nasal neoplasm (uncharacterised)	Meloxicam+amoxicillin clavulanic acid until 5 days ago
16	Mixed-breed	15.2	F	Mucous/haemorrhagic	Nasal adenocarcinoma	None
28	Weimaraner	6.9	F	Haemorrhagic	Nasal adenocarcinoma	None
4	Medium poodle	13.1	M	Seromucous	Oronasal defect, hard palate tumour	Doxycycline until 1 week ago
15	Mixed-breed	1	M	Mucohaemorrhagic	Nasal foreign body	Meloxicam, local NaCl
19	Mixed-breed	11	M	Mucopurulent	Nasal foreign body	Marbofloxacin until 10 days ago
13	Labrador	10.3	F	Purulent	Mucocutaneous pyoderma nasal planum	Amoxicillin clavulanic acid
9	Beauceron	2.3	F	Mucopurulent	Lower airway disease	None
14	Cocker spaniel	1.4	M	Mucopurulent	Lower airway infection	Amoxicillin clavulanic acid until 9 days ago
24	Brussels Griffon	11.7	F	Purulent	Lower airway infection	Amoxicillin clavulanic acid

When "none" is indicated in treatment section: no treatment within the last 2 weeks

two with sinonasal aspergillosis), oronasal defect and disease of the planum nasale. In three dogs, nasal disease was associated with bacterial bronchopneumonia.

Culture-based method

Three dogs had a negative culture on the four media; none of them had been treated with antimicrobials during the last 2 weeks before sampling.

Bacterial species isolated from each dog are shown in Tables 2-4. In the group of dogs with recent or ongoing antimicrobial treatment, six of nine dogs (66.7%) had ≤1 cultured colony type. In the group of dogs without recent or ongoing antimicrobial treatment, eight of 20 dogs (40%) had ≤1 cultured colony type.

The most commonly isolated species among all 29 samples included *Staphylococcus pseudintermedius* (isolated in 12 dogs; 41.4%), *Staphylococcus epidermidis* (3; 10.3%), *Klebsiella pneumoniae* (3; 10.3%), *Bordetella bronchiseptica* (2; 6.9%), *Streptococcus equi* (2; 6.9%), *E. coli* (2; 6.9%), *Neisseria dumasiana* (2; 6.9%) and *S. aureus* (2; 6.9%).

The remaining occasionally isolated species were *Neisseria canis*, *Staphylococcus sciuri*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, *Morganella morganii*, *Kluyvera ascorbata*, *P. aeruginosa*, *Enterobacter cloacae/xiangfangensis*, *Mannheimia haemolytica*, *Paenibacillus* sp., *Rothia nasimurium*, *Proteus mirabilis* and *Bacillus licheniformis*. In one dog (dog 15), the identification of one colony type (around 50 colonies of a coccobacillus Gram– Oxydase+ Lactose– growing on the MacConkey agar) was inconclusive by MALDI-TOF mass spectrometry.

Discrimination using MALDI-TOF mass spectrometry between members of the *E. cloacae* complex to which *E. cloacae and Enterobacter xiangfangensis* belong is known to be weak (Godmer et al., 2021).

16S rRNA gene amplicon sequencing method

From 3,625,695 raw reads in the 29 nasal swabs, we obtained 3,386,574 reads after cleaning (length and sequence quality). Finally, we retained 290,000 reads [10,000 reads per sample, minimal read length is 450 nucleotides with 0 ambiguous nucleotide (Kozich et al., 2013)] to adjust for uneven sequencing depth across samples. Suspected contaminants were removed by filtering them from the OTU. At the end of the analysis, we

obtained 15,840 OTUs among which 975 phylotypes at species level and 410 phylotypes at genus level. The two negative control swabs yielded no results.

Between 13 and 225 distinct OTUs were detected in the nasal samples. In dogs without antimicrobial treatment (n=20) the mean ±standard deviation of unique OTUs was 69 ±57 and in dogs with recent or ongoing antimicrobial treatment (n=9) it was 81 ±53. The total bacterial flora, intrinsic diversity values, beta-diversity and beta-dispersion did not significantly differ between treated/recently treated and untreated dogs.

