Appraisal of Extraction Protocols for Metagenomic DNA from Fish Gut Microbiota

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***Abstract*-The gut microbiota composition in fishes is highly diverse and complex. Complete cataloguing of this microbial community demands the use of metagenomic approaches. Obtaining DNA in sufficient quantity and purity is crucial in metagenomic analysis. The present study compares the existing DNA extraction protocols to ascertain the appropriate method for fish gut metagenomic analyses. Gut contents removed aseptically from intact *Sardinella longiceps* were used for DNA isolation. DNA isolation methods for fish flesh, saline soil, mouse gut and fish bacteria, coded as A, B, C and D were compared to the protocols for Gram positive and Gram negative bacteria of Qiagen DNeasy Blood & Tissue Kit and the protocol of QIAamp DNA Stool Minikit, coded as E, F and G, respectively. The concentration and purity of the DNA obtained was analyzed in terms of OD 260/280 and 260/230 ratios and the processing times were also compared. The 16S rDNA was amplified, to consider its suitability for further analysis. All the seven protocols yielded DNA, with different yields. Method A and B gave the maximum yield. In terms of purity, commercial kit based methods and method C were ideal. All the methods, except methods C and F gave PCR amplicons. Processing time was found to be the least for method G. The different methods varied widely in performance with respect to yield and purity, the final choice of method depends on the requirement of the researcher, which may be a high yield or purity or short processing time of the sample.**

Keywords:- Metagenomics; fish gut microflora; DNA extraction; PCR

I. INTRODUCTION

Marine environments cover about 71% of the earth's surface and contain approximately $10^6 – 10^9$ microbial cells per milliliter (1). However, only 0.001-0.1% of microbes in sea water is currently cultivable (2). Culture dependent analyses of environmental samples overlook a large majority of bacterial natural products that exist in nature (3). Hence, this approach is inadequate to tap the enormous potential of as-yet uncultured microbes.

According to FishBase (2012), 32,700 fishes have been described to date. This comprises nearly half of the total vertebrate diversity (4). Healthy fishes harbor bacterial populations on or in their skin (5,6), gills (7), digestive tract (8,9,10,11,12), internal organs such as liver, kidney, spleen and light emitting organs (13,14,15). The fish gut is a complex ecosystem populated by highly diverse group of microbes. The gut microbiota composition in fishes is influenced by various factors such as habitat salinity, trophic level, taxonomy and feeding habits (16). However, the gut microbiota composition in fishes is not a mere indication of the microorganisms in their natural setting but may also result from host selective pressures within the gut (17). Much of the investigations on fish gut flora were based on culture-dependent techniques (18). Like the other environmental samples, the total microbial community of GI tract cannot be characterized completely using these culture based techniques (19, 20, 21). In this backdrop, metagenomics approach has been emphasized to characterize the microbial biodiversity of fish gut flora for its application in ecological studies and bioprospecting.

Isolation of DNA is the first step in any metagenomic analysis. However, obtaining unbiased metagenomic DNA from an environmental sample is a daunting task (22). Comparison of DNA isolation protocols for environmental samples such as soil has been reported extensively (23, 24, 25, 26). Also, a number of DNA isolation protocols have been applied and compared on fecal samples to characterize the intestinal microbiota composition in humans and other animals (27, 28, 29, 30, 31). Irrespective of the existence of different metagenomic DNA isolation protocols for environmental samples with varying characteristics, no study has compared different metagenomic DNA extraction protocols for application to fish gut directly.

Obtaining high DNA yields of sufficient purity and integrity is pivotal to downstream applications of the extracted DNA, such as cloning or PCR. As the gut composition of fishes are highly heterogeneous, it is mandatory to perform an optimization of DNA extraction protocols for each sample analyzed. The present study employs intact gut samples of the Indian oil sardine to compare different community DNA extraction protocols so as to eliminate any bias or difficulties encountered during the

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use of fecal samples. Here, metagenomic DNA extraction protocols for fish, soil and mouse gut are compared to a culture based DNA isolation protocol of fish microbes and two other kit based methods.

