Manuscript Details

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Title	Occurrence of Aichi Virus in retail shellfish in Italy
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Abstract

AiV-1 is considered an emerging human enteric pathogens and foodborne transmission has been documented as an important source of exposure for humans, chiefly in relation to non-safe, risky food habits. We surveyed the presence of AiV-1 in retail shellfish, including oysters and mussles, identifying the virus in 3/170 (1.8%) of the analysed samples. The AiV-1 positive samples were of different geographic origin. Upon sequence analysis of a portion of the 3CD junction region, two AiV strains identified from harvesting areas in Northern Italy were characterised as genotype B and displayed 99-100% identity at the nucleotide level to other AiV-1 strains detected in sewages in Central Italy in 2012, suggesting that such strains are stably circulating in Italian ecosystems. Interestingly, a strain identified from mussles harvested in Southern Italy could not be characterised firmly, as inferred in the Bayesian analysis and by sequence comparison, indicating that different AiV strains are also circulating in Italy. Viral contamination in retail shellfish challenges the microbiological guidelines for food control and requires the development and optimization of additional diagnostic and prevention strategies.

Keywords	retail shellfish; molecular methods; Aichi virus

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UNIVERSITÀ DEGLI STUDI DI BARI ALDO MORO

dipartimento di medicina veterinaria

Valenzano, 7/9/2017

Food Microbiology

Dear Editor,

Please find attached the manuscript entitled "**Occurrence of Aichi Virus in retail shellfish in Italy"** by Valentina Terio*, Bottaro M., Di Pinto A., Fusco G., Barresi T., Tantillo G., Martella V., for publication as original research paper in *Food Microbiology*.

Awaiting a your reply, thanks very much for your attention.

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Comments for the Author

I thank the referee for the important remarks provided and can find below a point-by-point reply to the Editor's suggestions/criticisms.

R1: The manuscript describes a study on the presence of Aichivirus in retail shellfish in Italy. After analyzing 170 samples, the virus was detected in 3 (1.8%) samples, indicating a really low prevalence. However, this fact is ignored by the authors which insist in the importance of the detection, considering the increasing interest on emerging pathogens. But, can Aichivirus be considered as an emerging pathogen in Italy?

Reply 1.1:

There are several studies reporting the presence of AiV-like viruses, kobuviruses, in different animal species including goat, roe deer, pig, red fox, cats and dogs in Italy (Di Martino et al., 2013; Di Profio et al., 2013; Di Martino et al., 2014; Di Martino et al., 2015; DiBartolo et al., 2015). However, specific direct investigations for AiV in human patients have not been carried out, so it is not possible to assess the impact of AiVs on local population. Regardless, in some Italian regions, as in other European and extra-European countries, there are some dietary habits (i.e., consumption of row shellfish) that may influence the ecology of some foodborne pathogens. Filter-feeding animals (mussles, clams, oysters) are a good proxy to assess the presence of human enteric pathogens in local population. Our findings are supported by a 2013 study in Italy, demonstrating environmental contamination by those viruses. Di Martino et al. (2013) assessed the presence of AiV in untreated influent sewage samples collected at four wastewater treatment plants in central Italy. AiV was detected in 6 (12.5 %) of the 48 specimens and in all plants. Accordingly, we have indirect evidence that local population is exposed to infection by AiVs.

R1.2: On the other hand, the authors tried to link the positive detections with the original harvesting areas, omitting the possibility of contamination by handling during postharvesting operations. Such possibility should be mentioned and discussed in the manuscript.

Reply 1.2: The samples collected and included in the study are retail shellfish, so contamination by handling during post harvesting operations is very difficult. Shellfish in the depuration centre are placed in tanks containing controlled and purified water through ozone and UV. The most likely hypothesis, not only for us, is contamination of the harvesting areas.

R1.3: Conclusions are not fully supported by the results obtained. i.e. it is stated that the study confirms the lack of correlation between bacterial indicators and viral pathogens, but no estimations of E. coli numbers were made. I assume that this statement is based in the fact that are retail shellfish. However, the possibility of a bias in the food chain resulting in the non compliance of the regulations cannot be ruled out.