A total of 25 phyla were identified throughout all nasal samples among which Proteobacteria followed by Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria were the most frequently encountered. The most abundant families were *Enterobacteriaceae*, *Staphylococcaceae*, *Moraxellaceae*, *Porphyromonadaceae*, *Bacteroidaceae* and *Streptococcaceae* (Fig 1). Together they represent 57.4% of the total bacterial taxa. Among these families, the most abundant genera were *Staphylococcus* (mean relative abundance 16.5%), *Escherichia-Shigella* (12.8%), *Moraxella* (7.1%), *Porphyromonas* (6.2%), *Bacteroides* (5.2%) and *Streptococcus* (4.8%).

Comparison between culture- and molecular-based results

Taxa with a total relative abundance >50% (dominant), 10% to 50% (major) and <10% (minor) detected by sequencing were also found in culture in 46.7%, 20.0% and 0.4% of the cases respectively. Conversely, among the organisms that were cultured, 34.8% represented a major or dominant taxa by amplicon sequencing.

In nine samples (31%, samples 1, 2, 11, 13, 16, 18, 20, 24 and 28), a good agreement between the two methods was observed (Table 2). In most cases, this was associated with one overrepresented taxon (major or dominant taxon). Six samples in this group had a positive culture for different species of *Staphylococcus* (*S. aureus*, *S. pseudintermedius*, *S. sciuri* and *S. haemolyticus*) and amplicon sequencing detected the same species with a very high relative abundance (RA >80%) in four samples. Other samples were dominated by *S. equi* (90.3% RA) or *E. coli* (81.1% RA).

A moderate agreement between culture and sequencing results was present in five samples (17.2%, samples 9, 15, 21, 23 and 26, Table 2). In this group, samples showed a more heterogeneous profile without dominant taxa detected by sequencing. In three samples (9, 21 and 26), bacteria were cultured but not detected with sequencing. These were *S. epidermidis* (growth 1+), *N. canis* (1+) and *N. dumasiana* (2+).

A poor agreement between culture and sequencing results was assigned in eight samples (27.6%, Table 3). Heterogeneous profiles without dominant taxa were also observed. In all samples, *E. coli* was sequenced (minimum relative abundance: 10%, maximum relative abundance: 37.8%) but not cultured. In five cases, *E. coli* was associated with *Bacteroides fragilis* and/or *Lachnoclostridium* sp.

In four samples (4, 5, 7 and 29), species were isolated with culture but were undetected by sequencing. These species were *Klebsiella aerogenes* (growth 1+), *S. pseudintermedius* (growth 1+, isolated in three of the four samples), *P. mirabilis* (growth 1+) and *E. cloacae/xiangfangensis* (growth 3+). In three samples (8, 22, 29), cultured colony types were detected by sequencing but these only belonged to minor taxa, none of the most abundant species detected by sequencing were isolated by culture.

Among the moderate and poor agreement groups, taxa of the Pasteurellaceae family were detected by sequencing in five different samples (5, 18, 21, 26, 29), with a minimum and maximum relative abundance of 11.5% and 30.3%, respectively. In all five cases, however, taxa of this family were not cultured.

For seven samples, the growth requirements for the most abundant species detected by sequencing were not met (12, 14, 17, 19 and 27) or unknown (3 and 10, Table 4). In all cases, one dominant taxon was detected: *Moraxella* sp. (n=2), *Defluviitaleaceae* unclassified (n=1), *Porphyromonas cangingivalis* (n = 1), Gammaproteobacteria unclassified (n=1), *Actinomyces canis* (n=1), *Mycoplasma cynos* (n = 1). For five of the seven samples, all the cultured colony types, with a growth varying from 1+ to 3+, were not detected by sequencing.

DISCUSSION

The current study aimed to compare the results of bacterial identification from paired nasal swabs using agar-plate cultures and 16S rRNA gene amplicon sequencing. A moderate to good agreement and a poor agreement were observed in 48.3% and 27.6% of the samples, respectively. In 24% of the cases, the conditions required for the growth of the dominant species detected by sequencing were inadequate or unknown.

These discrepancies illustrate that standard culture does not reliably predict the bacterial profile detected by 16S rRNA gene amplicon sequencing.

Based on what has been described in the literature in healthy dogs (Ericsson et al., 2016; Isaiah et al., 2017;Tress et al., 2017; Vangrinsven et al., 2021), most of the dogs in this study had a nasal microbiota profile compatible with dysbiosis. Meaning that normally dominating taxa are underrepresented and substituted by normally low-abundance species. Although the type of profile associated with a bacterial infection has not yet been clearly defined, an unusual high abundance of pathogen-related taxa detected in sequencing analyses has been reported to be characteristic of bacterial infection (Singh et al., 2015).