II. MATERIALS & METHODS

A. Sample.

Fresh intact whole fish (*Sardinella longiceps*) were purchased from commercial vendors. The dissection of gut was performed by inserting a fine scalpel blade into the anus of the fish. The incision was extended anteriorly and the gut contents were removed under aseptic conditions.

B. DNA extraction.

200 mg tissue was weighed and used for DNA isolation. All the chemicals used in the present study were purchased from Sigma-Aldrich (USA) and Merck (Merck India Ltd, India). Seven different methods coded as method A to G were used, as detailed below. Methods A to D were followed with some modifications from the reported protocols.

C. Method A.

This method was originally employed for bacterial DNA isolation from fish flesh (32). Briefly, the gut tissue was homogenized in 1 mL lysis buffer (10 mM Tris-HCl, 50 mM Na_2EDTA, 100 mM NaCl, pH 8) containing 1% SDS , 1% Sarkosyl and 0.4 mg/mL of proteinase K. The mixture was incubated for 3 h at 45°C under agitation. The lysate was centrifuged at 10,000 X g, 10 min at 4°C and the supernatant was stored on ice. The pellet was kept at -80°C for 5 min and thawed for 30 s in a 400W microwave oven three times successively. The supernatant was collected and added to the supernatant stored on ice. The aqueous phase was extracted with equal volume of phenol: chloroform: isoamyl alcohol (P:C:I =25:24:1) by centrifugation. The aqueous layer was then extracted with chloroform: isoamyl alcohol (24:1). To the supernatant, equal volume of ice cold isopropanol was added and incubated overnight at -20°C.

D. Method B.

This is a method used for the isolation of metagenomic DNA from saline soil (24). Briefly, the gut tissue was suspended in 1 mL of extraction buffer (100 mM Tris–HCl (pH 8.2); 100 mM EDTA (pH 8); 1.5 M NaCl) and incubated for 24 h with shaking at150 rev/min. The supernatant was collected by low speed centrifugation. To 500 µL supernatant, equal volume of lysis buffer (5% SDS, lysozyme, 5 mg/mL, sarkosyl 1%, CTAB 1% and proteinase K, 5 mg/mL) was added and incubated at 65°C for 2 h with shaking every 15 min. The lysate was centrifuged at 12,000 rpm, 10 min at 4°C and the aqueous phase was extracted with P:C:I (25:24:1) and chloroform: isoamyl alcohol (24:1), as detailed in method A. To the supernatant, $1/10^{th}$ volume of 7.5M potassium acetate and equal volume of ice cold isopropanol was added and incubated overnight at -20°C.

E. Method C.

This is a method used for metagenomic DNA isolation from mouse gut, to study the mouse gastrointestinal microflora (33). Briefly, the gut tissue was homogenized in 1 mL TE buffer. To 500 µL homogenate, lysozyme (5 mg/mL) was added and incubated at 37°C for 1 h. Proteinase K (2 mg/mL) was added and incubated at 56°C for 1 h. Then, SDS (1% w/v) was added to the lysate and was incubated at 37°C for 30 min. Equal volume of P:C:I (25:24:1) was added and the aqueous phase was collected. It was extracted with chloroform: isoamyl alcohol (24:1) and to the supernatant, 0.1 volume of 3M sodium acetate and equal volume of chilled isopropanol was added and incubated overnight at -20°C.

F. Method D.

This is a method used for DNA isolation from culturable fish bacteria (34). The gut tissue was homogenized in Tris: Ethylenediaminetetracetate (EDTA), (TE) 50:50, pH 8 and to 500 µL homogenate, 20 µL of 10% SDS was added and was incubated for 30 min at 56°C. 250 µL of 7.5 M potassium acetate was added to the lysate and was incubated on ice for 15 min. The lysate was centrifuged at 12,000 rpm, 10 min at 4°C and the aqueous phase was extracted with P:C:I (25:24:1) and chloroform: isoamyl alcohol (24:1), as detailed in method A. To the supernatant, 350 µL isopropanol was added and incubated overnight at -20°C.

