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Original Article

Nitric Oxide Production in Probiotic Lactobacillus Plantarum : Revision of the Origin

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Abstract

Nitric oxide (NO) is formed in gastrointestinal tract by both intestinal mucosa and commensal microbiota. In this work, NO production by probiotic bacteria *Lactobacillus plantarum* 8PA3 was demonstrated by electron paramagnetic resonance (EPR)and NOspecificfluorescent dyes DAF-FM DA (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate) and DAA (1,2-diaminoanthraquinone sulfate). After we ruled out the generation of NO via denitrification, we showed that NO production by *L. plantarum*depends on L-arginine what is typical for NO-synthases (NOS), and thus proposed NOS-activity in *L. plantarum.* However,we were unable to inhibit the NO production with three specific NOS inhibitorsL-NAME, L-NIL, and nNOS Inhibitor I.We also demonstrated an increased level of NO production in lactobacilli with damaged cellular membranes suggesting stress-dependent NO production by *L. plantarum*.

Keywords: *Lactobacillus plantarum,* denitrification, nitric oxide synthase (NOS), electron paramagnetic resonance (EPR), fluorescent staining.

Introduction

In mammals, nitric oxide (NO) is generated by three isoforms of the enzyme called nitric oxide synthase (NOS) which catalyzes the oxidation of L-arginine to citrulline and NO. In these organisms, NO plays an important role in many biological functions that range from protection against pathogens and tumor cells to blood pressure regulation and nerve transmission (Aldertonet *al.*, 2001).

In contrast to eukaryotes, bacteria-derived NO has chiefly been regarded as an intermediate in the nitrogen cycle. In particular during dissimilatory denitrification, nitrite is reduced to NO by nitrite reductase and then further reduced to N_2O and N_2. There are two classes of nitrite reductases, namely those that contain either copper or haem as the cofactor. Neither of these enzymes is structurally or mechanistically similar to the mammalian NOSs (Cutruzzola,1999). Currently, it is known that bacteria, like eukaryotes, can realize L-arginine-dependent NOS-mediated NO production (Craneet *al.,* 2010). The bacterial NOS (bNOS) was first established for a number of bacterial species based

on biochemical detection of NOS activity (Chen& Rosazza,1994, 1995; Choi*et al.,*1997; Sari*et al.,* 1998; Choi*et al.,* 2000; Cohen& Yamasaki2003; Hong*et al.,* 2003). Genome sequencing revealed prokaryotes containing NOS homologs and resulted in cloning of these genes, followed by recombinant expression and characterization of NOS-like proteins: deiNOS (or drNOS) from *Deinococcus radiodurans*(Adak*et al.,* 2002b), saNOS from *Staphylococcus aureus*(Bird*et al.,* 2002; Chartier& Couture, 2004; Salard*et al.,* 2006),bsNOS from *Bacillus subtilis*(Adak et al., 2002a), baNOS from *Bacillus anthracis*(Midha*et al.,* 2005;Salard*et al.,* 2006),gsNOS from *Geobacillus stearothermophilus*(Sudhamsu& Crane,2006), stNOS from *Streptomyces turgidiscabies*(Kers*et al.,* 2004),and scNOS from *Sorangium cellulosum*(Agapie*et al.,* 2009).

Lactobacillus species represent a perspective object to research NO production due to their functional importance in the mammalian intestine as well as their considerable technological and commercial significance (Giraffa*et al.,*2010).Commensal microflora can be a strong source of NO in the human gastrointestinal tract,in particular in the presence of nitrate or nitrite and under anaerobic conditions (Sobko*et al.,*2005, 2006).Due to antimicrobial properties of nitric oxide, NO-producing *Lactobacillus* cells are used in NO-donating therapeutic devices, e.g. probiotic patches (Jones*et al.,* 2010).However, the mechanism of NO synthesisin *Lactobacillus* is still controversial. Nitrite reduction is a rare property of lactic acid bacteria. However, some lactobacilli may reduce nitrate to nitrite and NO under anaerobic conditions (Wolf*et al.,* 1990). Moreover, *L. fermentum* LF1 demonstrated denitrifying nitrite reductase activity under both anaerobic and aerobic conditions (Xu&Verstraete,2001). According to the results of a ^{15}N enrichment experiment, traces of (NO_2^-+NO_3^-)-N (total oxidized nitrogen), which seemed to be formed by the resting cells of *L. fermentum* IFO3956, appeared to be derived from L-arginine. Therefore, it was suggested that *L. fermentum* may possess a NOS (Morita*et al.,* 1997). Effects of L-arginine and probiotics on bacterial translocation and extent of liver failure have been studied in a rat acute liver injury model. The results indicated that *L. plantarum* DSM 9843 may containNOS (Adawi *et al.,* 1997), but experiments performed by Morita *et al.*(1997) and Adawi *et al.*(1997) are not enough to prove lactobacillar NO-synthase. Among the most grave drawbacks of the reports cited are that NO production from L-arginine was not investigated in pure culture of *L. plantarum* and the possibility of NO synthesis via denitrification was not assessed.The aim of the present study was toinvestigate if probiotic bacteria *L. plantarum* 8PA3 is able to synthesizeNO and to further elucidate the origin of detected NO.

Materials and methods

Materials

Sodium diethyldithiocarbamate (DETC) and L-arginine were obtained from Sigma-Aldrich (Germany). Fluorescent NO indicators DAF-FM DA (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate) and DAA (1,2-diaminoanthraquinone sulfate), and LIVE/DEAD *Bac*Light Bacterial Viability Kit L-7012 were purchased from Molecular Probes (Invitrogen).Inhibitors L-NAME (N^G-nitro-L-arginine methyl ester, hydrochloride), L-NIL (L-N^6-(1-imimoethyl)lysine, dihydrochloride), and nNOS Inhibitor I (4S)-N-(4-amino-5[aminoethyl]aminopentyl)-N'-nitroguanidine) were obtained from Calbiochem (Germany).

Bacterial strain and growth conditions

L. plantarum 8PA3 strain was isolated from preparation "Dry lactobacterin" produced by Research and Production Association "Biomed" (Perm, Russia). Unless otherwise specified, *L. plantarum* cultures were grown under microaerobic conditions in de Man-Rogosa-Sharpe (MRS) broth (Merck, Germany) for 48 hoursat 37°C, and harvested by centrifugation. In experiments investigating the effects of L-arginine and NOS inhibitors on NO production, these substances were dissolved in MRS broth, filter-sterilized and added at the time of inoculation.

NO determination by metmyoglobin method

Plates of MRS agar supplemented with metmyoglobin (MRS-Mb) were prepared as described in (Gundogdu*et al.,* 2006). Bacteria were inoculated onto MRS-Mb by stabbing and were incubated for 2–3 days at 37°C. NO production was registered by formation of dark red derivatives in medium indicating conversion of metmyoglobin to nitrosomyoglobin.

Determination of stable products of denitrification

The capacity of bacterial culture for denitrification was detected after its growth (37°C, 150 h) under micro-aerobic conditions in MRS broth supplemented with 100 mM KNO_3. Tests were performed with supernatant. Nitrites were quantified with Griess reagent from the photometric

measurement of the absorbance at 540 nm (Green*et al.*, 1982). Nitrates were measured spectrophotometrically with diphenylamine and concentrated sulfuric acid (Bartzatt&Donigan,2004). Gaseous products of denitrification were detected according their accumulation in a float.

EPR spectroscopy

For EPR studies, stationary phase cells were harvested by centrifugation and washed with 50 mM Tris buffer, pH 7.2. The resulting pellet was incubated in the same buffer containing 20 mM L-arginine, or 20 mM KNO_3, and diethyldithiocarbamate-ferrous complex $(DETC)_2$-Fe^{2+} as a spin trap at 37°C for 1 h. To detect NO resulting from denitrification, the reaction mixture was incubated anaerobically in a CO_2 atmosphere. Control samples did not contain bacteria. EPR spectra were recorded on a Bruker ESP-300 spectrometer (Germany) at liquid nitrogen temperature, microwave power 50 mW, modulation amplitude 1 G. The $(DETC)_2$-Fe^{2+}-NO concentration was determined by double integration of the EPR spectra and comparison with the reference concentration curve which was obtained using solutions with different concentrations of $(DETC)_2$-Cu^{2+} (10^{-3}- 10^{-6} M) in toluene (Iarullina*et al.*,2006).

Fluorescence assays

NO production was assessed with NO sensitive fluorescent dyes: DAF-FM DA, which indicates intracellular NO, and DAA, which can be used to monitor both intra- and extracellular NO. For fluorescent staining, the stationary phase bacteria were harvested by centrifugation and washed three times with sterile Hanks' buffer with calcium and magnesium (PAA Laboratories GmbH, Austria). The resulting pellet was resuspended in the same buffer andincubated for 1 h at 37°C with 10 mM DAF-FM DA, or 50 ìg/ml DAA. Then cells were washed three times with Hanks' buffer, 5 ml of cell suspension was mounted on glass microscope slides, covered with coverslips, and examined under oil immersion with a fluorescence micro-scope Leica DM6000B (Germany). Fluorescence intensities were measured and analyzed using the Leica FW4000 software.

Viability assay

Growth kinetics were determined at 590 nm on a Lambda 35 double-beam spectrophotometer, Perkin Elmer Instruments (USA). Viability was determined with LIVE/DEAD *Bac*Light bacterial viability kit L-7012, which is based on a mixture of the green fluorescence nucleic acid stain, SYTO9, that labels all cells in a population, and the red fluorescence nucleic acid stain, propidium iodide (PI), that penetrates only bacteria with damaged membranes and quenches the green stain SYTO9. When used in combination, intact cells are labeled green and cells with damaged membranes are labeled red. Images were obtained with fluorescent microscope Leica DM6000B (Germany) and analyzed using the Leica FW4000 software.

Statistics

The results were processed using Statistica 6.0 software. Three biological replicates were performed for each experiment and the average ± standard deviation was calculated. Student's t-test for paired values was used to determine the significance (≤ 0.05).

Results

Lactobacillus plantarum 8PA3synthesizes NO

First preliminary evidence of NO production in *L. plantarum* 8PA3 came from their capacity to convert metmyoglobin to nitrosomyoglobin. Due to this dark red derivative *L. plantarum* 8PA3 formed good visible rings around their colonies when grown on MRS agar supplemented with metmyoglobin (Fig. 1a). However, this result does not elucidate the origin of detected NO.

To traceNO inside *L. plantarum* 8PA3 cells directly, we took advantageof NO-specific fluorescent dyes DAA and DAF-FM DA. Lactobacilli showed very strong red (in the case of staining with DAA) and green (in the case of staining with DAF-FM DA) fluorescence (Fig. 1b)while the autofluorescence of the cells was below the detection limit (data not shown), indicating presence of NO in bacterial cells. We should note that NO-specific fluorescent dyes also does not clarify the mechanism of NO generation, but provide spatial resolution and possibilities for comparative quantitative evaluation of NO in probes. Surprisingly, bacteria with damaged membranes characterized as dead ones according to staining with PI, showed higher intracellular content of NO compared with intact cells (Fig. 1c), that could be an indicator of stress-induced NO synthesis in *L. plantarum* 8PA3.

NO is not a product of denitrification

Because some *Lactobacillus* species can carry out the dissimilatory nitrate reduction (Wolf*et al.*, 1990;

Xu & Verstraete, 2001), we studied the ability of *L. plantarum* 8PA3 to synthesize NO via denitrification. When incubated micro-aerobically in the medium supplemented with nitrates, bacteria produced trace amounts of nitrites (0.2 - 0.4 ìM) (Table 1), thus indicating nitrate reductase activity of lactobacilli. Gas products of denitrification were not synthesized. It should be noted that NO, as an intermediate of denitrification, cannot be revealed with the method performed, because NO is rapidly transformed in the cells to non-toxic products thus avoiding potential toxicity of the molecule (Goretski *et al.,* 1990). To detect NO possibly formed via denitrification we took advantage of the highly specific NO detection method based on EPR spectroscopy. Bacteria were incubated micro-aerobically with KNO_3 and $(DETC)_2$-Fe^{2+}complex as a spin trap, and NO was quantified. As NO is a highly pervasive hydrophobic molecule that readily penetrates cell walls and membranes (Gusarov*et al.,* 2008), no additional procedures to sonicate cells were performed. Characteristic triplet EPR signal with g-factor close to 2.035 produced by $(DETC)_2$-Fe^{2+}-NO complex was revealed. However, the complex is likely to have abiogenic origin since NO concentrations with and without bacteria did not significantly differ (Fig. 2). As NO can be chemically generated from nitrites at acidic pH (Sobko *et al.,* 2005), a certain level of abiogenic NO could be expected. We therefore conclude that *L. plantarum* 8P-A3 is incapable of NO synthesis via denitrification and suggest that they lack nitrite reductase activity.