In healthy dogs, the nasal microbiota has been described as being dominated by the phylum Proteobacteria representing between 50% and 80% of the total bacterial taxa in previous studies (Ericsson et al., 2016; Isaiah et al., 2017; Rodrigues Hoffmann et al., 2014; Tress et al., 2017; Vangrinsven et al., 2021) and largely dominated by the genus *Moraxella* (Tress et al., 2017; Vangrinsven et al., 2021). In the present study, Proteobacteria was also the dominating phylum, but consisting mainly of the genus *Escherichia-Shigella*. The other predominant genus was *Staphylococcus*. These two genera are among the most commonly isolated by culture in dogs with nasal disease (Meler et al., 2008). They are also encountered in healthy dogs

Table 2. Comparison of culture and predominant taxa for sequencing results in nasal cavities: samples with a moderate to good agreement

Dog	Strain	Culture			16S rDNA sequencing			
		MALDI-TOF mass spectrometry	Media	Growth	Closest 16S rRNA match (compared to culture results)	Relative abundance %	Most abundant species (>10% relative abundance) in sample	Relative abundance %
13		*Staphylococcus aureus*	MSA	4+	*Staphylococcus aureus*	87.7	*Staphylococcus aureus*	87.7
28	A	*Streptococcus equi*	EDW	4+	*Streptococcus equi*	90.3	*Streptococcus equi*	90.3
	B	*Klebsiella pneumoniae*		3+	*Klebsiella pneumoniae*	0.24		
16	A	*Escherichia coli*	MCC	4+	*Escherichia coli*	81.1	*Escherichia coli*	81.1
	B	*Morganella morganii*	MCC	2+	Undetected			
20	A	*Staphylococcus pseudintermedius*	MSA	4+	*Staphylococcus pseudintermedius*	16.6	*Kluyvera ascorbata*	61.8
	B	*Kluyvera ascorbata*	MCC	1+	*Kluyvera ascorbata*	61.8	*Staphylococcus pseudintermedius*	16.6
1	A	*Pseudomonas aeruginosa*	MCC	3+	*Pseudomonas aeruginosa*	2.6	*Staphylococcus pseudintermedius*	97.1
	B	*Staphylococcus pseudintermedius*	MSA	1+	*Staphylococcus pseudintermedius*	97.1		
11		*Staphylococcus pseudintermedius*	MSA	3+	*Staphylococcus pseudintermedius*	85.5	*Staphylococcus pseudintermedius*	85.5
24		*Staphylococcus pseudintermedius*	MSA	2+	*Staphylococcus pseudintermedius*	29.8	*Staphylococcus pseudintermedius*	29.8
							Corynebacterium sp.	15.1
2		*Staphylococcus pseudintermedius*	MSA	2+	*Staphylococcus pseudintermedius*	98.5	*Staphylococcus pseudintermedius*	98.5
18	A	*Staphylococcus sciuri*	MSA	2+	*Staphylococcus sciuri*	21.9	*Staphylococcus sciuri*	21.9
	B	*Klebsiella pneumoniae*	MCC	1+	*Klebsiella pneumoniae*	5.1	*Pasteurella canis*	11.5
	C	*Staphylococcus haemolyticus*	MSA	1+	*Staphylococcus haemolyticus*	11.0	*Staphylococcus haemolyticus*	11.0
							Acinetobacter soli	10.1
9	A	*Bordetella bronchiseptica*	MCC	4+	*Bordetella bronchiseptica*	20.9	*Bacilli* unclassified	32.3
	B	*Staphylococcus epidermidis*	MSA	1+	Undetected		*Bordetella bronchiseptica*	20.9
							Escherichia coli	13.6
21	A	*Streptococcus equi*	EDW	1+	*Streptococcus equi*	20.8	*Pasteurellaceae* unclassified	23.3
	B	*Neisseria canis*	COL	1+	Undetected		*Streptococcus equi*	20.8
							Escherichia coli	18.3
							Bacteroides fragilis	10.7
23		*Staphylococcus pseudintermedius*	MSA	2+	*Staphylococcus pseudintermedius*	14.3	*Escherichia coli*	20.6
							Staphylococcus pseudintermedius	14.3
							Moraxella sp.	12.9
							Bacteroides fragilis	11.9
							Lachnoclostridium sp.	10.3
26	A	*Neisseria dumasiana*	COL	2+	Undetected		*Pasteurella canis*	30.3
	B	*Escherichia coli*	MCC	1+	*Escherichia coli*	10.7	*Escherichia coli*	10.7
15	A	*Neisseria dumasiana*	COL	4+	*Neisseria* sp.	10.6	*Porphyromonas cangingivalis*	46.9
	B	Unidentified*	COL	3+			*Porphyromonas gulae*	11
							Neisseria sp.	10.6