Reply 1.3: The main objective of the work was the research of AiV in bivalve molluscs. We analyzed retail shellfish, and we take for granted that the products were in strict compliance with bacteriological parameters lay down by EU Reg 2073/2005. As for the lack of correlation between bacterial indicators and viral pathogens, there are a lot of studies that have demonstrated the lack of this correlation including Di Pinto et al., 2004; Croci et al., 1999; Goyal et al., 1979; Koopmans and Duizer, 2004.

R1.4: Again, the potential role of Aichivirus in food-related gastroenteritis (as stated in the conclusions) is not firmly supported by the results obtained. Were the shellfish tested for other

viruses? If so, information on the existence of mixed contaminations would be of interest for the readers. Some information is repeated in different parts of the manuscript. Please, avoid such iterations

Reply 1.4: As suggested by the referee, we have changed the conclusions of the manuscript, in order to get rid of repetitions.
At p. 10, line 235-237 and 237-242, the sentences were either deleted or replaced.

We agree with the Referee's comments that our findings do not demonstrate firmly the role of AiV in human gastro-enteritis. We had already disclosed this in the discussion at p 8-9, line 199-202, stating that "caution must be taken when considering the public health implications, since only molecular methods were used in our study. It will be necessary in the future to define the correlation between the level of viral contamination detected by PCR in shellfish and virus residual infectivity."

We toned down throughout the manuscript claims on "the potential role of Aichivirus in food-related gastroenteritis".
At p 10, line 239-241, the sentence " Our finding suggest the potential role of Aichivirus in food-related gastroenteritis caused by contaminated bivalves, chiefly in countries or population (as in Southern Italy where consumption of raw or slightly cooked seefood is common" was replaced with "We were successful to identify AiV-1 RNA in shellfish at retail, i.e. in products at the end of the production chain and destined to direct human consumption without any further action/control by the health bodies".

The specific task of this work was to gather information on the presence of AiV in food destined to human consumption. We agree that screening for other enteric pathogens can provide useful information, but this was not in the scopes of the study.

R1.5: The distribution of genotypes in the tree is incorrect. Sequences AB034659 and AB092832 belong to genotype B.

Reply 1.5: The referee's observation is correct. We have corrected the brackets in the tree to display the correct information for the two sequences AB034659 and AB092832 of genotype B.

Highlights:

- Presence of Aichi virus in retail shellfish was evaluated
- Shellfish were analysed using validated methods
- Aichi virus were detected in 1.8% of the 170 samples

Occurrence of Aichi Virus in retail shellfish in Italy

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Abstract

AiV-1 is considered an emerging human enteric pathogens and foodborne transmission has been documented as an important source of exposure for humans, chiefly in relation to non-safe, risky food habits. We surveyed the presence of AiV-1 in retail shellfish, including oysters and mussles, identifying the virus in 3/170 (1.8%) of the analysed samples. The AiV-1 positive samples were of different geographic origin. Upon sequence analysis of a portion of the 3CD junction region, two AiV strains identified from harvesting areas in Northern Italy were characterised as genotype B and displayed 99-100% identity at the nucleotide level to other AiV-1 strains detected in sewages in Central Italy in 2012, suggesting that such strains are stably circulating in Italian ecosystems. Interestingly, a strain identified from mussles harvested in Southern Italy could not be characterised firmly, as inferred in the Bayesian analysis and by sequence comparison, indicating that different AiV strains are also circulating in Italy. Viral contamination in retail shellfish challenges the microbiological guidelines for food control and requires the development and optimization of additional diagnostic and prevention strategies.

Keywords: retail shellfish, molecular methods, Aichi virus

1. Introduction

Aichi virus, a human enteric virus of the genus *Kobuvirus*, family *Picornaviridae*, was first recognized in 1989 as the cause of oyster-associated non-bacterial gastroenteritis in humans in Aichi Prefecture, Japan (Yamashita et al., 1991).

The *Kobuvirus* genus includes three species, Aichivirus A (formerly Aichi virus) (Yamashita et al., 1998), Aichivirus B (formerly Bovine kobuvirus) (Yamashita et al., 2003) and Aichivirus C (porcine kobuvirus) (Reuter et al., 2009). The species Aichivirus A includes the prototype Aichi virus 1 (AiV-1) identified in humans, along with canine kobuvirus 1 (Kapoor et al., 2011; Li et al., 2011), feline kobuvirus 1 (Chung et al., 2013) and murine kobuvirus 1 (Phan et al., 2011).