NOS substrate L-arginine induces NOproduction

When incubated aerobically with NOS substrate L-arginine *L. plantarum* 8PA3 gave rise to a EPR signal of $(DETC)_2$-Fe^{2+}-NO complex, indicating high amount of NO synthesized by lactobacilli. Notably, NO production in bacteria continued during incubation with $(DETC)_2$-Fe^{2+}. Perhaps, trapping of NO molecule by $(DETC)_2$-Fe^{2+}complex allowed to escape toxic effects of NO, determined mainly by its free radical nature.Since nitrite reductase-derived origin of lactobacillar NO was earlier ruled out, we suggest that *L. plantarum* 8PA3 produce NO through NOS system.

Yet again, NO was present in the cell-free samples, but in considerably smaller amounts than in bacteria-containing samples (Fig. 2). Abiogenic NO output was much higher in anaerobic conditions than in aerobic ones, probably because NO is rapidly oxidized by atmospheric oxygen to form nitrite and nitrate. Registered in cell-free samples under aerobic conditions NO is likely non-enzymatically derived from L-arginine and H_2O_2(Nagase*et al.,* 1997). Indeed, some lactobacilli are known to generate hydrogen peroxide (Eschenbach*et al.,*1989).

Growth of *L. plantarum* 8PA3 with 100 ìM L-arginine resulted in a two-fold increase of overall NO production, registered by DAA, whereas intracellular NO content, indicated by DAF-FM DA, did not change (Fig. 1d). We showed that L-arginine has no influence on bacterial viability (Fig. 3a) and growth (Fig. 3b). Thus, the increase in NO production was not due to the effect of the amino acid on cell amount, but was caused by NOS induction.

Inhibitors of eukaryotic NOSs are not effective towards lactobacillar NOS

To evaluate mammalian NOS inhibitors for their effect on NO production by *L. plantarum* 8PA3, bacteria were grown with three NOS inhibitors added into the growth medium in concentrations which were effective towards eukaryotic NOSs. 100 ìM L-NAME, 100 ìM L-NIL, or 10 ìM nNOS Inhibitor I had no effect on NO synthesis in *L. plantarum* 8PA3 as was indicated in fluorescence assays with DAA and DAF-FM DA (Fig 1e). To exclude the difference in viability of inhibitor-treated and control cells, we performed viability assay. The calculated ratio between living (green) and dead (red-orange) cells in allinhibitor-treated variants did not differ from control untreated cells (Fig. 3a) and thus did not depend on the presence of NOS inhibitors. So, we showed that inhibitors of eukaryotic NOSs affect neither bacterial viability, nor NO production.

Discussion

Commensal bacteria can be a significant source of NO in the gut (Sobko*et al.,*2005, 2006), yet the exact mechanism of NO production by intestinal microflora is not clear.Here we show that probiotic *L. plantarum* 8PA3 is able to synthesize NO and provide some evidence for bNOS-derived origin of this metabolite.

Lactobacilli are potentially capable of NO production via denitrification (Wolf*et al.,* 1990; Xu & Verstraete,2001). However, as follows from the accumulation of nitrites in growth medium (Table 1), among all the enzymes of the denitrification pathway only nitrate reductase was detected in *L. plantarum* 8PA3. These results are in agreement with experimental data of Xu and Verstraete (2001), who also found out nitrate reductase activity of lactobacilli. Additionally, this finding was supported

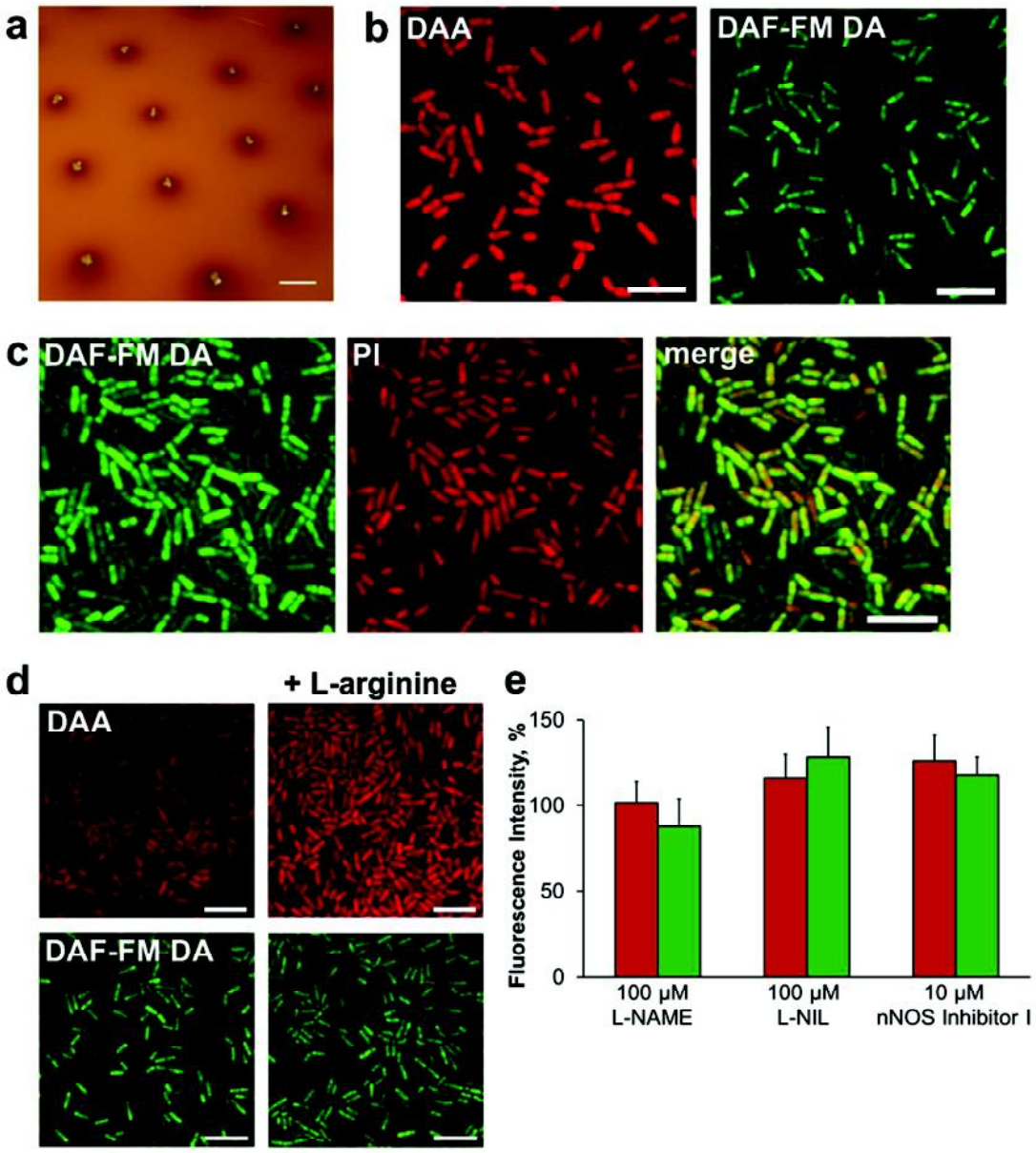

Fig. 1: NOS-mediated production of nitric oxide by *L. plantarum* 8PA3. **a** Formation of nitrosomyoglobin detected by red colored medium around the colonies of *L. plantarum* 8PA3 grown on MRS-Mb. Scale bar = 5 mm. **b** Visualization of NO in *L. plantarum* 8PA3 by fluorescent staining with DAA and DAF-FM DA. **c** Detection of intracellular NO (staining with DAF-FM DA, green color) in bacterial cells with damaged membranes (recognized by staining with PI, red color), presented as a merge image (yellow colored cells). **d** Induction of NO production in lactobacilli by 100 ìM L-arginine added to growth medium. Bacterial cultures in both variants were grown for 48 h and fluorescent stained with DAA or DAF-FM DA. Scale bars (b, c, d) = 5 ìm. **e** NO production in *L. plantarum* 8PA3 grown in the medium with NOS inhibitors measured by fluorescent staining with DAA (red columns) and DAF-FM DA (green columns). Values are expressed as percent of NO levelin control cells grown without any additional compounds.

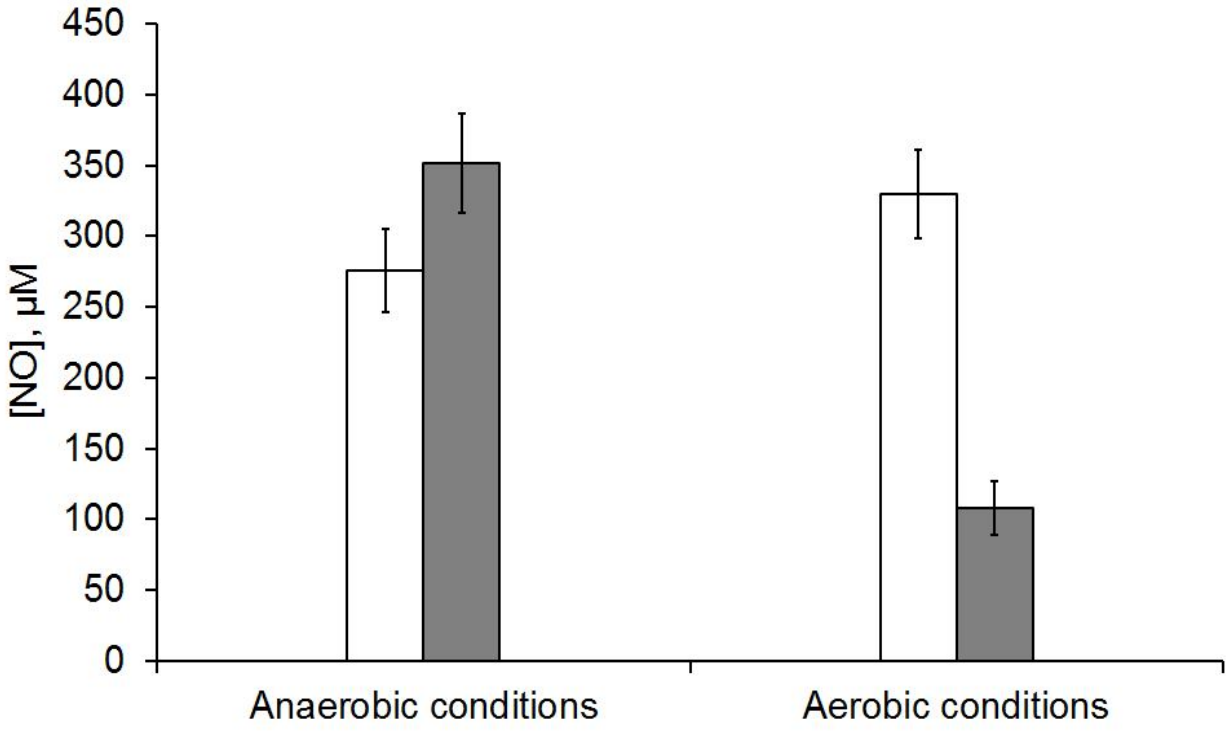

Fig. 2: EPR-measurement of NO content in probes incubated 60 min aerobically with 20 mM L-arginine or anaerobically with 20 mM KNO_3 in the presence (blank columns) and absence (filled columns) of *L. plantarum* 8PA3. Each value is the mean of three independent experiments and is expressed as mean ± SD.

Table 1: Detection of denitrification products in *L. plantarum* 8PA3after 150 h growth in MRS broth supplemented with 100mM [NO_3^-]

Strain	Substrate and products of denitrification		
	NO_3^-, mM	NO_2^-, µM	Gaseous products* (N_2, N_2O), ml^3
L. plantarum 8PA3	50 ± 8	0.3 ± 0.1	0

*NO is not accumulated because of the rapid reduction (Goretski *et al.*, 1990).

by our recent genomic screening of *L. plantarum* for homologs of known bacterial nitrate and nitrite reductases. We revealed a cluster of *nar* genes coding for nitrate reductase and no genes for nitrite reductase (Iarullina & Il'inskaia 2007). It is believed, that the predominant pathway for NO production *in vitro* by lactic acid producing lactobacilli is an acidic nonenzymatic reduction of nitrite (Sobko *et al.*, 2005). Using EPR we also detected chemically generated NO in probes incubated anaerobically with nitrate (Fig. 1b).