COL Columbia blood agar, MCC MacConkey agar, MSA Mannitol salt agar, EDW Edward agar
*Unconclusive identification by MALDI-TOF mass spectrometry

Table 3. Comparison of culture and predominant taxa for sequencing results in nasal cavities: samples with a poor agreement

Dog	Strain	Culture			16S rDNA sequencing			
		MALDI-TOF mass spectrometry		Growth	Closest 16S rRNA match (compared to culture results)	Relative abundance %	Most abundant species (>10% relative abundance) in sample	Relative abundance %
8		*Staphylococcus pseudintermedius*	MSA	1+	*Staphylococcus pseudintermedius*	6.6	*Escherichia coli*	32.1
							Lachnoclostridium sp.	15.3
							Bacteroides fragilis	13.4
22		*Moraxella canis*	COL	1+	*Moraxella canis*	1.2	*Escherichia coli*	35.9
							Bacteroides fragilis	12.2
							Lachnoclostridium sp.	12.0
29	A	*Staphylococcus epidermidis*	MSA	4+	*Staphylococcus epidermidis*	0.05	*Pasteurella canis*	13.6
	B	*Enterobacter cloacae/ xiangfangensis**	MCC	3+	Undetected		*Escherichia coli*	13.5
7		*Staphylococcus pseudintermedius*	MSA	1+	Undetected		*Escherichia coli*	37.2
							Lachnoclostridium sp.	14.0
							Bacteroides fragilis	10.2
5		*Staphylococcus pseudintermedius*	MSA	1+	Undetected		*Escherichia coli*	25.4
							Pasteurellaceae unclassified	14.4
							Bacteroides fragilis	11.8
4	A	*Klebsiella aerogenes*	MCC	1+	Undetected		*Mycoplasma canis*	16.6
	B	*Staphylococcus pseudintermedius*	MSA	1+	Undetected		*Escherichia coli*	10.8
	C	*Proteus mirabilis*	MCC	1+	Undetected			
6		No growth					*Escherichia coli*	37.8
							Bacteroides fragilis	19.7
							Lachnoclostridium sp.	13
25		No growth					*Conchiformibius* sp.	31.6
							Escherichia coli	10

COL Columbia blood agar, MCC MacConkey agar, MSA Mannitol salt agar
*Weak discrimination between these two species with MALDI-TOF mass spectrometry

Table 4. Comparison of culture and predominant taxa for sequencing results in nasal cavities: samples for which growth conditions (culture) of the most abundant species (sequencing) were inadequate or unknown

Dog	Strain	Culture			16S rDNA sequencing			
		MALDI-TOF mass spectrometry		Growth	Closest 16S rRNA match (compared to culture results)	Relative abundance %	Most abundant species (>10% relative abundance) in sample	Relative abundance %
17	A	*Mannheimia haemolytica*	COL	3+	Undetected		*Moraxella* sp.	91.7
	B	*Staphylococcus epidermidis*	MSA	2+	Undetected			
3	A	*Paenibacillus* sp.	COL	2+	Undetected		*Defluviitaleaceae* unclassified	88.7
	B	*Staphylococcus pseudintermedius*	MSA	1+	Undetected			
	C	*Klebsiella pneumoniae*	MCC	1+	Undetected			
12		*Staphylococcus saprophyticus*	MSA	2+	Undetected		*Porphyromonas cangingivalis*	90.2
19		*Rothia nasimurium*		1+	Undetected		*Moraxella* sp.	78.0
10	A	*Staphylococcus aureus*	MSA	1+	Undetected		Gammaproteobacteria unclassified	97.9
	B	*Bacillus licheniformis*	COL	1+	Undetected			
27		No growth					*Actinomyces canis*	76.7
14	A	*Staphylococcus pseudintermedius*	MSA	2+	*Staphylococcus pseudintermedius*	0.7	*Mycoplasma cynos*	84.8
	B	*Bordetella bronchiseptica*	MCC	2+	*Bordetella bronchiseptica*	7.5		

COL Columbia blood agar, MCC MacConkey agar, MSA Mannitol salt agar

but are mostly around tenfold less abundant (Ericsson et al., 2016; Tress et al., 2017; Vangrinsven et al., 2021). These results are in agreement with previous studies demonstrating alterations of the nasal microbiota in dogs with nasal diseases (Tress et al., 2017; Vangrinsven, 2021).