In all the above methods, overnight incubation was followed by a centrifugation step to collect DNA. The DNA pellet was washed twice with 70% cold ethanol, air dried and resuspended in 20 µL of 1X TE buffer.

G. Methods E and F.

These methods of DNA extraction employed Qiagen DNeasy Blood & Tissue Kit (Qiagen India Pvt. Ltd, India). Separate protocols, as specified by the manufacturers for Gram positive and Gram negative bacteria were followed and were coded as methods E and F.

H. Method G.

QIAamp DNA Stool Minikit (Qiagen India Pvt. Ltd, India) was used following manufacturer's instructions for DNA isolation from stool.

DNA extracts were analyzed on a 0.8% agarose gel run in 1X TAE buffer at 60 V. Gels were stained with 0.5 µg/mL ethidium bromide and the image was captured using gel documentation system (Syngene, USA).

Quantification of the extracted DNA was done using Biospec-nano spectrophotometer (Shimadzu, Japan). The

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purity of the extracted DNA was determined in terms of 260/280 and 260/230 ratios. All the experiments were performed in triplicates.

I. PCR assay.

The 16S rDNA sequences of the community DNA were amplified using universal primers 5'AGAGTTTGATCCTGGCTCAG 3' and 5' ACGGCTACCTTGTTACGACTT 3' (35) to assess its suitability for PCR. The reaction mixture contained 10X PCR buffer, 200 µM dNTP mix, 0.02 mM each of forward and reverse primers, 2mM $MgCl_2$ and 0.1U of *Taq* polymerase in a volume of 20 µL. The thermal regime consisted of an initial denaturation step of 5 min at 94^0C, 34 cycles of 0.5 min at 94^0C, 0.5 min at 56^0C, 1 min at 72^0C, and a final extension of 10 min at 72^0C. PCR was performed in a thermal cycler (Bio Rad, USA). The amplicons were electrophoresed on 1.2% agarose gel and visualized using gel documentation system (Syngene, USA).

J. Statistical analysis.

Statistical analysis was done using InStat (Ver.2.04a). One way ANOVA with Tukey post-hoc test was used to compare the means and p values <0.05 were considered significant. The graphs were plotted with the help of Sigma plot for Windows Version 12.5 (Systat Software inc., Germany).

III. RESULTS

Seven different DNA extraction methods were compared in the present study. DNA isolation protocols for fish, soil and mouse gut were compared to a culture based DNA isolation protocol of fish microbes and two other kit based methods. All the methods yielded DNA (Figure 1).

The results from ANOVA indicated significant difference between the yields of all the methods, except between Method D and F ($p>0.05$), and method E and G ($p>0.05$). As shown in figure 2, method A, outlined by Giamozzi et al. (2005) gave the highest yield (392.15±1.14 ng/mg) followed by method B (304.66±37.21 ng/mg). The yields obtained using other methods were three to seven folds lower than these methods.

The purity of the isolated DNA was checked in terms of absorbance ratios at 260/280 nm, indicative of protein contamination (36) and 260/230 nm, indicative of organic contaminants (37) (Figure 3). Method E gave the preferred 260/280 ratio of ~1.8, followed by method C (1.69±0.03). Pure DNA, with least organic contamination was obtained using methods F (2.26±0.03) and G (2.21±0.03).