After we excluded denitrification as a source of NO in *L. plantarum* 8PA3 we further investigated NO production by *L. plantarum* 8PA3 using EPR (Fig. 2) and specific fluorescent NO staining (Fig. 1b). It is likely that under our experimental conditions, NO synthesis could occur through an NO-synthase (NOS)-like activity as described earlier by Adawi *et al.* (1997).For NOS to be responsible for NO production in *L. plantarum* 8PA3, it should have activity characteristic of NO-synthases. Consistently, NOS-mediated bacterial NO production should be suppressed by NOS inhibitors and activated by NOS substrate L-arginine according to L-arginine paradox(Tsikas *et al.*, 2000). As expected, in *L. plantarum* 8PA3 NO production was activated by

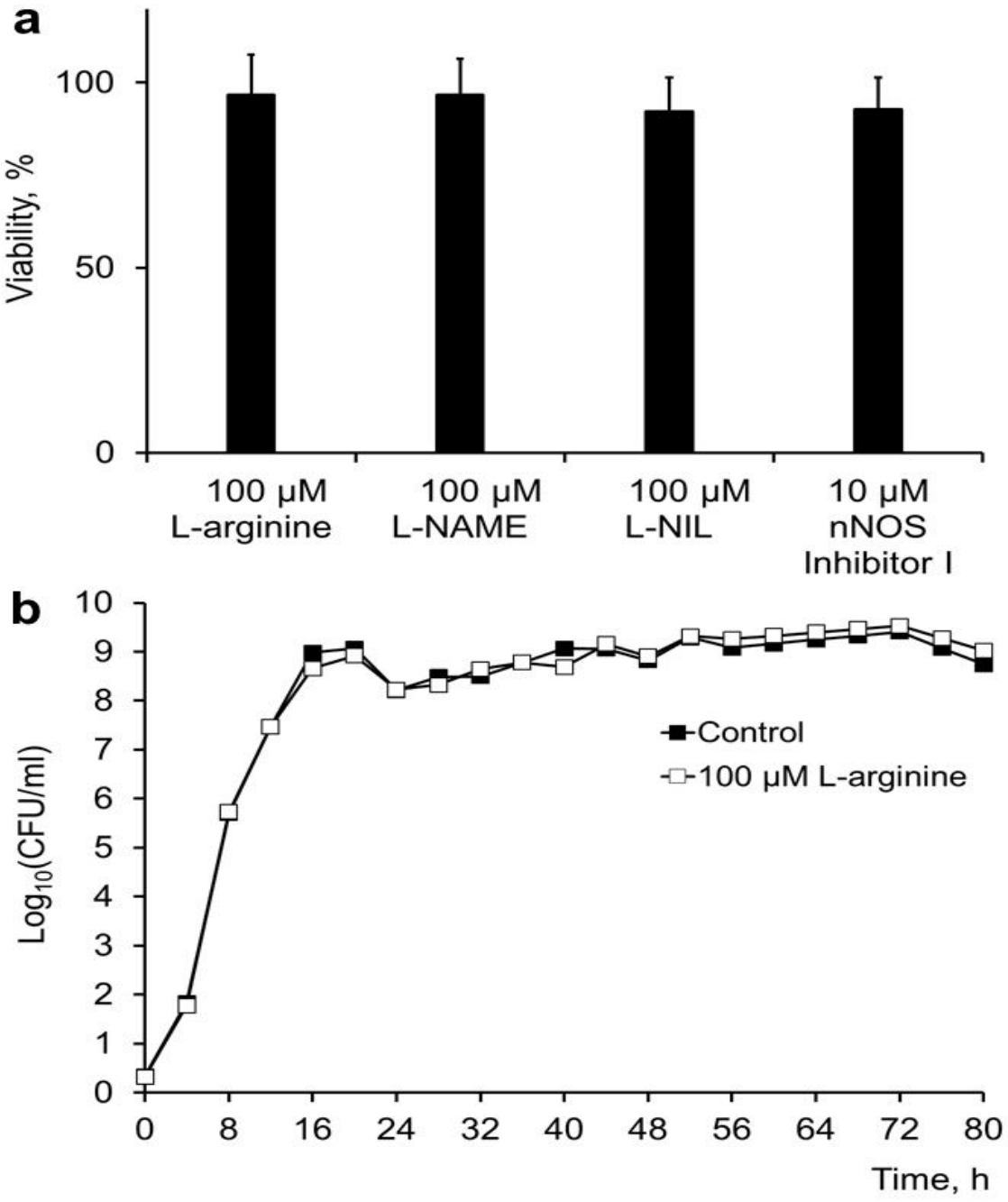

Fig. 3: :The non-effect of L-arginine and NOS inhibitors on *L. plantarum* 8PA3 viability and growth. **a**Viability of *L. plantarum* 8PA3 (48 h, MRS medium) in the presence of L-arginine and NOS inhibitors according to staining with LIVE/DEAD *Bac*Light Bacterial Viability Kit. Values are expressed as percentage viability of control cells grown without any additional compounds. **b** Time course of *L. plantarum* 8PA3 growth in the presence (open squares) and absence (filled squares) of 100 ìM L-arginine. Each value is the mean of three independent experiments. SI ≤8%.

exogenous L-arginine (Fig. 1d) thus suggesting bNOS-mediated NO production.We used inhibitors with different selectivity for the three human NOS isoforms: L-NIL is more selective for the inducible NOS, nNOS Inhibitor I – for neuronal NOS, and L-NAME is slightly more selective for endothelial NOS, but none of these NOS inhibitors was efficient towards NO production by *L. plantarum* 8PA3 (Fig. 1e). These results are probably caused bydegradation of the inhibitors by bacteria or membrane impermeability for them.For example,the inhibitors of mammalian NOSs inhibited the activity of bacterial NOSs in crude homogenates or purified preparations, but not in intact *Staphylococcus aureus* (Choi *et al.,* 1998) and *Rhodococcus* sp. R312 cells (Cohen & Yamasaki,2003).It should be noted that currently it is difficult to assign detected NO synthesis in*L. plantarum* 8PA3 to the certain NOS homolog. In genome of *L. plantarum* we revealed the flavodoxin protein with high homology to the C-terminal reductase domain of eukaryotic NOS – essential redox partners of the catalytic NOS oxygenase domain (NOS_{ox}) (Iarullina and Il'inskaia 2007). Nevertheless, none of the *Lactobacillus* species sequenced contain the NOS_{ox}homolog(Crane *et al.,*2010).Probably, unefficiency of NOS inhibitors against NO synthesis in *L. plantarum* 8PA3 results from significant differences in structure of lactobacillar NOS and known NOSs. Further biochemistry and genetics studies of *L. plantarum* must clarify protein and gene determinantsof detected NO production.

Fluorescent staining with DAA and DAF-FM DA demonstrated NO presence in almost all cells of the population. This finding points to the significance of NO function inside bacteria. Interestingly, lactobacilli were labeled with DAF-FM DA to a different extent, indicating non-equal NO content in different cells of the culture (Fig. 1b). It is well-established that cells in non-synchronized culture are in different stages of cell-cycle. Using the red fluorescence nucleic acid stain PI we revealed an increased level of NO production in lactobacilli with damaged cellular membranes (Fig. 1c), that could be due to the stress-dependent NO production. Earlier it was already shown that bNOS protects*Bacillus subtilis*and *B. anthracis*against oxidative stress (Gusarov & Nudler, 2005; Shatalin *et al.,* 2008). Because NO is a potent cytotoxic agent, it may be suggested that NO itself may cause cell damage. However, activation of NO synthesis by L-arginine did not affect bacterial growth and viability (Fig. 3). When lactobacilli were grown with L-arginine overall NO production increased, while intracellular NO content stayed at a basal level (Fig. 1d). It is known that NO molecules have high permeability through the membranes of cells due to their small sizes and the absence of charge (Gusarov *et al.,* 2008). Currently, we can only speculate how lactobacilli keep a steady-state concentration of potentially pervasive and toxic NO inside their cells. Probably, they possess an adaptive system which regulates NO concentration and is important for the viability of bacteria in their natural environment in the mammalian intestine.

In summary, we demonstrated L-arginine dependent aerobic NO production by*L. plantarum* 8PA3, thus including the strain to a cohort of Gram-positive NOS-containing bacteria. The discovery of NO producingpathway in commensal probiotic bacteria similar to the one performed in surrounding intestinal mucosa is rather promising. Practical application of bNOS-containing probiotic bacteria offers a novel approach for a regulated and continuous delivery of physiological amounts of NO for research and medical purposes. As the same molecule appears to serve different masters, the interplay between probiotic- and host-derived NO is particularly engaging.In this context, an important problem is the role of bacteria-derived NO inboth producers and eukaryotes. Our results indicate NO association with stress-response in *L. plantarum* 8PA3. Whether there is any connection between NO production and probiotic activity of *Lactobacillus* species are still essential questions to be addressed. It is quite likely that NO formation by probiotic bacteria contributes to their health promoting effects on the organism, mechanisms of which are not entirely understood yet.

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Original Article

Phylogenetic Analysis of BTV1 from Northern and Southern States of India

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Abstract

Bluetongue (BT) is an economically important viral disease for sheep and goat causing high morbidity and mortality. In the present study, eight Bluetongue virus (BTV) isolates of goat origin from Utter Pradesh (UP) and four BTV isolates of sheep origin from Andhra Pradesh (AP) adapted in BHK-21 cell line were used. After appearance of 75% of cytopathic effect in BHK-21 cells the virus along with cells were pelleted down and nucleic acid (dsRNA) was extracted using Tri Reagent. All the isolates were confirmed as BTV based on characteristic cytopathic effect in BHK-21 cell culture, RNA-PAGE study (3:3:3:1 pattern) and 366bp amplicon size with group specific ns1 gene based RT-PCR. All the isolates were confirmed as BTV 1 based on segment 2 based serotype specific RT-PCR showing specific amplicon of 605bp size. The nucleotide sequencing of vp2 gene followed by BLAST search also confirmed the isolates as BTV1. The phylogenetic study revealed the eastern topotype origin of these isolates. The phylogenetic analysis also revealed that BTV1 isolates from north India form more closely related sub cluster with other Indian isolates of BTV1. However, BTV1 from south India form separate sub cluster with BTV1 from Greece with in same eastern cluster. The presence of a common *culicoides* vector species *C. oxystoma* has been reported in all these states and could be the possible cause for spread of virus in these parts of the India.

Keywords: Bluetongue virus serotptype-1, cytopathic effect, Phylogenetic analysis, vp2 gene

Introduction

Bluetongue (BT) is an economically important viral disease of domestic and wild ruminants. BT is non-contagious, infectious and insect borne (*Culicoides*) disease (Maclachlan, 1994). The BT disease is caused by Bluetongue virus (BTV) which belongs to genus *Orbivirus* and family *Reoviridae*. BT disease has high economic impact on livestock industry. It leads to high morbidity, mortality, abortion, foetal abnormality, still birth, weight loss, reduced milk and meat yield, wool break etc. in affected animals. It causes severe clinical signs such as lameness, fever, swelling and cyanosis of lips and tongue. The more severe forms of the disease are primarily seen in sheep and in white-tailed deer (Howerth et al., 1988; Darpel et al., 2007). However, Buffalo, cattle and goats act as silent reservoirs and remain viraemic for several months (Maclachlan et al., 2009). The disease has high potency to spread among large and small ruminants, which may cause socioeconomic problems in view of mandatory trade barrier on movement of animals, their embryos, germplasm and other animal products from BT endemic countries to BT free countries. BT affects

many ruminant animals. Therefore it is listed as a multiple species diseases by Office International des Epizooties (OIE, 2013).

There are twenty-four distinct serotypes (BTV1 to BTV24) of BTV have been identified worldwide (Mertens et al., 2004). However, due to rapid reassortment and mutations in genome, BTVs are consistently evolving new serotypes globally. Recently two more serotypes i.e., BTV25 from Switzerland (Hofmann et al., 2008) and BTV26 from Kuwait have been isolated (Maan et al., 2011).

In India, 21 different BTV serotypes have been reported from different states of the country based upon serology and virus isolation (Prasad et al., 2009). Recently, the 22nd BTV serotype i.e. BTV21 have been reported from Andhra Pradesh state (Susmitha et al., 2012). Similarly, the BTV12 has been isolated from Andhra Pradesh state of India (Rao et al., 2013). The nucleotide sequence analysis revealed that most of the BT viruses can be classified into two major groups i.e., 'eastern' or 'western' topotypes, and also into a number of geographic subgroups based on its geographical distribution (Balasuriya et al., 2008).