AiV-1 is a small non-enveloped virus of approximately 27–30 nm in diameter with a single-stranded, positive polarity RNA genome of 8,280 nucleotides (nt) in length. The single large open reading frame encodes a polyprotein of 2,432 amino acids that is cleaved into the structural proteins VP0, VP3 and VP1 and non-structural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D (Sasaki et al., 2001; Yamashita et al., 1998).

Upon sequencing of a short genome fragment of the 3C and 3D (3CD) junction region, AiV-1 has been further classified into at least 2 main phylogenetic lineages or genotypes, indicated with the letters A and B (Di Martino et al., 2013; Le Guyader et al., 2008; Yamashita et al., 2003; Yamashita et al., 2000) and this is exploited for epidemiological investigations and molecular tracking of sporadic cases and outbreaks of gastro-enteritis. An AiV-1 strain identified in France has been proposed as a distinct lineage/genotype, C (Ambert-Balay et al., 2008).

AiV-1 has been suspected to play a role as human gastroenteric pathogen. AiV-1-related clinical signs and symptoms include diarrhea, abdominal pain, nausea, vomiting and fever (Yamashita et al., 1991).

Virological surveys suggest that AiV-1 is responsible for sporadic cases of gastroenteritis (0.5%-1.8%) (Kitajima et al., 2015). However, serological investigations have revealed high antibody prevalence in humans of different age groups, suggesting that AiV-1 infections are quite common

(Khamrin et al., 2014; Ribes et al., 2010). Serological studies in Spain, Germany, and Tunisia have revealed that 70, 76, and 92 % of the population across all age groups has antibodies specific for AiV-1 (Oh et al., 2006; Ribes et al., 2010; Sdiri-Loulizi et al., 2010).

AiV-1 has been detected in Asia, Africa, South America and Europe in various types of environmental samples, such as sewage, river water, groundwater, and shellfish, indicating a worldwide distribution and suggesting a complex ecology, as observed for other enteric viruses (Atmar et al., 1995; La Rosa et al., 2017). Accordingly, AiV-1 has been proposed as an emerging viral pathogen associated with environmental contamination and water and foodborne infections (Kitajima et al., 2015). AiV-1 transmission occurs through direct contact, by faecal-oral routes, or through consumption of contaminated food or water. Importantly, AiV-1 has been associated with human gastroenteritis outbreaks related to consumption of oysters or other shellfish (Hansman et al., 2008; Le Guyader et al., 2008; Sdiri-Loulizi et al., 2010).

Filter-feeding shellfish are an important source for transmission of enteric viral diseases, since they are able to accumulate and concentrate waterborne pathogens, especially when they are grown in coastal areas contaminated by sewage (Le Guyader et al., 2000; Terio et al., 2010).

In the European Countries, the microbiological quality of commercially harvested shellfish intended for human consumption must comply with the EU Food Hygiene Regulations (EC 2073/2005 and subsequent amendments), which rely exclusively on bacterial indicators (*Escherichia coli* and *Salmonella* spp). The microbiological requirements do not include human viral pathogens and therefore fulfilment of the parameters established by the regulations does not rule out the presence of viral pathogens in retail shellfish (Terio et al., 2010). Moreover, bacterial indicators are not correlated with the presence of enteric viruses (Croci et al., 2000; Goyal et al., 1979; Koopmans and Duizer, 2004).

Although the faecal indicator system has been in place for many years, it has been understood that this system does not adequately index for the presence of viral agents transmitted by shellfish, such

as norovirus and hepatitis A virus (Formiga-Cruz et al., 2003) or of other food-borne viruses such as Aichi virus.

Information on contamination of shellfish by AiV-1 is limited to a few countries (Rivadulla et al., 2017; Sdiri-Loulizi et al., 2010), whilst it is scanty or absent for several geographical areas, including a number of European countries. This information may be relevant, chiefly in regions where consumption of raw shellfish is more common, thus enabling additional epidemiological cycles for enteric viruses and favouring the spread of foodborne pathogens (La Bella et al., 2017). In this study, we assessed the presence of AiV-1 in retail shellfish in Apulia region, Italy, between April 2016 and April 2017.