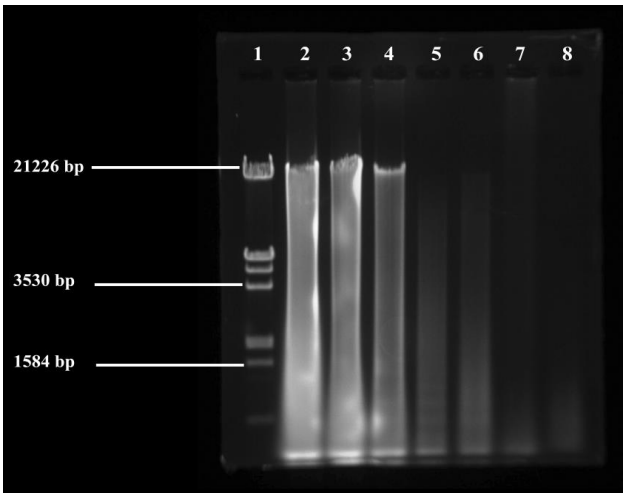

Fig 1: Agarose gel electrophoresis of metagenomic DNA isolated by various protocols
Lane 1: λ/Eco RI + Hind III double digest ladder
Lanes 2 to 8: DNA isolated by methods A, B, D, E, F, C and G respectively

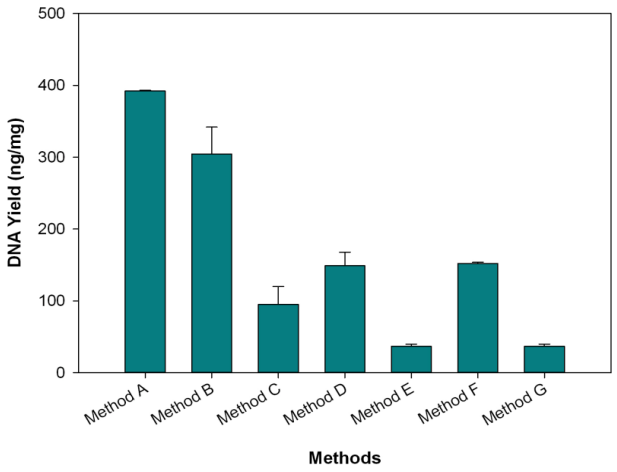

Fig 2: Yield (ng/mg) of DNA from metagenomic DNA extraction protocols
Values are mean ± SD of 3 independent experiments

The other strategies used to compare the DNA extraction protocols were PCR amplification and processing time. The DNA was subjected to PCR amplification of 16S rDNA to check the suitability of DNA for further analyses (Figure 4). All DNA, except that obtained from methods A and C gave PCR amplicons. The present study also considered processing time of the samples to consider its feasibility for the analysis of large sample numbers. Method G was found to take the least processing time (1h). This was followed by methods E (1.4 h), and F (3.4 h). The phenol-chloroform based methods B (39 h), A (16 h), C (15 h) and D (13.5 h) took longer and had higher processing times.

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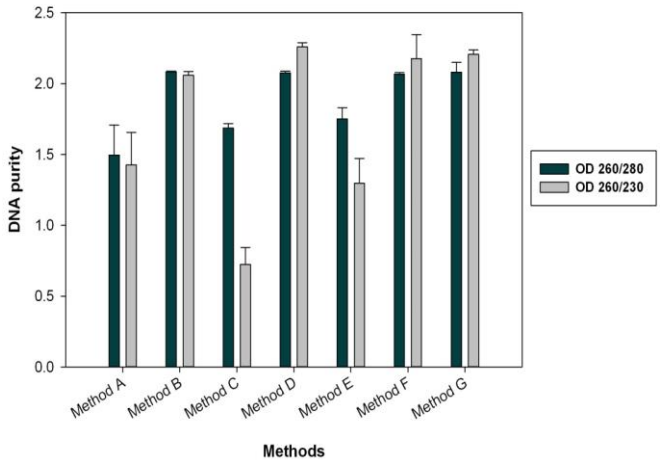

Fig 3: Purity of DNA extracted by various protocols
Values are mean ± SD of 3 independent experiments