BTV is an icosahedral virus. The genome of virus consists of ten-segmented, double stranded RNA (dsRNA) molecule. Each of the ten segments codes for at least one viral protein. Seven proteins (VP1 to VP7) are structural and form virus particle. The virus also encodes four non-structural proteins NS1, NS2, NS3 and NS3a which are expressed in virus infected host cells (Mertens et al., 1989). However, recently segment 9 encoded another non-structural protein (i.e., NS4) has been reported (Ratinier et al., 2011; Belhouchet et al., 2011). The inner capsid of BTV is composed of five polypeptides: three minor proteins (VP1, VP4, and VP6) and two major proteins (VP3 and VP7) (Roy, 1989). The outer capsid is composed of serotype specific two viral proteins, VP2 and VP5 (Ghiasi et al., 1987).

The vp2 gene sequences are serotype specific and can be used for determination of respective serotypes. The vp2 gene based serotype–specific RT–PCR is a rapid assay that has shown perfect agreement with the serotyping by conventional virus neutralization methods (Mertens et al., 2007). The BTV1 is one of the most prevalent serotype in India. Recently, an outbreak of BTV1 was reported in a flock of goats in Mathura district of Uttar Pradesh (Biswas et al., 2010). The present study was carried out for vp2 gene based serotype confirmation and determining the phylogenetic relationship of Indian isolate of BTV1 with global isolates.

Materials and methods

Viral sample origin

A total of twelve BTV isolates collected in 2008 from various geographical regions of India were obtained under All India Network Programme on Bluetongue. The virus samples were propagated in BHK-21 cell line in our lab by passaging in BHK21 cell line. Out of these twelve, four samples were of sheep origin collected from Andhra Pradesh (NRT35/IND, NRT39/IND, CHT1/IND and BT1/IND) and remaining eight samples (MKD18/IND, MKD19/IND, MKD20/IND, MKD21/IND, MKD22/IND, MKD23/IND, MKD24/IND and MKD25/IND) were of goat origin from Mathura district, Uttar Pradesh.

Isolation of viral nucleic acid

The virus samples produced BTV specific cytopathic effect (CPE) in infected BHK-21 cell culture within 48 hours. After appearance of 75% CPE, the BHK-21 cell culture along with virus was harvested and pelleted down at 5,000x g for 5 minutes in table top refrigerated centrifuge (REMI, India). Supernatant was decanted carefully and BTV dsRNA was isolated from cell pellet using Tri Reagent method (Sigma, USA) (Chomoczynski and Sacchi, 1987). Using 4M Lithium Chloride and 7.5 M Ammonium acetate, single stranded RNA was selectively precipitated and removed. The dsRNA pellet was washed with prechilled 70% ethanol, air dried and dissolved in nuclease free water.

RNA Poly acrylamide Gel Electrophoresis (RNA–PAGE) of viral dsRNA

The viral dsRNA was subjected to RNA-PAGE using 8% poly acrylamide gel electrophoresis. The RNA–PAGE was allowed for silver staining to visualize the BTV specific nucleic acid segment (Svensson et al., 1986) (data not shown).

Preparation of cDNA and PCR

Viral genomic dsRNA of all the BTV isolates were subjected to cDNA synthesis by Reverse Transcription using Mo-MuLV reverse transcriptase enzyme (Promega, USA) and random decamer (Ambion, USA) in thermal cycler (Biorad i Cycler) as per manufacturer's instruction. The cDNA from all the twelve isolates were subjected to group specific (ns1 gene specific) PCR for confirmation of samples as BTV (Kovi et al., 2005). To confirm the serotype of

virus isolates the cDNA were further subjected to serotype specific PCR using vp2 genes specific primers of all the BTV serotypes.

Cloning of vp2 gene PCR product

The vp2 gene PCR products were purified using QIA quick gel extraction kit (Qiagen, USA) to remove primer dimmers and other PCR ingredients. The purified PCR products were cloned using Pjet 1.2 cloning vector and JM107 cell as host system (Fermentas, USA) as per the manufacturer's instruction. The positive clones were selected by colony touch PCR using vp2 gene specific primer pair. For nucleotide sequencing, the plasmids from positive clones were extracted using Quiaprep kit (Quiagen, USA) as per the manufacturer's instruction.

Nucleic acid sequencing and sequence data analysis

The plasmids from positive clones of all the twelve isolates were allowed to nucleic acid sequencing using vector specific primer by Genetic Analyser ABI PRISM TM 3130 XL machine in our laboratory. The vector sequence from nucleotide sequence obtained was trimmed using online available Vecscreen software (http: / / www. ncbi. nlm.nih. gov / tools / vecscreen /). The nucleotide sequences of vp2 gene of all the isolates were subjected to BLASTN+ 2.2.30 (Zhang et al., 2000) analysis for serotype confirmation. The percent nucleotide as well as its deduced amino acid sequence identity matrix with global isolates was calculated using BioEdit 7.2.5 (Hall, 1999). The phylogenetic analysis of vp2 gene nucleotide as well as its deduced amino acid sequence in study along with other global sequences of same serotype was done using MEGA6 software (Tamura et al., 2013).

Result and Discussion

India is endemic for BTV infection. A large number of BTV serotypes were reported from different states of India. Conventionally, diagnosis of BT includes serological tests, virus isolation and serotype identification by virus neutralization, polymerase chain reaction (PCR), plaque inhibition and fluorescence inhibition tests. The vp2 gene sequences are serotype specific and can be used for determination of respective serotypes.

In present study 12 BTV samples from Uttar Pradesh and Andhra Pradesh states were used. These viruses were grown in BHK-21 cell line. All the viruses showed the characteristic cytopathic effect of BTV such as rounding off and increase in number of floating cells in medium followed by formation of empty areas in BHK-21 monolayers (Sekar et al., 2009). The viral dsRNA was prepared from cell culture pelleted material.

In RNA–PAGE analysis, all the 12 isolates showed the characteristics 10 segmented migration pattern (3:3:3:1) of dsRNA which is specific to BTV (data not

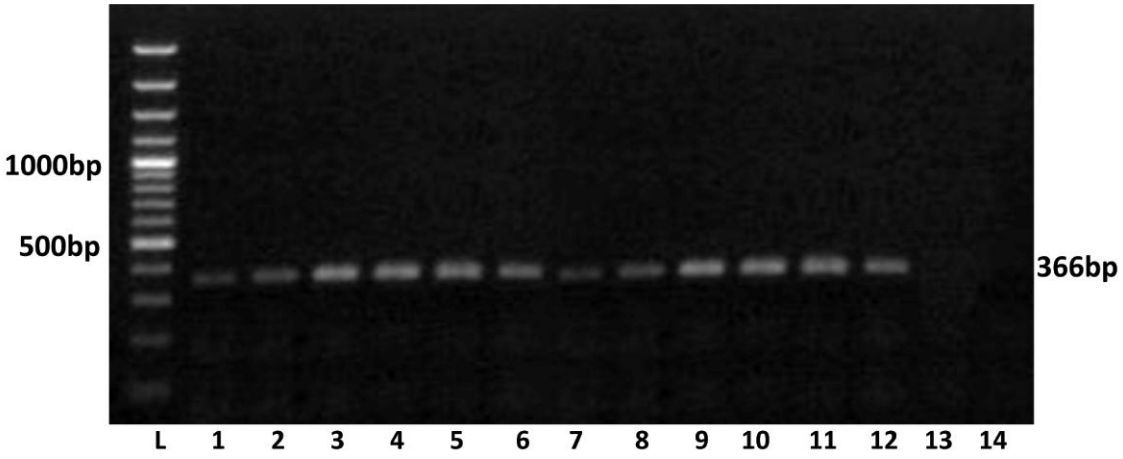

Fig. 1: Ns1 gene specific RT-PCR of twelve BTV isolates showing characteristic 366bp amplicon size in 1% agarose gel electrophoresis. Lanes: L: 100bp DNA ladder, 1: MKD18 / IND, 2: MKD19 / IND, 3: MKD20 / IND, 4: MKD21 / IND, 5: MKD22 / IND, 6: MKD23 / IND, 7: MKD24 / IND, 8: MKD25 / IND, 9:NRT35 / IND, 10: NRT39 / IND, 11:CHT1 / IND, 12: BT1 / IND, 13: BHK-21 cell negative control and 14: Nuclease free water negative control.

Table 1: BTV1 nucleotide and amino acid identity

Percent amino acid identity

BTV1 isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1 India.MKD18/IND.JQ037800	ID	100	99.5	100	99.5	100	98	97.5	98	99	99	98.5	100	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
2 India.MKD19/IND.JQ037801	100	ID	99.5	100	99.5	100	98	97.5	98	99	99	98.5	100	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
3 India.MKD20/IND.JQ037802	99.8	99.8	ID	99.5	99	99.5	97.5	97	97.5	98.5	98.5	98	99.5	92	88	89	99.5	80.5	80.5	81	98.5	81	94.5	94.5	94.5	94	94.5
4 India.MKD21/IND.JQ037803	100	100	99.8	ID	99.5	100	98	97.5	98	99	99	98.5	100	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
5 India.MKD22/IND.JQ037804	99.6	99.6	99.5	99.6	ID	99.5	98	98	97.5	98.5	98.5	98	99.5	92	88	89	99.5	80.5	80.5	81	98.5	81	94.5	94.5	94.5	94	94.5
6 India.MKD23/IND.JQ037805	100	100	99.8	100	99.6	ID	98	97.5	98	99	99	98.5	100	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
7 India.MKD24/IND.JQ037806	99.1	99.1	99	99.1	99	99.1	ID	99	96	97	97	96.5	98	90.5	86.5	87.5	98	79.1	79.1	79.6	97	79.6	93	93	93	92.5	93
8 India.MKD25/IND.JQ037807	98.8	98.8	98.6	98.8	98.8	98.8	99.5	ID	95.5	96.5	96.5	96	97.5	90	86.5	87.5	97.5	78.6	78.6	79.1	96.5	79.1	92.5	92.5	92.5	92	92.5
9 India.BT1/IND.JQ037811	98.6	98.6	98.5	98.6	98.3	98.6	97.8	97.5	ID	97	97	96.5	98	90.5	86.5	87.5	98	81	81	81.5	97	81.5	93	93	93	92.5	93
10 India.CHT1/IND.JQ037812	98.8	98.8	98.6	98.8	98.5	98.8	98	97.6	97.5	ID	99	97.5	99	91.5	87.5	88.5	99	80.5	80.5	81	98	81	94	94	94	93.5	94
11 India.NRT35/IND.JQ037810	97.6	97.6	97.5	97.6	97.3	97.6	96.8	96.5	96.3	97.8	ID	98.5	99	91.5	87.5	88.5	99	80.5	80.5	81	98	81	94	94	94	93.5	94
12 India.NRT39/IND.JQ037809	98.5	98.5	98.3	98.5	98.1	98.5	97.6	97.3	97.3	97.3	97.8	ID	98.5	91	87	88	98.5	79.6	79.6	80	97.5	80	93.5	93.5	93.5	93	93.5
13 India/Meerut01/India/2010/KC954625	100	100	99.8	100	99.6	100	99.1	98.8	98.6	98.8	97.6	98.5	ID	92.5	88.5	89.5	100	81	81	81.5	99	81.5	95	95	95	94.5	95
14 India/Chennai/AY559061	91.7	91.7	91.5	91.7	91.4	91.7	90.9	90.7	91.2	90.5	89.7	90.2	91.7	ID	86.1	86.1	92.5	75.6	75.6	75.6	91.5	76.1	87.5	87.5	87.5	87.5	87.5
15 India/Sirsa3/Y559060	88.4	88.4	88.2	88.4	88.2	88.4	87.6	87.4	87.7	87.2	86.4	86.9	88.4	81.4	ID	98.8	88.5	72.6	71.6	72.6	87.5	72.6	86.2	86.1	86.3	86.3	86.3
16 India/Avikanagar/AY559058	88.7	88.7	88.5	88.7	88.5	88.7	87.9	87.7	88	87.6	86.7	87.2	88.7	81.8	99.6	ID	89.5	72.6	72.6	72.6	88.5	72.6	86.1	86.2	86.2	86.2	86.3
17 India/India A/AJ585111	100	100	99.8	100	99.6	100	99.1	98.8	98.6	98.8	97.6	98.5	100	91.7	88.4	88.7	ID	81	81	81.5	99	81.5	95	95	95	94.5	95
18 Morocco/MOR2006/06/EU625362	72.8	72.8	72.7	72.8	72.8	72.8	72	71.7	73	73	72.8	72.5	72.8	68.4	68.4	68.6	72.8	ID	100	97.5	82	99.5	79.1	79.1	79.1	78.6	79.1
19 Algeria/ALG2006/01/EU625361	72.8	72.8	72.7	72.8	72.8	72.8	72	71.7	73	73	72.8	72.5	72.8	68.4	68.4	68.6	72.8	100	ID	97.5	82	99.5	79.1	79.1	79.1	78.6	79.1
20 South Africa-ref/South Africa/AJ585122	73.8	73.8	73.7	73.8	73.7	73.8	73	72.7	74	73.7	73.5	73.5	73.8	68.7	68.4	68.4	73.8	94.5	94.5	ID	82.5	98	80	80	80	79.6	80
21 Greece/GRE2001/07/IN635334	95.8	95.8	95.7	95.8	95.5	95.8	95	94.8	95	94.7	93.5	94.7	95.8	88	85.2	85.6	95.8	72.8	72.8	73.8	ID	82.5	96	96	96	95.5	96
22 Portugal/BTV1/PT/29058/07/ EU498674	73.2	73.2	73	73.2	73.2	73.2	72.3	72	73.3	73.3	73.2	72.8	73.2	68.5	65.6	65.9	73.2	99.6	99.6	94.7	73.2	ID	79.6	79.6	79.6	79.1	79.6
23 Australia/DPP8304/KM099539	87.7	87.7	87.6	87.7	87.4	87.7	86.9	86.6	87.2	87.6	87.2	87.6	87.7	80.8	78.8	79.1	87.7	74.3	74.3	74.3	87.9	74.3	ID	100	100	99.5	100
24 Australia/DPP8086/KM099538	87.1	87.1	86.9	87.1	86.7	87.1	86.2	85.9	86.6	86.9	86.6	86.9	87.1	80.1	78.2	78.5	87.1	74.2	74.2	74	87.2	74.5	98.1	ID	100	99.5	100
25 Australia/DPP7137/KM099537	87.4	87.4	87.2	87.4	87.1	87.4	86.6	86.2	86.9	87.2	86.9	87.2	87.4	80.6	78.3	78.6	87.4	74.2	74.2	73.8	87.6	74.5	98.5	98.3	ID	99.5	100
26 Australia/DPP6504/KM099536	87.6	87.6	87.4	87.6	87.2	87.6	86.7	86.4	87.1	87.4	87.1	87.4	87.6	80.8	78.5	78.8	87.6	74	74	74	87.7	74.3	98.6	98.5	99.5	ID	99.5
27 Australia/DPP6112/KM099535	87.6	87.6	87.4	87.6	87.2	87.6	86.7	86.4	87.1	87.4	87.1	87.4	87.6	80.6	78.5	78.8	87.6	74	74	74	87.7	74.3	98.6	98.5	99.5	99.6	ID