The main difficulty with culture interpretation of the nasal cavities is the high degree of contamination by the rich and diverse normal commensal nasal flora. Moreover, the close contact between nasal passages and the external environment in association with the dog sniffing/nose-licking behaviour makes contamination by environmental and oral flora also possible. As a result, a positive culture cannot automatically be the synonym of nasal bacterial infection.

So far, bacteria isolated by nasal swab culture were considered to be healthy flora when there was only a sparse growth of bacteria that have been reported cultured from the nasal passages of healthy dogs (Lobetti, 2009; Plickert et al., 2014). Bacteria have been considered pathogenic if there was a pure and heavy growth and if they were bacteria not previously reported cultured from the nasal passages of healthy dogs (Lobetti, 2009). Based on the current results, however, these criteria do not seem to be applicable for all cases. A first obstacle is the absent or weak growth of some bacteria by culture, such as *A. canis*, *P. cangingivalis*, *M. cynos* and *Moraxella* sp. among others, while they were detected at a high relative abundance by sequencing. For these bacteria, growth conditions were not ideal or even inadequate. *Moraxella* sp. need CO_2 to allow and support growth, *Porphyromonas* sp. are strict anaerobic bacteria, *Actinomyces* sp. grow better in anaerobiosis and *Mycoplasma* sp. do not develop on agar medium. For the unclassified taxa, *Defluviitaleaceae* and Gammaproteobacteria unclassified, adequate growth conditions are difficult to predict. The second obstacle is the heavy growth (3+ or 4+) of bacterial species which were undetected or were sequenced with a low relative abundance such as *E. cloacae/xiangfangensis* (undetected), *K. pneumoniae* (RA 0.24%), *P. aeruginosa* (RA 2.6%), *S. epidermidis* (RA 0.05%) and *M. haemolytica* (undetected). One explanation could be related to the sampling method that we used, which consisted of two consecutive swabs. The material collected with swab 1 and used for culture could differ from the material collected with swab 2 which was used for sequencing. For undetected species, deeper sequencing of specimens may also have increased the sensitivity for molecular detection. Excessive growth of a weakly abundant species or selection for fast-growing bacteria over pathogens are other potential explanations for these discrepancies between the two methods. Assuming that pathogens are more likely to be a major or dominant species, the dilution of the sample before inoculation on the culture media could be an alternative to detect only major/dominating flora.

In the present study, poor culture specimen handling and misidentification are very unlikely since the specimens were handled immediately, by the same operator in the same laboratory and MALDI-TOF MS is a very accurate identification technique. In practical conditions, however, such obstacles render culture interpretation, as a sole method to detect nasal bacterial infection, challenging. It must also be remembered that identification of particular organisms with either approach does not imply that this organism is the causative agent of disease, which further complicates result interpretation.

More intriguing were the sequenced bacterial species for which growth requirements were met but still bacteria were not isolated by culture, which was repeatedly the case for *E. coli*. A possible explanation could be, again, the fact that two consecutive swabs were used. Other possibilities include competition for nutrients with other bacterial species, detected bacteria that are dead or inability of culture to replicate the nasal conditions. In all but one of these cases, there was no growth on the MacConkey agar excluding competition with other species for nutrients on the media where *E. coli* is