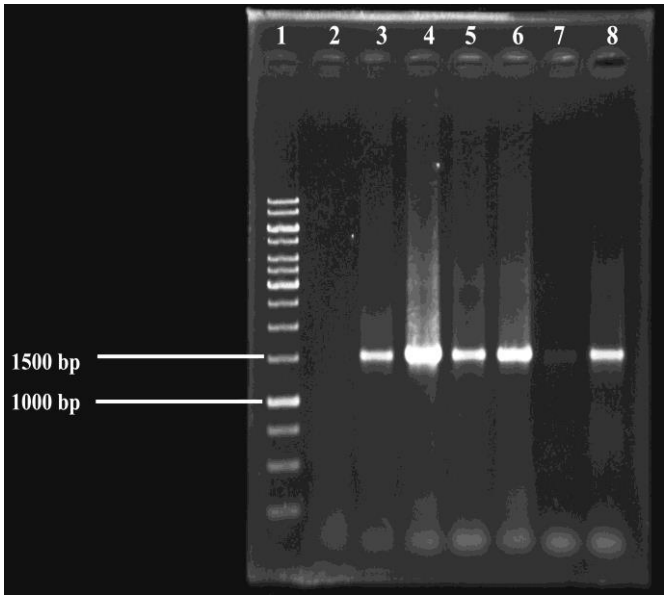

Fig 4: Agarose gel electrophoresis after 16S rDNA amplification
Lane 1: GeneRuler 1 kb DNA ladder
Lanes 2 to 8: 16S PCR products of DNA isolated by methods A,B, D, E, F, C and G respectively

IV. DISCUSSION

Metagenomics is a rapidly progressing discipline that provides valuable insight into the vast metabolic potential lurking in the unculturable majority of microbes. The enormous biodiversity locked within the marine environment has been investigated extensively using metagenomics approaches, however, fish gut microbiome, representing a 'world within world' is less studied and is certain to give payoffs. Metagenomic analysis mandates sufficient amounts of good quality and truly representative DNA, to proceed with downstream applications such as PCR or library construction and sequencing. Regardless of the existence of various DNA extraction protocols, this step continues to be a major bottleneck when it comes to a different sample and optimization becomes mandatory. The present study, therefore aims to compare various DNA extraction protocols for metagenomic analysis of fish gut microflora.

All the DNA extraction protocols used in the present study yielded DNA. ANOVA results indicated a significant statistical difference between the yields of all the methods compared. The difference in the modes of cell lyses and purification steps explains the difference in yield. Methods A and B employed harsh lyses procedures and gave very high yields, when compared to the other methods. Method A has an additional freeze thaw step for complete cell lysis, in addition to the various detergents used for disrupting the cell membrane. The high yield obtained using method B is on account of the use of high concentrations of detergents like CTAB and SDS, and enzymes, proteinase K and lysozyme. The yields by the kit based methods E, F and G were very low, when compared to the conventional phenol-chloroform extraction protocols. This study thereby supports the earlier reports that silica based purification methods gives many fold lower yields than phenol-chloroform extraction–procedures (27, 38, 39, 40).

Though DNA yield is a major consideration in metagenomic analyses, the success of further analyses, such as PCR on the extracted DNA depends on the quality of DNA. Carryover of phenolic compounds from the sample (41) or from the extraction procedure inhibits PCR reaction by binding to or denaturing the DNA polymerase (42). Improper inactivation of proteolytic enzymes or denaturants used in cell lyses also leads to the inactivation of polymerases (43). Hence, the quality of the DNA was checked in terms of 260/280 and 260/230 ratios. All the methods, except A, C and D were found to give satisfactory ratios. Protein contamination was a major issue only with method A. This is because it employed very low concentrations of proteinase K (0.4 mg/mL). Method C also gave DNA with protein contamination. This may be ascribed to the fact that it does not use proteinase K for protein removal. Organic acid contamination was a major problem with methods A, C and D. This reflects a carryover of phenol or other such contaminants during the procedure.

The DNA was further checked for its amenability to PCR reaction to ascertain its suitability for downstream applications, such as sequence based functional screening and diversity analysis. PCR is used in sequence based metagenomic analyses wherein, a gene of interest is PCR amplified to identify the clones of interest. Also, 16S PCR is still the most widely used method for the bacterial diversity

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analysis of metagenomic samples. Hence, the samples were subjected to 16S PCR amplification. Methods A and C did not give any amplicons. This result are supported by the poor 260/230 ratios obtained, that suggests the presence of PCR inhibitors in DNA.