2. Methodology

2.1 Sampling and processing of shellfish samples

A total of 112 mussels (*Mytilus galloprovincialis*), 36 oysters *(Ostrea edulis*) and 22 clams (*Venus gallina*) batches were collected from open-air markets, hypermarket and fish chops in the Apulia region (SE Italy) from April 2016 to April 2017. All the samples were harvested in class A marine areas. After collection, batches (each composed of 10 individual mollusks) of digestive glands were processed according to ISO/TS 15216-2:2013 method.

RNA extraction was carried out with Nuclisens® Magnetic Extraction Kit – NucliSENS® easyMAG system (BioMérieux, Marcy I'Etoile, France) following manufacturer's instructions after adding an extraction control, following the guidelines of ISO/TS 15216-2:2013 method.

2.2 Detection of AiV-1 by Reverse transcriptase-polymerase chain reaction (RT-PCR)

AiV-1 was detected by RT - PCR using SuperScript® OneStep RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, Ltd, Paisley, UK) and the primer set Ai6261 (5' ACACTCCCACCTCCCGCCAGTA 3') and Ai6779 (5' GGAAGAGCTGGGTGTCAAGA 3'),

targeting a 519-bp fragment at the 3CD junction region (viral protease and RNA-dependant RNA polymerase) (Pham et al., 2007; Yamashita et al., 2000).

The thermal profile was comprised of 50 °C for 60 min and 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 1 min, with a final extension at 68 °C for 10 min.

A nested PCR was performed with the primer pair C94b-246k (C94b, 5' GACTTCCCCGGAGTCGTCGTCT 3'; 246k, 5' GACATCCGGTTGACGTTGAC 3') to amplify a 223-bp fragment within the 3CD junction region (Pham et al., 2007; Yamashita et al., 2000) using the HotStarTaq Master mix kit (Qiagen, Hilden, Germany). The thermal profile consisted of 95 °C for 15 min and 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

2.5 Sequencing and phylogenetic analysis

Nested PCR products (223-bp fragment) were separated by electrophoresis in a 1.5 % agarose gel and appropriately sized bands were excised and purified on column (Qiaquick Gel extraction Kit, Qiagen, Gmbh, Germany). Cycle sequencing was carried out using BigDye Terminator Cycle chemistry (Applied Biosystems, Foster City, California, US). Raw sequences were edited using the Geneious software version 10.0.5 (Biomatters Ltd, New Zealand).

The sequences were analysed using free access sequence databases by BLAST (http://www.ncbi.nlm.nih.gov) and therefore compared to a selection of sequences representative of recent epidemic strains with reference strains circulating worldwide.

The Enterovirus Genotyping Tool version 0.1 (http://www.rivm.nl/mpf/typingtool/enterovirus) (Kroneman et al., 2011) was also used for correct classification of the AiV-1 sequence.

The phylogenetic analysis were performed by using Geneious software package (Geneious version 10.0.5 created by Biomatters).

3. Results and Discussion

In this study, 170 shellfish samples collected in Italy over a 12-month period were screened for AiV-1. All the samples tested negative in the first-round RT-PCR with primer pair Ai6261 - Ai6779. However, in the second-round PCR with primers C94b-246k, AiV RNA was detected in 3/170 (1.8%) bivalve molluscan samples. The samples of *Mytilus galloprovincialis* species were purchased from fish markets located in the North of Apulia (Foggia) in April 2016 and April 2017. However, the harvesting areas, all which were of class A, were located in Ravenna (Northern Italy, Adriatic sea) and Taranto (Southern Italy, Ionian sea), i.e. in two completely different ecosystems.

In a 2014-2015 Italian study, AiV-1 RNA was detected in 13/108 (12.04%) mussels obtained from both class A and class B harvesting areas in Campania region, Tirrenian sea (Fusco et al., 2017). Also, analysis of untreated influent sewage samples collected from four wastewater treatment plants in central Italy identified AiV in 6 (12.5 %) out of 48 samples and 4 out of 4 plants (Di Martino et al. 2013). Overall, these scattered pieces of information suggest that AiV is present in different Italian ecosystems.

Interestingly, in our study there was no difference in the prevalence of various enteric viruses between class-A and class-B harvesting areas, suggesting that virus contamination is not strictly related to bacteriological contamination, as also observed elsewhere (La Bella et al., 2016; Terio et al., 2010; Loisy et al., 2005; Romalde et al., 2002).