Percent nucleotide identity

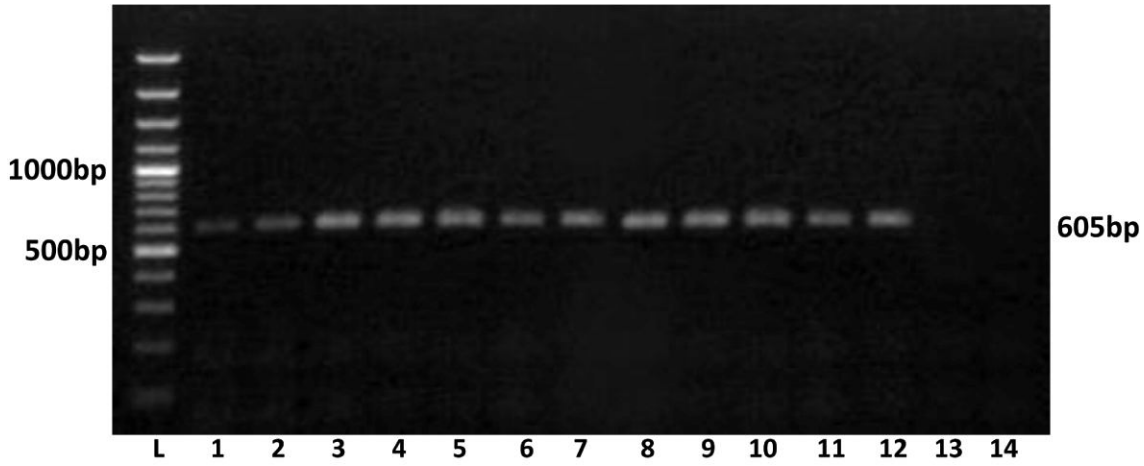

Fig. 2: Vp2 gene specific RT-PCR based serotyping of different BTV 1 isolates showing 605bp amplicon size in 1% Agarose gel electrophoresis. Lanes: L: 100bp DNA ladder, 1: MKD18/IND, 2: MKD19/IND, 3: MKD20/IND, 4: MKD21/IND, 5: MKD22/IND, 6: MKD23/IND, 7: MKD24/IND, 8: MKD25/IND, 9:NRT35/IND, 10: NRT39/IND, 11:CHT1/IND, 12: BT1/IND, 13: BHK-21 cell negative control and 14: Nuclease free water negative control.

shown). Upon group specific ns1 gene based RT-PCR all the isolates yielded an expected size of 366 bp of amplicon on 1% agarose gel electrophoresis (Figure 1). The characteristics cytopathic effect in BHK–21 cell culture, migration pattern in RNA–PAGE and group specific ns1 gene based RT-PCR further confirmed the samples as BTV. The BTV isolates grown in BHK–21 cells were evaluated by different RT–PCR assays by several other workers earlier (Aradabib et al., 1998; Biswas et al., 2010).

For serotype confirmation, all the isolates were subjected to vp2 gene specific RT-PCR using primers specific to all the BTV serotypes. All the isolates in study had shown specific PCR amplicon of 605bp with BTV1 vp2 gene primer only (Figure 2) without showing any amplification with remaining serotype specific primers. Thus, all the isolates were serotyped as BTV1. The cloned product of vp2 gene of all the isolates was allowed for nucleic acid sequencing.

BLASTN+ 2.2.30 analysis of all the isolates showed that vp2 gene sequences of all the isolates align only with BTV1 isolates from India and different parts of the world. It confirmed the serotype of all the BTV isolates as BTV1. The sequence data obtained was submitted to NCBI and accession numbers assigned to different isolates MKD18/IND, MKD19/IND, MKD20/IND, MKD21/IND, MKD22/IND, MKD23/IND, MKD24/IND, MKD25/IND, NRT39/IND, NRT35/IND, BT1/IND and CHT1/IND were JQ037800, JQ037801, JQ037802, JQ037803, JQ037804, JQ037805, JQ037806, JQ037807, JQ037809, JQ037810, JQ037811 and JQ037812 respectively.

The percent nucleotide and its deduced amino acid sequences identity of the isolates in study along with other BTV1 isolates from different parts of the world were calculated using Bioedit 7.2.5 (Hall, 1999). The nucleotide and its deduced amino acid sequence identity study revealed that BTV1 isolates from north India (MKD18/IND, MKD19/IND, MKD20/IND, MKD21/IND, MKD22/IND, MKD23/IND, MKD24/IND, and MKD25/IND) showed 98.8-100%/97-100% nucleotide/amino acid (nt/aa) identity among themselves (Table 1). Similarly, BTV1 isolates from south India (NRT39/IND, NRT35/IND, BT1/IND, and CHT1/IND) showed 96.3-97.8%/96.5-99% nt/aa identity among themselves. However, overall nt/aa identity of BTV1 isolates in study was found to be 96.5-100%/95.5-100%.

The BTV1 isolates in study showed 78.2-100%/86.1-100% nt/aa identity with eastern topotype BTV1 isolates from India (Meerut 01, India A, Chennai, Sirsa 3and Avikanagar isolates), Australia (DPP8304, DPP8086, DPP7137, DPP6504 and DPP6112) and Greece (GRE2001). However, they showed only 68.4-74.5%/72.6-81.5% nt/aa identity with western topotype of BTV1 viruses from Morocco (isolate MOR2006/06), Algeria (ALG2006/01), South Africa (South Africa-ref) and Portugal (BTV1/PT/29058/07). These topotype results agree with previous study made (Maan et al., 2010). It confirmed the eastern origin of segment 2 of BTV1 isolates in study.

The nucleotide sequence based phylogenetic analysis of these isolates along with other BTV1

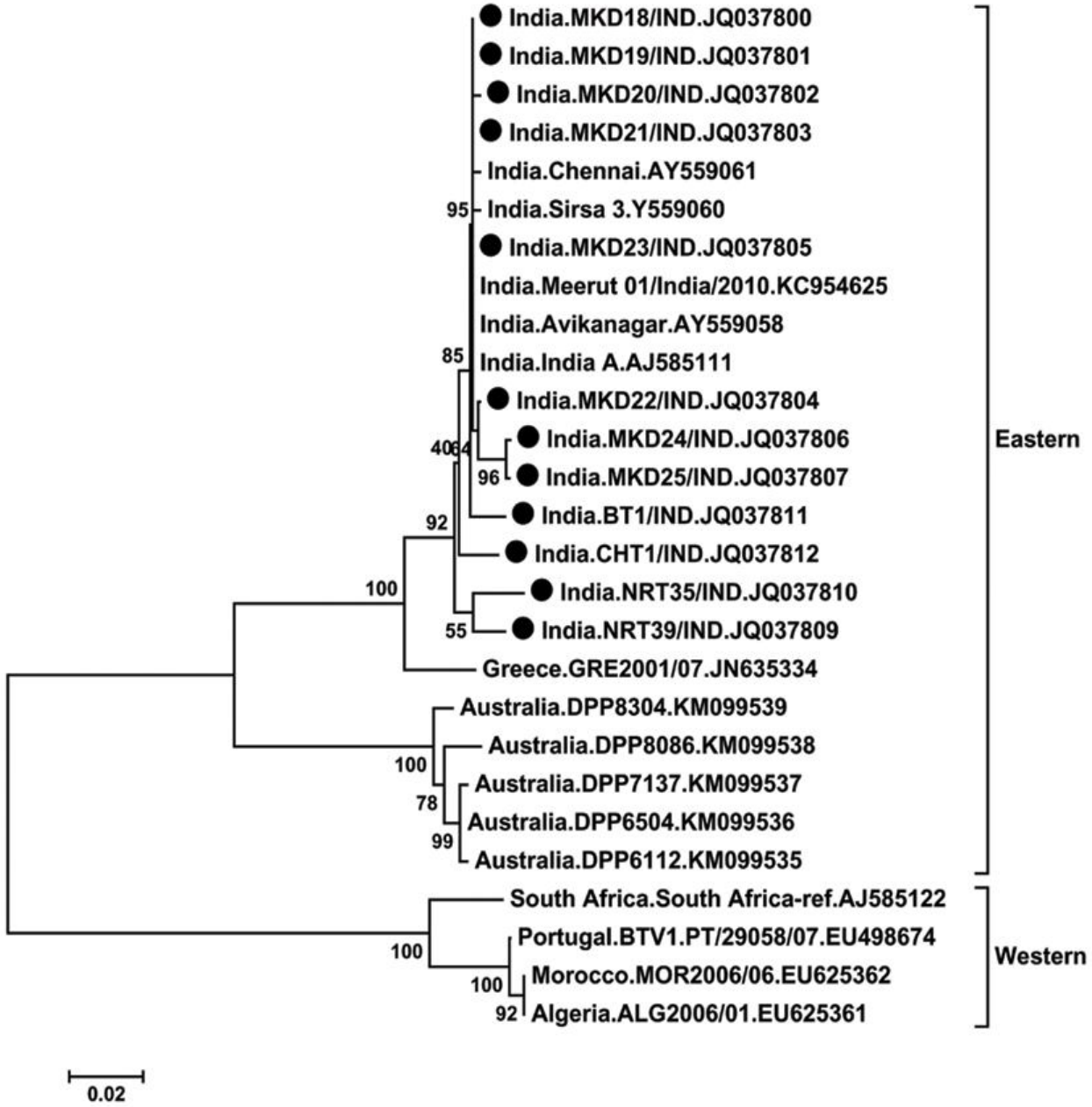

Fig. 3: Vp2 gene nucleotide sequence based phylogenetic analysis of Indian isolates of BTV1 with other global isolates of BTV1. ● Indian isolates used in this study

isolates using Mega 6 software programme revealed that BTV1 isolates in study form a separate eastern cluster with eastern topotype BTV1 isolates from India, Australia and Greece (Figure 3). The western cluster was consists of BTV1 viruses of western topotype from Morocco, Algeria, South Africa and Portugal. However, within eastern cluster BTV1 isolates from north India (MKD18/IND, MKD19/IND, MKD20/IND, MKD21/IND, MKD22/IND, MKD23/IND, MKD24/IND, and MKD25/IND) formed a more closely related sub cluster along with other Indian isolates. Similarly, south Indian BTV1 isolates (NRT39/IND, NRT35/IND, BT1/IND, and CHT1/IND) formed a separate more closely related sub cluster with BTV1 from Greece. The similar result was observed with deduced amino acid sequence based phylogenetic analysis (Figure 4).