Processing time is another pertinent criterion in metagenomic analysis, when the sample size is very large. Time constraints are a major problem with traditional phenol chloroform extraction procedures as additional clean up processes are required to remove the carryover of phenol and other such chemicals that inhibit PCR. This problem can be resolved by the use of commercial DNA extraction kits mostly based on the method developed by Boom *et al* (44) that uses silica matrix for DNA binding, allowing the washing off of proteins and a preferential DNA elution in the end. In the present study, processing time was the lowest for method G, followed by E and F, all of which are kit based.

V. CONCLUSION

In conclusion, the study compared the efficacy of seven DNA isolation protocols for fish gut metagenomic analysis. Though high yields were obtained using methods A and B, other considerations such as purity and amenablity to PCR ward off the use of method A. When purity is the major consideration, the kit based methods E, F and G are preferable. However, method G may be adopted when processing time is the prime concern. Thus, methods B, E, F and G can be considered as likely candidates for metagenomic research considering yield, purity, amenability to PCR and processing time. The outcome of this study holds promise to those working in the area of gut metagenomics and to others disposed to explore the diversity and bioactive potential of fish gut microflora.

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REFERENCES

1. Fenical W., Jensen P.R**.** 2006. Developing a new resource for drug discovery: Marine actinomycete bacteria. Nature Chemical Biology **2**: 666–673. DOI:10.1038/nchembio841

2. Hugenholtz P., Goebel B.M., Pace N.R. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology **180** (18): 4765-4774.

3. Rappé Michael S., Stephen J. Giovannoni. 2003. The Uncultured Microbial Majority. Annual Review of Microbiology 57: 369–394

4. Nelson J.S. 2006. Fishes of the World. 4th edn. Wiley, New York, USA

5. Horsley R.W. 1973. The bacterial flora of the Atlantic salmon (*Salmo salar* L.) in relation to its environment. Journal of Applied Bacteriology 36 (3): 377–386. DOI: 10.1111/j.1365-2672.1973.tb04119.x

6. Diler O., Altun S., Calikusu F., Diler, A. 2000. A study on qualitative and quantitative bacterial flora of the rainbow trout (*Oncorhynchus mykiss)* living in different fish farms. Turk Veterinerlik ve Hayvanclk Dergisi 24 (3): 251–259

7. Shewan J.M. 1961. The microbiology of sea-water fish. Vol. 1. Pp. 487–560. *In*: Borgstrom G. (ed) Fish as Food. Academic Press, New York, USA.

8. Trust T.J., Sparrow R.A.H. 1974. The bacterial flora in the alimentary tract of freshwater salmonid fish. Canadian Journal of Microbiology 20 (9): 1219–1228. DOI: 10.1139/m74-188

9. Yoshimizu M., Kimura T., Sakai M. 1976. Studies on the intestinal microflora of salmonids,1: The intestinal microflora of fish reared in freshwater and seawater. Bulletin of the Japanese Society for the Science of Fish 42: 91–99

10. Campbell A.C., Buswell, J.A. 1983. The intestinal microflora of farmed Dover sole (*Solea solea*) at different stages of fish development. Journal of Applied Bacteriology 55 (2): 215–223. DOI: 10.1111/j.1365-2672.1983.tb01318.x

11. Yano Y., Nakayama A., Yoshida K. 1995. Population sizes and growth pressure responses of intestinal microfloras of deep-sea fish retrieved from the abyssal zone. Applied and Environmental Microbiology 61 (12): 4480–4483

12. Savas S., Kubilay A., Basmaz N. 2005. Effect of bacterial load in feeds on intestinal microflora of seabream (*Sparus aurata*) larvae and juveniles. Israeli Journal of Aquaculture - Bamidgeh 57 (1): 3–9.