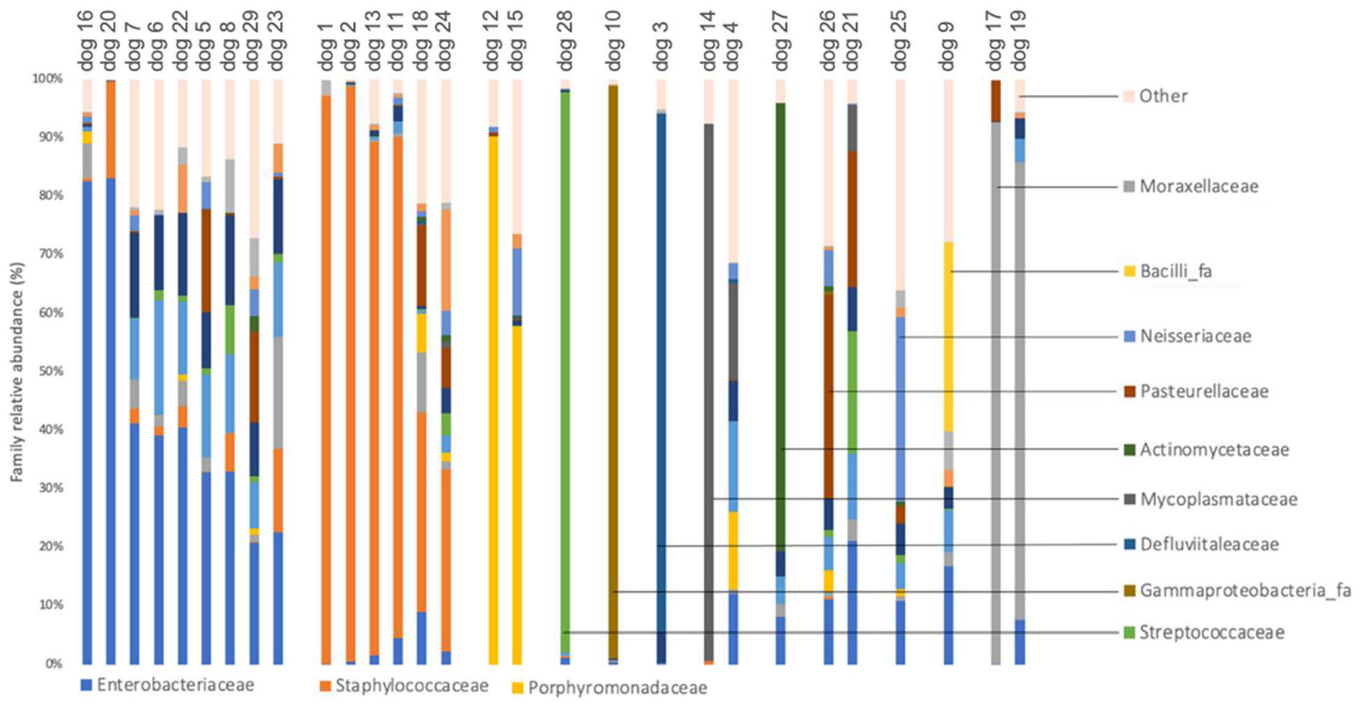

FIG 1. Relative abundance at family level. Samples were grouped based on major families

expected to grow. In five cases, the uncultured *E. coli* was associated with other bacteria typically found in the faecal flora such as *Bacteroides fragilis* and *Lachnoclostridium* sp. This could reflect a previous inspiration of faeces with bacteria that are dead and therefore do not grow. However, previous studies in healthy dogs (Isaiah et al., 2017; Rodriguez et al., 2019;Tress et al., 2017; Vangrinsven et al., 2021), with presumed similar sniffing behaviour, failed to show similar findings, which does not support this hypothesis.

In 5 cases, taxa of the *Pasteurellaceae* family were also detected by sequencing but not cultured. These taxa should grow on the Columbia agar +5% sheep blood medium in aerobic conditions. However, these were not dominant taxa and *Pasteurella* species grow best with CO_2 or in microaerophilic conditions, the absence of CO_2 during incubation could potentially explain their absence in the culture results.

Bacteria with higher abundance and growing easily on selected media were more likely to be identified by culture. If we assume that pathogens, once established, multiply and become dominant or major taxa, culture should be able to highlight them, at least when they grow on the selected media. Yet, the concept that low-abundance bacteria (minor taxa) may play a significant role in disease processes has also been put forward in human beings (de Cena et al., 2021). Current knowledge in terms of canine nasal microbiota is not yet advanced enough to know if such microbes play a role in canine nasal diseases, but if they did, culture would probably not be sensitive enough to detect them.