Whether the observed differences also reflect a temporal/geographical variation or a different sensitivity of the diagnostic instruments used in the various studies remains to be assessed. Also, the fact that we only tested products at retail, and therefore fulfilling the severe production criteria, could have somewhat biased the results.

The presence of AiV-1 in mussels from class-A harvesting area is of particular importance since shellfish from these areas may be destined to direct human consumption, resulting in a potential public health risk. However, caution must be taken when considering the public health implications, since only molecular methods were used in our study. It will be necessary in the future to define the

correlation between the level of viral contamination detected by PCR in shellfish and virus residual infectivity.

Despite several efforts, viral contamination in shellfish remains a serious problem and recent papers have demonstrated contamination of different bivalve molluscs worldwide (Benabbes et al., 2013; Terio et al., 2010; Woods et al., 2016). According to an EFSA report (2015), in 2014 viruses were, for the first time, the most commonly detected (20.4%) causative agent of foodborne outbreaks. Although norovirus and hepatitis A virus are regarded as the most common causes of foodborne infections, in recent years other viruses with zoonotic potential, including AiV-1, have been identified in shellfish.

Based on the existing literature, geographical patterns can be osberved in the distribution of AIV-1 genotypes. Genetic analysis of the AiV-1 identified in gastroenteritis outbreaks in several European countries has revealed that genotype A is the most common genotype circulating in Europe. Genotype A is predominant in Germany (Oh et al., 2006), France (Ambert-Balay et al., 2008), Sweden (Jonsson et al., 2012) and Finland (Kaikkonen et al., 2010). Genotype A was also predominant in Japan (Pham et al., 2007; Yamashita et al., 2000). Genotype B seems predominant in Pakistan (Yamashita et al., 2000), Bangladesh (Pham et al., 2007), Malaysia (Yamashita et al., 2000) and Brazil (Oh et al., 2006). Analysis of sewages and waste water in Itay 2012 identified only AiV-1 strains of genotype B (Di Martino et al., 2013). Two of the sequences (samples #7 and #15) determined in this study were characterised as genotype B. Upon sequence comparison with cognate sequences available in the databases, they displayed the highest nt identity (99-100%) to the Italian strains detected in sewages in 2012 (Di Martino et al., 2013), suggesting that such genotype B AiV-1 strains are circulating in Italian environments. One of the three sequences, sample #29, could not be characterized firmly in the Bayesian analsysis, as it was not rooted strictly with genotype A and B AiV-1 strains. In our analysis, other AiV-1 strains selected from the databases also acted as genetic outlier between genotype A and B (*Figure 1*). By interrogation of the sequence databases using web-based tools BLAST and FASTA, the strain #7 displayed the

highest nt identity (97%) to AiV-1 strains detected in Japan (accession AB092832). Whether the sub-classification scheme into genotypes A to C developed in the literature is not adequate to summarize the genetic heterogeneity of AiV-1 strains can not be ruled out and should be assessed by full-genome sequencing of the viruses.

Interestingly, strains #7 and #15 were identified in mussles harvested in Northern Italy, whilst strain #29 was from mussles harvested in Southern Italy, i.e. in two different ecosystems.

4. Conclusion

The observed increase in food-borne diseases related to the consumption of raw or lightly cooked mussels, requires continuous monitoring of common, emerging and neglected enteric viral agents, including AiV-1, in order to assess more precisely the risks for human health. In polluted environments, shellfish can play an important role as *reservoirs* and/or vehicles of enteric viruses. We were successful to identify AiV-1 RNA in shellfish at retail, i.e. in products at the end of the production chain and destined to direct human consumption without any further action/control by the health bodies. Monitoring of viral contamination in shellfish can be useful to gather, indirectly, information on the circulation of human enteric pathogens in local population. This will also be important to improve safety of food products and to plan more effective campaigns in consumers.