13. Toranzo A.E, Novoa B., Romalde J.L., Nunez S., Devesa S., Marino E., Silva R., Martinez E., Figueras A., Barja J.L. 1993. Microflora associated with healthy and diseased turbot *(Scophthalmus maximus*) from 3 farms in Northwest Spain. Aquaculture 114: 189–202. DOI: 10.1016/0044-8486(93)90295-A.

14. Decostere A., Haesebrouck F., Devriese L., Ducatelle, R. 1996. Identification and pathogenic significance of *Shewanella* sp. from pond fish. Vlaams Diergeneeskundig Tijdschrift 65 (2): 82–85.

15. Haygood M.G., Distel D.L. 1993. Bioluminescent symbionts of flashlight fishes and deep-sea anglerfishes form unique lineages related to the genus *Vibrio*. Nature 363: 154–156. DOI: 10.1038/363154a0.

16. Sullam K.E., Essinger S.D., Lozupone C.A., O'Connor M.P., Rosen G.L., Knight R., Kilham S.S., Russell J.A. 2012. Environmental and

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ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Molecular Ecology 21 (13): 3363–3378. DOI: 10.1111/j.1365-294X.2012.05552.x

17. Bevins C.L., Salzman N.H. 2011. The potter's wheel: the host's role in sculpting its microbiota. Cellular and Molecular Life Sciences 68 (22): 3675–3685. DOI: 10.1007/s00018-011-0830-3

18. Denev S., Staykov Y., Moutafchieva R., Beev G. 2009. Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. International Aquatic Research 1: 1-29. DOI: 10.1111/j.1749-7345.2005.tb00390.x

19. Onarheim A.M., Raa J. 1990. Characteristics and possible biological significance of an autochthonous flora in the intestinal mucosa of sea-water fish. Pp. 197–201. *In*: Lesel R. (ed) Microbiology in Poecilotherms. Elsevier Science Publishers, Amsterdam.

20. Ringo E., Strom E., Tabachek J.A.1995. Intestinal microflora of salmonids. A review. Aquaculture Research 26: 773–789

21. Sugita H., Tanaka K., Yoshinami M., Deguchi Y. 1995. Distribution of *Aeromonas* species in the intestinal tracts of river fish. Applied and Environmental Microbiology 61 (11): 4128–4130

22. Miller D.N. 2001. Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soil and sediments. Journal of Microbiological Methods 44 (1): 49-58.

23. Santos H.F., Carmo F.L., Leite D.C., Jesus H.E., Maalouf Pde C., Almeida C., Soriano A.U., Altomari D., Suhett L., Vólaro V., Valoni E., Francisco M., Vieira J., Rocha R., Sardinha B.L., Mendes L.B., João R.R., Lacava B., Jesus R.F., Sebastian G.V., Pessoa A., van Elsas J.D., Rezende R.P., Pires D.O., Duarte G., Castro C.B., Rosado A.S., Peixoto R.S. 2012. Comparison of different protocols for the extraction of microbial DNA from reef corals. Brazilian Journal of Microbiology 43 (2): 517-27. DOI: 10.1590/S1517-83822012000200012.

24. Purohit M.K., Singh S.P. 2009. Assessment of various methods for extraction of metagenomic DNA from saline habitats of coastal Gujarat (India) to explore molecular diversity, Letters in Applied Microbiology 49 (3): 338-44. DOI: 10.1111/j.1472-765X.2009.02663.x.

25. Nahid S., Yasir S., Ali A. 2012. Defining optimum metagenomic procedure for microbial diversity analysis in wheat rhizosphere. Advances in Applied Science Research 3 (1): 407-411.