The phylogenetic analysis study and sequence identity revealed that the segment 2 of BTV1 isolates in study might be originated most probably from India or Greece. However, to know the origin of individual segments of the BTV genome, sequencing of the all

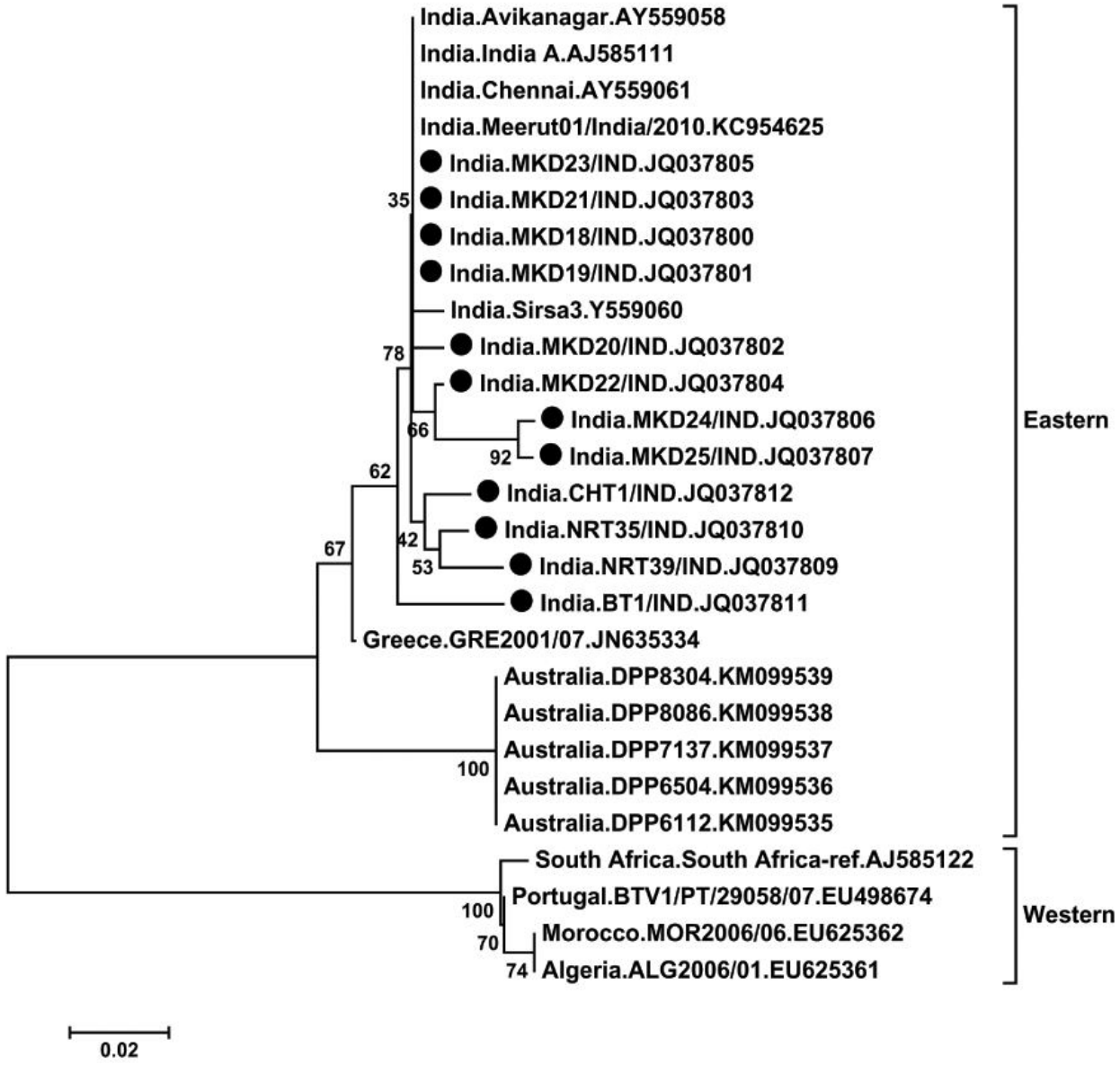

Fig. 4: Vp2 gene deduced amino acid sequence based phylogenetic analysis of Indian isolates of BTV1 with other global isolates of BTV1. ● Indian isolates used in this study

the segments is required. Recently, reassortment in the segment 6 of BTV16 and BTV21 has been reported in the Indian BTV16 isolate (Shafiq et al., 2013). The transmission of BTV takes place along with live animals or its product transport and its *Culicoides* vector. Previous studies have suggested prevalence of BTV serotypes 2, 4, 6, 9, 12, 13, 14, 17, 18 and 19 in Andhra Pradesh whereas BTV 23 in Uttrakhand state. The circulation of BTV1 has been reported from many states of India including Rajasthan, Gujarat Maharashtra, Tamil Nadu and Karnataka (Prasad et al., 2009). Therefore, migration of BTV 1 within adjoining states is quite possible due to movement of animal from one state to another. The activity of the *Culicoides* vector may also play role in transmission and spread of the infection in adjoining areas. BT viruses undergo continuous genetic reassortment and are transmitted by Culicoides vector, posing the disease control a challenge. The knowledge of prevalence of various circulating serotypes could be used for development of sensitive diagnostics to study epidemiology and effective vaccination to control the disease in field. Rapid molecular based diagnosis of BTV isolates is required to improve surveillance of BTV serotypes prevalent and to facilitate the choice of an appropriate serotype–specific vaccine in a disease outbreak.

Conclusions

BT is one of the major infectious diseases of small ruminants in India. BT is primarily a disease of sheep. In this study sequence based serotyping and phylogenetic study of twelve isolates of BTV from north and south India has been reported. All the BTV isolates were serotyped as BTV1. Phylogenetic study confirmed the eastern origin of these isolates. The sequence and phylogenetic study revealed that all the isolates might be originated either from BTV1 isolates from India or Greece. The segment 2 based RT–PCR used here can be used for diagnosis of BTV from variety of biological samples such as blood, tissue samples and *Culicoides* vectors. The present study suggested that RT-PCR could be a reliable and rapid method for detection and determination of serotypes of Indian isolates of BTV. The information thus generated regarding circulation of BTV1 in various part of the India could be very useful for development of rapid and sensitive diagnostics and suitable vaccine candidates for control BT disease.

Acknowledgements

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Original Article

Isolation, Identification and Antibiotic Susceptibility of *Salmonella* Species from Seafood Sold in Local Markets of Goa

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Abstract

Present short work was designed to study incidences of *Salmonella* species in the raw seafood from local markets and fish collection jetties of Goa. Study observed 12% (24/200) of the raw sea foods (9.5% fish, 2% prawns and 0.5% crabs) contaminated with *Salmonella* spp. All the 24 isolates were tested for antimicrobial susceptibility by disc diffusion assay. Isolates were found resistant to trimethoprim-sulfamethoxazole (100%), ampicillin (41%) and tetracycline (25%). All the isolates were sensitive to ciprofloxacin, chloramphenicol, streptomycin and norfloxacin. Intermediate phenotypes were observed against nalidixic acid, kanamycin and gentamicin.The study shows occurrence of antibiotic resistant *Salmonella* species in retail fish markets and collection jetties in Goa, India.

Introduction

Seafood has high nutritional value and seafood products are increasingly popular worldwide. The biological agents which are involved in sea food contamination includes bacteria, viruses and parasites which can cause range of symptoms like mild gastroenteritis to life threatening diseases (Amagliani et al. 2012). Bacterial contamination of seafood products are being a major risk factor for transmission of pathogens (Iwamoto et al. 2010).

Salmonellae are Gram-negative, facultative anaerobes, non-spore forming rods that belong to the family *Enterobacteriaceae.* Salmonellosis has two distinct syndromes; it can be detected as systemic disease or gastroenteritis. Gastroenteritis is more often associated with food borne transmission (Knodler 2014). *Salmonella* has been highlighted as the potential outbreak causing infection in European countries (EFSA 2010), US (CDC 2014) and other countries worldwide including India (Kumar et al. 2009). *Salmonella* serovars are widely present and can enter in water bodies through wild animals, domestic stock, poor sanitation and inappropriate disposal of human and animal waste (Amagliani et al. 2012). Salmonellosis constitutes a major public health burden and represents a significant cost to society in many countries. Very fewcountries report data on the economic cost of the disease. In case of USA, an estimated 1.4 million non-typhoidal*Salmonella* infections were reported annually resulting in 168,000 visits to physicians, 15,000 hospitalizations and 580 deaths (Voetsch et al. 2004). Vast range of sea food products were reported with *Salmonella* infections worldwide which includes molluscs, mussels, oysters, and shrimps (Iwamoto et al. 2010). Nearly 10% of import and 2.8% of domestic raw sea food were positive for *Salmonella* (Heinitz et al. 2000).Particularly in India, Kumar et al. (2009) has studied*Salmonella* extensively and food type such as Finfish, Shrimps, Clams, Crab, Lobster,

Octopus, Squid Mussel and Oyster have been reported to be contaminated with *Salmonella*. In Mangalore, Indian seafood associated food poisoning outbreaks were observed causing 34 personsto be infected (Antony et al. 2009).

Goa which lies on the West coast of India is a smallest Maritime time State with a coastline of 104 Km. It has 48 Fishing villages along the coast line however Fish landing takes place at 88 Centres.The continental shelf of Goa has been estimated at 10,000 square meters. Approximately, 40,000 to 50,000 people are dependent on fish harvesting, processing and marketing in Goa (Gaonkar 2008). In Goa (India), seafood is highly preferred due to ease of availability. These raw seafood are sold locally without any further processing in order to minimise bacterial load. Therefore, occurrence of bacterial pathogens such as seafood can't be denied. In this preliminary study we made an attempt to determine the incidences of *Salmonella* species among the fresh seafood catch and seafood that gets sold locally in state of Goa.

Material and Methods

Sample collection

A total of 200 raw seafood samples comprising fish (143), prawns (47) and crabs (10) were collected in sterile zip-lock bags. Samples were collected from two jetties (Vasco and Betim) and two local fish markets (Panjim and Mapusa) of Goa. Samples were transported to the laboratory in sterile containers at 4°C and processed immediately.

Isolation and Identification

Isolation of *Salmonella* species was carried out according to the method defined by United States Department of Agriculture (USDA 2014). Accordingly, the samples were washed with sterile water and macerated by stomacher in sterile bags. Approximately one gram of macerated sample was inoculated into 10 ml of buffered peptone water and incubated at 35 ± 2°C for 22-26 h. The enriched broth was transferred (0.5 ml) into 10 ml tetrathionate broth (TT) broth. The samples were incubated at 42 ± 0.5°C for 22-24 h. A loopful of enriched broth was streaked on Hektoen Enteric agar (HKE) and incubated at 37°C for 24 h. Green colonies with dark centres were suspected as *Salmonella* spp. Presumptive isolates were stored at 4°C in Nutrient broth. These presumptive *Salmonella* isolates were further confirmed by biochemical tests. Isolates were tested for Gram staining, catalase activity, oxidase activity L-lysine decarboxylase and α -galactosidase (ONPG) activity. Biochemical tests such as Voges Proskauer and Indole were performed and typical growth on TSI and Urea agar (Christensen) slants were observed.

Antimicrobial susceptibility testing (AST)

A panel of 10 antibiotics namely Ampicillin, Trimethoprim-Sulfamethoxazole, Ciprofloxacin, Chloramphenicol, Streptomycin, Tetracycline, Norfloxacin, Nalidixic acid, Kanamycin and Gentamicin were selected for AST which are generally used in human diseases prophylaxis to treat bacterial infections. For tests, two to three typical colonies of each isolate from XLD or HE agar were enriched in 5 ml of BHI broth at 37°C for 12-18 h. After incubation growth was pelleted by centrifugation at 5000g for 5 min and adjusted to 0.5 McFarland with 0.85 % NaCl. Adjusted bacterial suspension was spread evenly over Muller Hinton agar plates with sterile cotton swabs. Antimicrobial discs (Hi-Media, Mumbai) were placed by keeping appropriate distance between discs. The plats were immediately kept in the refrigerator at 4°C for 10-15 min in order to diffuse the antimicrobial concentration evenly before the initiation of bacterial growth phases. Later the plates were incubated at 37°C for 18 to 24 h. The zones of inhibition were measured and compared with Clinical and Laboratory Standard Institute (CLSI) standards.