In two dogs, a high abundance of *Moraxella* sp. was observed resembling the type of profile that has been described in healthy dogs (Ericsson et al., 2016; Isaiah et al., 2017; Tress et al., 2017; Vangrinsven et al., 2021). Yet, clinical symptoms were similar to the other dogs of the study and secondary bacterial infection was suspected. In these two dogs culture results were positive for *M. haemolytica+S. epidermidis* and *R. nasimurium*, results that, taken separately, could have led to antimicrobial treatment while this treatment would have been considered unnecessary based on sequencing results. Moreover, as mentioned earlier, culture of *Moraxella* sp. was not attempted while it would be interesting to detect its presence. Indeed, when *Moraxella* sp. is the dominant species, bacterial infection and significant dysbiosis are unlikely since this is the profile most often found in the nose of healthy individuals (Tress et al., 2017; Vangrinsven et al., 2021).

Among the high-abundance species detected by sequencing, which could reflect bacterial infection, only *S. pseudintermedius* was detected more than once. When present, it was detected with culture, albeit not always with a significant growth. For most of the others (*M. cynos*, *P. cangingivalis* and *A. canis*), as standard growth conditions are not adequate, targeted PCR could be an alternative. But such a PCR panel would probably be quite broad, difficult to determine and not very practical for use. Alternatively, a PCR for *Moraxella* sp. with a low cycle threshold would likely reflect a healthy nasal flora without dysbiosis.

Like it can be observed in the sample of dogs that have been included in this study, systemic antimicrobials are regularly prescribed by veterinarians in dogs with nasal disease in which secondary bacterial infection is suspected. The usefulness of systemic antimicrobials in this context is however unclear and, in the authors opinion, the influence of systemic antimicrobials on the nasal microbiota is likely to be negligible. The total bacterial flora, intrinsic diversity values, beta-diversity and beta-dispersion did not significantly differ between dogs treated with antimicrobials, ongoing or recent, compared to untreated dogs, suggesting

a limited effect of such treatments on the nasal flora. This would be in accordance with previous studies suggesting that the ability of systemic antimicrobials to affect the nasal bacterial flora may be less than assumed (Hauser et al., 2016; Jain et al., 2018; Tress et al., 2017). The number of colony types was lower in treated dogs possibly reflecting the effect of antibiotic treatment on culture results. However, the present study was not designed to assess the effect of antimicrobial treatment on the nasal microbiota and nasal swab culture results so no final conclusions can be drawn based on these results.

As mentioned earlier, the use of two consecutive swabs may have influenced the results and is one of the limitations of this study. Alternatively, the material of one single swab could have been suspended in an adequate medium then aliquoted for culture on one hand and PCR on the other. Another limitation is the absence of specific growth conditions to improve culture of microbes with particular growth requirements. However, since the goal of this study was to reproduce routine clinical conditions, conventional culture media were used. Propidium monoazide could have been used to mask the DNA of dead bacteria before amplification and limit the bias of this DNA in the sequencing results. However, this method does not mask 100% of the dead bacteria with a variating effectiveness from one taxon to another, making interpretation of clinical samples challenging. A group of healthy dogs was not included in this study because previous studies describing the nasal microbiota in healthy dogs yielded comparable main results. One of these previous publications (Vangrinsven et al., 2021) was conducted by the same team using identical swabs, sampling technique, DNA extractions sets, primer sets, bioinformatic pipelines and database. However, data were not processed together in one unique table which could have led to some differences but likely minor ones.

In conclusion, the present results do not support the routine use of standard nasal swab for the diagnostic workup in dogs with nasal diseases. First it should be kept in mind that, whatever the method used, the true clinical implication of the identified bacteria(s) is difficult to establish. Then, various observations in the present study are interesting to point out because they reveal discrepancies between culture and sequencing results which further complicates the interpretation of nasal swab culture results. Identification by sequencing of dominant or major species potentially contributing to nasal disease and requiring specific growth conditions is not a rare event. Positive culture, even with a pure and heavy growth, does not invariably mean that this colony type is a dominant or even major taxa. When amplicon sequencing and culture results are interpreted together, a more comprehensive and interpretable information is available. Nasal swab culture can be considered under specific conditions such as in dogs with recurring purulent nasal discharge of unknown origin despite investigation of the underlying condition although interpretation of the results remains challenging. Clinical application of amplicon sequencing may be employed in these cases if culture is negative or if targeted antimicrobial therapy against the cultivable bacteria fails to produce any disease improvement.

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Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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