26. Delmont T.O., Robe P., Clark I., Simonet P., Vogel T.M. 2011. Metagenomic comparison of direct and indirect soil DNA extraction approaches. Journal of Microbiological Methods **86** (3): 397-400. DOI: 10.1016/j.mimet.2011.06.013

27. Salonen A., Nikkilä J., Jalanka-Tuovinen J., Immonen O., Rajilić-Stojanović M., Kekkonen R.A., Palva A., de Vos W.M. 2010. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: Effective recovery of bacterial and archaeal DNA using mechanical cell lysis. Journal of Microbiological Methods 81 (2): 127–134. DOI: 10.1016/j.mimet.2010.02.007

28. McOrist A.L., Jackson M., Bird A.R. 2002. A comparison of five methods for extraction of bacterial DNA from human faecal samples. Journal of Microbiological Methods 50 (2): 131–139

29. Smith B., Li N., Andersen A.S, Slotved H.C., Krogfelt K.A. 2011.Optimising bacterial DNA extraction from faecal samples: comparison of three methods. The Open Microbiology Journal 5: 14-17. DOI: 10.2174/187428580110501001

30. Claassen S., du Toit E., Kaba M., Moodley C., Zar H.J., Mark P., Nicol A. 2013. Comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. Journal of Microbiological Methods 94 (2): 103–110

31. Tang J., Zeng Z., Wang H., Yang T., Zhang P., Li Y., Zhang A., Fan W., Zhang Y., Yang X., Zhao S., Tian G., Zou L. 2008. An effective method for isolation of DNA from pig faeces and comparison of five different methods. Journal of Microbiological Methods 75 (3): 432–436. DOI: 10.1016/j.mimet.2008.07.014.

32. Giacomazzi S., Leroi F., Joffraud J.J. 2005. Comparison of three methods of DNA extraction from cold-smoked salmon and impact of physical treatments. Journal of Applied Microbiology 98 (5): 1230-1238.

33. Salzman N.H., de Jong H., Paterson Y., Harmsen H.J.M., Welling G.W., Bos N.A. 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiology **148**: 3651–3660

34. NBFGR Training manual 2012

35. Shivaji S., Bhanu N. V., Aggarwal R. K. 2000. Identification of *Yersinia pestis* as the causative organism of plague in India as determined by 16S rDNA sequencing and RAPD-based genomic fingerprinting. FEMS Microbiology Letters 189 (2): 247-252

36. Sambrook J, Russell DW. 2001. Molecular Cloning: A laboratory manual. 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA

37. Daneshwar P., Khoyratty S.S.S. 2004 .Genomic DNA Extraction from *Victoria amazonica*. Plant Molecular Biology Reporter 22 (2): 195a–195j.

38. Morita H., Kuwahara T., Ohshima K., Sasamoto H., Itoh K., Hattori M., Hayashi T., Takami H. 2007. An improved DNA isolation method for metagenomic analysis ofthe microbial flora of the human intestine. Microbes and Environments 22: 214–222.

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39. Scupham A.J., Jones J.A., Wesley I.V. 2007. Comparison of DNA extraction methods for analysis of turkey cecal microbiota. Journal of Applied Microbiology 102 (2): 401–409.

40. Kaser M., Ruf M.T., Hauser J., Marsollier L., Pluschke G. 2009. Optimized method for preparation of DNA from pathogenic and environmental mycobacteria. Applied and Environmental Microbiology 75 (2): 414–418. DOI: 10.1128/AEM.01358-08

41. Simon M. C., Gray D.I., Cook N. 1996. DNA extraction and PCR methods for the detection of *Listeria monocytogenes* in cold-smoked salmon. Applied and Environmental Microbiology 62 (3): 822–824.

42. Jacobsen C. S., Rasmussen O. F. 1992. Development and application of a new method to extract bacterial DNA from soil based on separation of bacteria from soil with cation-exchange resin. Applied and Environmental Microbiology 58 (8): 2458–2462.

43. Saulnier P., Andremont A. 1992. Detection of genes in feces by booster polymerase chain reaction. Journal of Clinical Microbiology. 30 (8): 2080–2083.

44. Boom R.C., Sol C.J.A., Salimans M.M.M., Jansen C.L.E., Wertheim-van Dillen P.M.E., van der Noordaa J. 1990. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology 28 (3): 495–503.

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