Results and discussion

Fishery sector has been playing very important role in improving the socioeconomic status of over 14 million fishers in the country, whose livelihood is depending on fishing and allied activities (Gaonkar 2008). This is emerging as a viable sector contributing towards employment generation in fishing and allied activities, supplementing food supply,raising nutrition level and earning foreign exchange through exports.The Marine Fisheries resources of Goan waters provide valuable food and support livelihoods to fishermen and their families (Gaonkar 2008). The fresh catch obtained by fisherman is sold locally without any effort for microbicidal treatments. On the other hand recent reports suggest that such

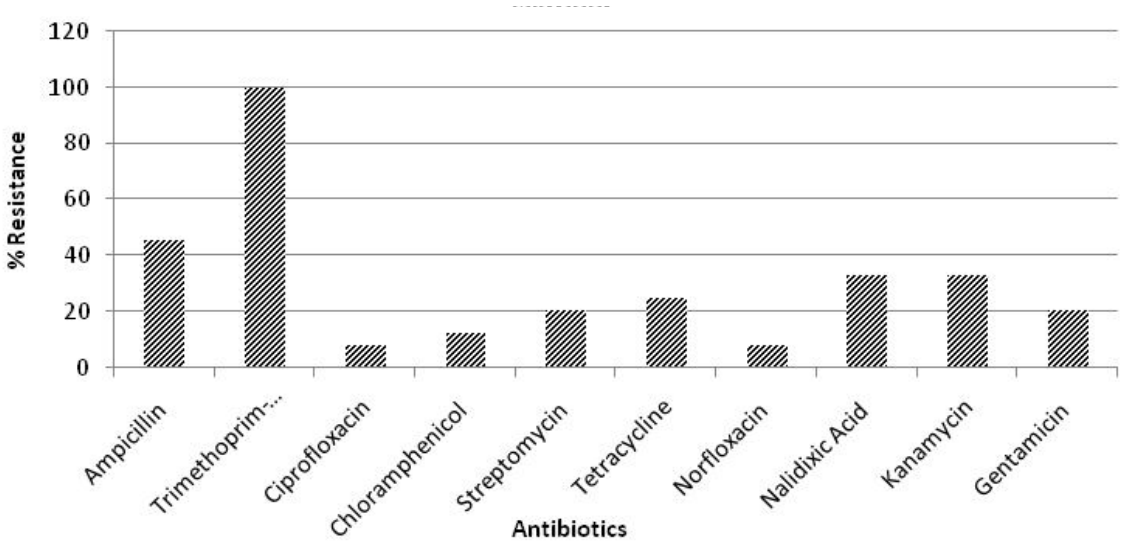

Fig. 1: Antibiotic resistance profile of *Salmonella* species obtained from seafood

seafood may possess bacterial pathogens contamination (Poharkar et al., 2014; Poharkar et al., 2013). Therefore it is necessary to understand the incidences of pathogens in such seafoods.

In this study, an attempt was made to observe the incidences of *Salmonella* species among the fish catch from coast areas of Goa and local market. To determine the incidences, a total of 200 samples comprising of fish (143), prawns (47) and crabs (10) were collected and processed for isolation of *Salmonella* species. The isolation and identification was performed as per method defined by USDA. Accordingly, 24 samples were found to contain *Salmonella* species. Of these 24, 19 were from fish, 4 from prawns and 1 from crab. Several previous studies indicates such incidences of *Salmonella* from seafood (Amagliani et al. 2012). *Salmonella* species are widely present in nature. The main source for the addition of *Salmonella* species to waterbodies are domestic waste, wild animals and direct disposal of sewages (Amagliani et al. 2012). Once *Salmonella* gets introduced to the environment it can survive for longer period (Winfield & Groisman 2003). Such longer persistence ability of *Salmonella*, in water bodies increases the chances of contamination of associated biota such as fish. Contamination of seafood is commonly observed and therefore frequently reported. In addition, several types of fish have been reported to contain *Salmonella* in their intestinal content, which may spread to organs (Nesse, Løvold, et al. 2005; Nesse, Refsum, et al. 2005). Huss et al. (2000) have classified seafoods in different categories, in which raw seafood such as fish have been show at most risk. Goa is known for tourism and therefore several different types of dishes are prepared which utilise raw sea foods. Therefore, there is obvious increased risk of *Salmonella* infection. Also, several seafood gets served as semi-cooked dishes which may lead to gastrointestinal disease. One of the reasons for such gastrointestinal diseases could be occurrence of *Salmonella* in such foods.

Emerging antibiotic resistance among pathogenic bacteria is concerning matter. Pathogens obtained from humans, animals, food as well as environment has been shown to possess antibiotic resistance (Castanon 2007). Isolates obtained in this study were checked for the commonly used antibiotics. All the isolates were found to be resistant to sulphamethoxazole-trimethoprim (Fig. 1). Generally, *Salmonella* species are sensitive to sulphamethoxazole-trimethoprim and very few reports show the resistance to sulphamethoxazole-trimethoprim. A recent study with *Salmonella* enterica obtained from dairy products from Egypt observes 91.5% resistant isolates to sulphamethoxazole-trimethoprim (Ahmed et al. 2014). Comparatively, several other studies which attempts sulphamethoxazole-trimethoprim sensitivity analysis with *Salmonella* species shows lower range (11-35%) resistant isolates (Adesiyun et al. 2014; Ertas Onmaz et al. 2014; Onyango et al. 2014). Ampicillin and tetracycline were not effective against 41% and 25% of the isolates. Occurrence of antibiotic resistant *Salmonella* in seafood is concerning. Further study is necessary to determine the genetical constituent causing antibiotic resistance.

This preliminary study shows prevalence of the antibiotic resistant *Salmonella* spp. in the raw seafood that sold in the markets of Goa. More study is necessary to carry out on all other markets and jetties to have overall incidences occurring across Goa. Also, a study with sea and estuarine water may help

to trace out the source. Microbiological quality control and surveillance programs should be carried out in order to restrict or minimise the incidences of *Salmonella*. Also, fisherman and seafood processing personnel should make aware for the incidences of the Salmonellosis and its consequences. Also, generation of area specific antibiogram will help in prudent treatment of food borne salmonellosis. Efforts should be taken for the implementation of hazard analysis and critical control points to reduce the incidence of *Salmonella*s pecies in seafood.

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Review Article

Ebola Hemorrhagic Fever

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Abstract

Ebola virus disease (EVD) also known as Ebola haemorrhagic fever is a severe contagious disease affecting humans and non-human primates. It is usually transmitted to humans through direct contact with blood, tissue, body fluids and secretions from an infected animal or human. The causative agent is classified in the genus Ebolavirus of the Filoviridae family. Filoviruses are filamentous enveloped viruses containing a non-segmented, negative-strand genomic RNA of approximately 19 kilobases. Patients are diagnosed by testing of body fluids and serum with an ELISA test / PCR; however the results are not always accurate. There is currently no treatment for Ebola hemorrhagic fever. Vaccines are in clinical trials and hold the future in preventing this disease. Ebola is on the United States' list of possible bioterrorism agents because no humans have been found to have immunity to it.

Background

Ebola is a member of the Filoviridae viral family, characterized by the long, thin filaments it is a RNA virus (19-kb negative-sense) which codes for seven proteins. The seven filoviral proteins are the glycoprotein (GP), the polymerase (L), the nucleoprotein (NP), a secondary matrix protein (VP24), the transcriptional activator (VP30), the polymerase cofactor (VP35), and the matrix protein (VP40). It was first discovered near the Ebola River and was named accordingly. Filoviruses are infrequently encountered, and their natural history is only now being understood. Because of the lack of predictive information about them, and serious human disease they cause, they require our attention. Filoviruses cause a severe, unrelenting viral hemorrhagic fever with high mortality [1, 2, 3]. Discovered in late 1970s, the international community was startled as it caused major outbreaks of hemorrhagic fever in the Democratic Republic of the Congo (DRC) [4] and Sudan [5].

Outbreaks chronology

Ebola virus was first discovered in 1976 when an outbreak of Ebola hemorrhagic fever occurred in Zaire in Democratic Republic of the Congo (DRC) [6]. Since then five different strains of Ebolavirus have been discovered, namely Zaire ebolavirus (EBOV), Sudan ebolavirus (SUDV), Tai Forest ebolavirus (TAFV), Bundibugyo ebolavirus (BDBV) and Reston ebolavirus (RESTV),with fruit bats considered as the most likely reservoir host [7].

The Zaire Ebola virus has one of the highest fatality rates of any pathogenic virus affecting

humans. In the 1976 outbreak, it killed 88 percent of patients, 81 percent in 1995, 73 percent in 1996, 80 percent in 2001-2002, and 90 percent in 2003, although none of these outbreaks were as large as the original [6]. However March 2014-Present outbreak involving multiple countries in Africa has reported a total of 21206 cases and 8386 deaths [8].

Sudan Ebola virus had a fatality rate of 53 percent in 1976, 65 percent in 1979, 53 percent in the over 400 patients infected in 2000, 41 percent in 2004, 36.4% in June-October 2012 and 50% in November 2012-January 2013 which occurred in Uganda [6, 9].

Ivory Coast Ebola (TAFV) virus was first discovered in 1994 when a scientist conducting autopsies on chimpanzees contracted Ebola hemorrhagic fever. This has been the only case of Ivory Coast Ebola known to have occurred in humans [6].

In 1989 Crab-eating macaques that were imported from the Philippines to Reston, Virginia were found to have a virus similar to Ebola. Around 6 out of 150 animal handlers developed antibodies to this virus, however none of which actually developed Ebola hemorrhagic fever. It was later classified as Reston Ebola virus and CDC concluded that this strain had a low infection rate for humans [6]. Other episodes occurred in Italy in 1992 and in the United States in 1996. All these events were traced to the facility of a single exporter, but the ultimate source of the virus has never been ascertained, although Mindanao was the origin of the monkeys taken for conditioning and resale. In 2009, an outbreak of Reston Ebola virus was discovered in pigs in the Philippines, and antibody evidence of human infection was also found however the source has not been found [10].

In November 2007, CDC confirmed EHF in diagnostic samples associated with an outbreak of illnesses with unknown etiology in Bundibugyo District, Uganda. Genetic sequencing demonstrated that infections were caused by a novel fifth Ebolavirus species, BEBOV [11], marking the first time a new filovirus species had been identified since 1994 [12] mortality rate being 25%. Then in June-November 2012 another outbreak occurred in Democratic Republic of Congo having a mortality of around 36% [9].

Epidemiology and Transmission

EVD occurred mainly in the rainforest areas of Central Africa (DRC, Sudan, Gabon, and Uganda) up to 2013. *Taï Forest ebolavirus* (TEBOV) affected only West Africa in 1994. The severe epidemics, starting in 2013-14, affected a large West African region (Guinea, Sierra Leone, and Liberia) with imported cases in Nigeria and Senegal. Another alarming event is that the epidemics penetrate densely populated areas including capital cities.

Ebola is transmitted through body fluids and / or direct contact with infected individuals. It is believed to spread to human populations through contact with infected primates, as opposed to directly from natural reservoirs. Fruit bats are the suspected natural sources of the virus. They themselves are asymptomatic but have been found to carry the virus, making them good candidates for natural reservoirs.

Transmission of Ebola virus among non-human animals is a little different. It is proposed that after partially eating the fruits, fruit bats drop these fruits that carry viruses in the bat saliva. Gorillas or other monkeys then eat the fruit, and therefore the virus as well. Decomposing bodies only remain infectious for three to four days after death, and gorillas do not typically interact among different groups, which mean the victims were probably infected by several animal host reservoirs [7]. In addition to high titres of virus in blood, the skin of patients, including fibroblasts and other dermal structures, is extensively infected [13]; this probably accounts for the additional risk to those participating in traditional burial preparation of the cadaver [4] and mourners touching the cadaver [14]. Nevertheless, Ebola has been shown to spread through the air under carefully controlled laboratory conditions [15].

Pathogenesis

Ebola virus disease has findings that are similar in human patients and nonhuman primate models. Viremia occurs in the acute period, and its disappearance is marked by clinical improvement and usually the appearance of antibodies in blood [16]. Humoral immune response is probably not effective because passive convalescent antibody transfer does not protect against experimental inoculation [17, 18]. Possible explanations for the failure to mount an effective immune response in fatal cases include the presence of a putatively immunosuppressive amino acid sequence in the filovirus glycoprotein [19], the secretion of a soluble glycoprotein by Ebola virus–infected cells [20], and the extensive lymphoid damage evident in postmortem examination [21]. In addition, Ebola-infected cells have a deficient response to added

interferon [22, 23], induction of the antiviral state, and induction of interferon or activation of downstream pathways. One major filovirus protein, VP35, is known to be responsible for the latter [24].

Clinical features

The incubation period of EVD in humans is usually 2-21 days [25]. Ebola virus begins to effect infected individuals with flu-like symptoms like fever, myopathy, and headache, followed by hemetemesis and diarrhoea, nausea and vomiting, anorexia, body weakness, abdominal pain, arthralgia, back pain, mucosal redness of the oral cavity, dysphagia, conjunctivitis, rashes on the body [26].