5. Acknowledgments

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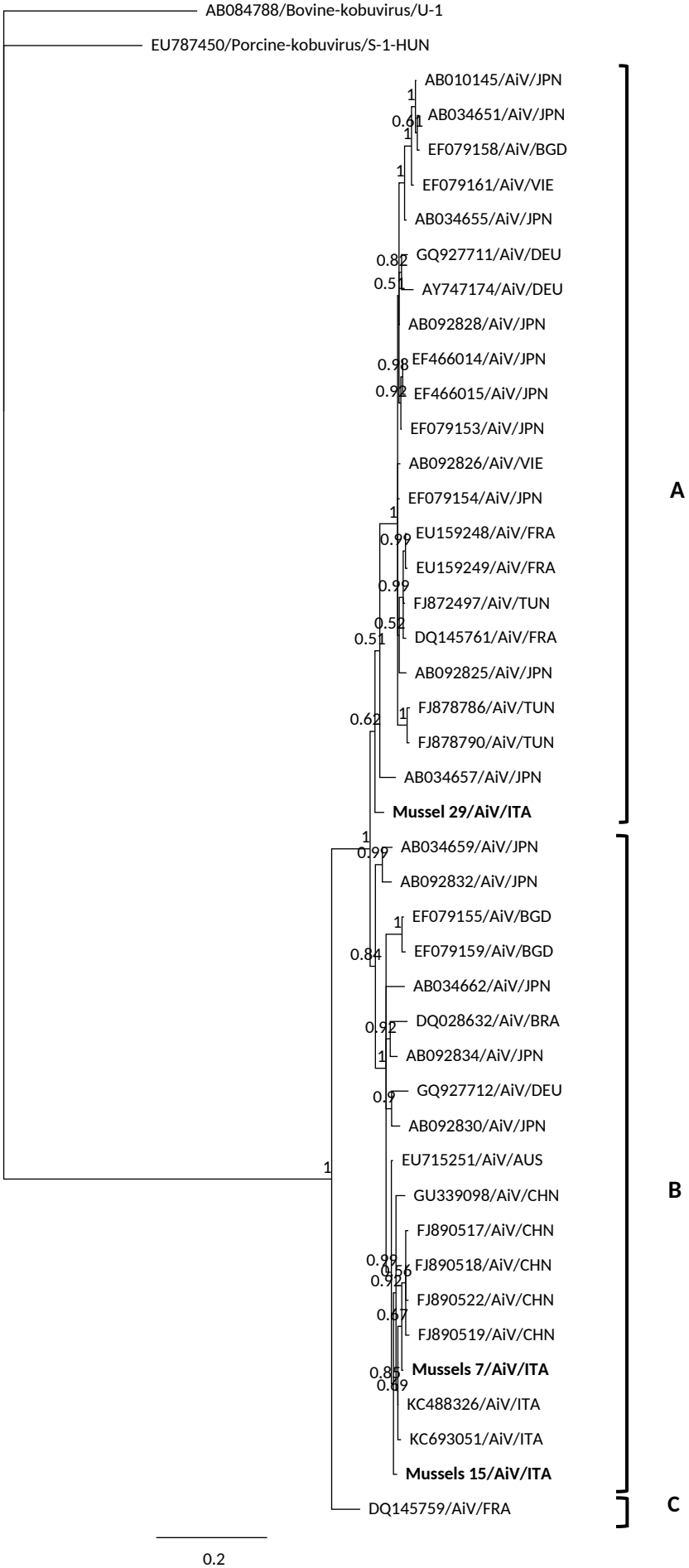
Figure 1: Phylogenetic tree of AiV-1
Bayesian phylogenetic analysis of AiVs based on the 519-nt 3CD long fragment of 3CD. The viruses detected in this study are in bold and in a box. Tree was generated using the Bayesian inference with Generalized Time-Reversible (GTR) model and gamma rate variation and supplying statistical support with subsampling over 1000 replicates. Numbers on the tree branches indicate the posterior probability values. Values lower than 0,8 are not shown. The scale bar indicates the number of substitutions per site. Genotypes are indicated with letters A to C.


AB084788/Bovine-kobuvirus/U-1
EU787450/Porcine-kobuvirus/S-1-HUN
AB010145/AiV/JPN
AB034651/AiV/JPN
EF079158/AiV/BGD
EF079161/AiV/VIE
AB034655/AiV/JPN
GQ927711/AiV/DEU
AY747174/AiV/DEU
AB092828/AiV/JPN
EF466014/AiV/JPN
EF466015/AiV/JPN
EF079153/AiV/JPN
AB092826/AiV/VIE
EF079154/AiV/JPN
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Mussel 29/AiV/ITA
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Mussels 15/AiV/ITA
DQ145759/AiV/FRA
A
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C
0.2