As the disease progresses, wasting becomes evident, and bleeding manifestations such as petechiae, hemorrhages, ecchymoses around needle puncture sites, and mucous membrane hemorrhages occur in half or more of the patients. Around day 5, most patients develop a maculopapular rash, prominent on the trunk. In the second week, the patient defervesces and improves markedly or dies in shock with multiorgan dysfunction, often accompanied by disseminated intravascular coagulation, anuria, and liver failure. Convalescence may be protracted and accompanied by arthralgia, orchitis, recurrent hepatitis, transverse myelitis, or uveitis [17].

Diagnosis

General approach

The approach to evaluating patients with possible Ebola virus disease depends upon whether or not the individual displays appropriate signs and symptoms, how likely it is that the exposure will result in disease (ie, the level of risk), and when the exposure occurred.

1. Patients who present with signs and symptoms consistent with Ebola virus disease should be immediately assessed to determine their risk of exposure to Ebola virus [27].

- For all symptomatic patients who may have been exposed to Ebola virus, infection control precautions should be used and also for patients in whom risk of exposure is unclear at the time of their initial presentation, until a medical evaluation can be performed.
- Testing for Ebola virus should generally be performed for patients who have symptoms consistent with Ebola virus disease and have had an exposure that puts them at risk.

2. Asymptomatic individuals with a possible exposure to Ebola should be monitored so that they can be isolated if signs or symptoms occur.

Indications for initial testing for Ebola virus infection

All patients with suspected Ebola virus disease should be evaluated in conjunction with local and state health departments [28-30].

- Testing for Ebola virus infection is performed in symptomatic patients with any possible risk of exposure to Ebola virus (high, some, or low risk).[27]
- In patients who have an identifiable risk but no signs or symptoms of Ebola virus disease testing is not warranted. These patients should be monitored and tested if they become ill.
- Testing is not warranted for patients without any identifiable risk of exposure to Ebola virus.

Laboratory diagnosis

The laboratory diagnosis of Ebola virus infection is made by the detection of viral antigens or RNA in blood or other body fluids and by viral isolation.

Diagnostic tests

For Ebola virus infection rapid diagnostic tests are the most commonly used tests for diagnosis.

- Most acute infections are diagnosed through the use of RT-PCR which generally detects viral RNA within three days after the onset of symptoms [31, 32].
- For patients with symptoms for fewer than three days duration repeat testing may be needed [32].
- A negative RT-PCR test that is collected e”72 hours after the onset of symptoms rules out Ebola virus disease [31, 33].
- The demonstration of genetic diversity and rapid accumulation of sequence changes of Ebola virus in the West African epidemic indicates that careful monitoring will be needed to ensure the continued sensitivity of RT-PCR diagnostics [34].

- In past outbreaks, testing for viral antigens by enzyme-linked immunosorbent assay (ELISA) was also frequently performed [20, 35-40].
- IgM antibodies detection by capture ELISA is useful in early convalescence [16]. IgG serologic testing has not been reliable. When an indirect fluorescent antibody test is applied, false-positive and irreproducible results are common. For this reason, confirmation, even of apparent seroconversions, is desirable. The IgG ELISA appears to have decreased this problem but still requires further verification [17, 41].

Timeline of Infection	Diagnostic tests available
Within a few days after symptoms begin	Antigen-capture enzyme-linked immunosorbent assay (ELISA) testingIgM ELISAPolymerase chain reaction (PCR)Virus isolation
Later in disease course or after recovery	IgM and IgG antibodies
Retrospectively in deceased patients	Immunohistochemistry testing PCR Virus isolation

Other laboratory findings

Patients with Ebola virus disease typically develop leukopenia, thrombocytopenia, and serum transaminase elevations, as well as renal and coagulation abnormalities. Other findings include a marked decrease in total plasma protein (reflective of a capillary leak syndrome) and elevated amylase levels [42].

Current Treatments

1. Supportive Care

Since there are currently no FDA-approved treatment strategies the current clinical standard for filoviral infection is supportive care. It consists of oral fluid rehydration, oral medication, nutritional supplementation, and psychosocial support [43]. Nasogastric feeding tubes and i.v. administration of both fluids and medication are increasingly considered supportive care where possible during outbreak scenarios to prevent dehydration and facilitate support of blood pressure [43, 44]. Platelet transfusion and replacement of coagulation factors is indicated. Heparin or other treatment should be used for treatment of DIC. In the severe Zaire Ebola virus monkey model, activated protein C improves survival, and this licensed drug should be considered for human therapy [45]. In addition, a recombinant inhibitor of the tissue factor–activated factor VII complex {recombinant nematode anticoagulant protein c2 (rNAPc2)} improves survival and should also be considered [46].

2. Immunotherapy

Transfer of immune serum for the treatment of filovirus infection in humans has previously been attempted. However, interpretation of these results has been cautious due to the study conditions as well as the uncertainty of the disease stage at which the individuals were treated [47]. As a result, much attention has focused on animal studies evaluating candidate products. Both polyclonal and monoclonal passive therapies have been shown to be efficacious in rodents for filovirus infection [27, 48, 49]. These sources of monoclonal antibodies have ranged from murine monoclonal antibodies to recombinant-derived cloned human monoclonal antibodies from survivors of filovirus infection [27, 50].

Vaccines

A DNA vaccine prepared against glycoprotein (GP) induces protective cellular immunity against Zaire strain challenge in guinea pigs but not in monkeys [18]. A vesicular stomatitis virus–based construct using Ebola Zaire GP successfully protected mice and monkeys, including postexposure treatment in some settings [51]. While this type of construct has never been used in humans, no ill effects were seen. A prototype adenovirus vaccine expressing this antigen has successfully protected monkeys and demonstrated a good safety profile in phase I studies in humans [52, 53]

Prevention

If you travel to or are in an area affected by an Ebola outbreak, make sure to do the following:

- Practice careful hygiene. For example, wash your hands with soap and water or an alcohol-based hand sanitizer and avoid contact with blood and body fluids.
- Avoid funeral or burial rituals that require handling the body of someone who has died from Ebola.

- Avoid contact with bats and nonhuman primates.
- Avoid facilities in West Africa where Ebola patients are being treated.
- After you return, monitor your health for 21 days and seek medical care immediately if you develop symptoms of Ebola.
- Healthcare workers who may be exposed to people with Ebola should follow these steps:
- Wear appropriate personal protective equipment (PPE).
- Practice proper infection control and sterilization measures.
- Isolate patients with Ebola from other patients.
- Avoid unprotected contact with the bodies of people who have died from Ebola.
- Notify health officials if you have had direct contact with the blood or body fluids, of a person who is sick with Ebola.

Conclusion

The main goals currently being addressed with Ebola virus are finding ways of treatment for Ebola hemorrhagic fever and finding safe and effective vaccines for the virus that can be applied to humans. If an approved vaccine could be developed for Ebola virus, it would save many people from the painful effects of Ebola hemorrhagic fever. Although it is not a problem right now for most populations outside of Africa, Ebola virus has the potential to be dangerous from the point of view of global health in the future. With more research and a greater understanding of the virus, Ebola will hopefully become a less pressing matter in global health.

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Case Report

A Case of Septicaemia Caused by Shewanella Putrefaciens in a 9 Month Old Infant

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Abstract

Background: Shewanella putrefaciens is as yet rarely responsible for clinical syndromes in humans. It is a gram negative non fermentative, oxidase positive, motile bacillus that is found mainly in marine environments. It produces hydrogen sulfide. It is a facultative anaerobe which grows quickly on both solid and liquid media. It was first isolated from dairy products in 1931 by Derby and Hammer. **Aim** : Infections with Shewanella putrefaciens in humans is on the rise in recent years and it mainly causes soft tissue infections, otitis media, bacteremia and pneumonia with other pathogens like E.coli. **Materials and Methods**: Here we report a case of septicaemia caused by Shewanella putrefaciens in a 9 month old infant. . Blood sample was collected in paediatric blood culture broth in a ratio of 1:5 (blood: broth) and was incubated in BactT/Alert (Biomeriux) and then subcultured on blood and MacConkey agar plates. The growth of gram negative,non fermentative,oxidase positive bacteria was identified as Shewanella putrefaciens by Microscan (Siemens) and was found to be susceptible to Amoxy/clav,cefipime, cefoperazone/sulbactum,cefotaxime,ceftazidime, ciprofloxacillin.doripenum,ertapenem,imipenemmeropenem,piperacillin/tazobactum,tetracycline,ticarcillin/clavulinic acid,tigecycline and Trimethoprim/sulpha. **Conclusion:** Shewanella putrefaciens is as yet rarely responsible for clinical syndrome in humans. However the infection with this organism is on the rise in recent years. So attention should be devoted to unusual pathogens.

Keywords: Shewanella putrefaciens antibiotic susceptibility, identification, infections, septicaemia.

Introduction

Shewanella putrefaciens is a gram negative non fermentative, oxidase positive, motile bacillus that is found mainly in marine environments. It produces hydrogen sulfide. It is a facultative anaerobe which grows quickly on both solid and liquid media. It was first isolated from dairy products in 1931 by Derby and Hammer.[5]

Shewanella putrefaciens can be found in fresh water, brackish and salt water ecosystems. Most research done on Shewanella putrefaciens in relation to marine life concentrates on the prevention of bacterial outbreaks in fisheries.

Shewanella putrefaciens as a human pathogen is very rare. It is typically only seen to effect in humans in combination with other bacterial infections such as E.coli pneumonia and Streptococcus[3,,3,4]

Infections from Shewanella putrefaciens mainly occurs in soft tissues such as skin, intraabdominal areas or in the blood[1,2,6]. It is most commonly thought of as a contaminant along with other bacteria or as a saprophyte surviving with other organisms on previously damaged tissues in the body. Here we report a rare case of septicaemia caused by Shewanella putrefaciens in a 9 month old infant

Case report

A 9 month old infant from a tribal village was admitted in government hospital for pyrexia of unknown origin. Clinically he had high grade fever (39.4^0C), there was no organomegaly, his chest X-ray was normal and his blood reports showed a leucocyte count 29000 / cumm with 85% neutrophils, platelets count 1.80 lakhs / cumm, CRP 18.6 mg / dl (N-0-6). He was empirically put on injectable antibiotics but did not show much improvement. Blood culture was received in Microbiology department of Sampurna Sodani Daignostic Clinc, Indore a standalone diagnostic centre of central Madhya Pradesh in paediatric BacT / Alert bottle. The blood culture bottle was loaded in BacT / Alert system which indicated a growth in the bottle after 24 hours. Subculture was done on Blood agar and MacConkey Agar plates. There was a growth of motile, gram negative non fermentative, oxidase positive bacteria which was identified by MicroScan(Siemens) as Shewanella putrefaciens . NBP panel was used for antibiotic susceptibility testing. On review of literature [11] and according to culture and sensitivity results, therapy of our patient was changed to cefipime. Patient improved dramatically within 48 hours and was discharged on the 10th day.

Materials and Methods

Blood sample was collected in paediatric blood culture broth under aseptic precautions in a ratio of 1:5 (blood: broth) and was incubated in BactT / Alert (Biomeriux) and then subcultured on blood and MacConkey agar plates.On the basis of colony morphology , gram staining, motility NBPC panel was selected for identification and sensitivity of the micro organism. Following criteria was used for identification

Colony morphology:- small 2-3mm diameter ,non lactose fermenting .

1. Grams Staining :- Gram negative bacilli,
2. Motility – motile bacteria in hanging drop preparation
3. Biochemical reaction:- performed on automated Microscan (Siemens)
4. Antimicrobial sensitivity tests:- performed on automated Microscan (Siemens)

Results

The growth of gram negative, non fermentative, oxidase positive bacteria was identified as Shewanella putrefaciens by Microscan (Siemens) and was found to be susceptible to Amoxy / clav, cefipime, cefoperazone / sulbactum, cefotaxime, ceftazidime, ciprofloxacillin.doripenum, ertapenem, imipenemmeropenem, piperacillin / tazobactum, tetracycline, ticarcillin / clavulinic acid, tigecycline and Trimethoprim / sulpha. The MIC pattern of the antibiotics is shown in table 1

Discussion

The infection from Shewanella putrefaciens most commonly involves skin and soft tissue associated with damage to skin (trauma, cut, ulcer)and otitis media. Primary bacteremia with fulminant course is also seen in immunocompromised patients. The source of contamination with Shewanella putrefaciens in our patient could not be confirmed. However parents denied being exposed to any fresh or sea water or trauma or recent travel history. The association between a positive culture and the clinical symptoms with the improvement in clinical picture after the initiation of treatment leaves no doubt about the pathogenic character of the isolate.

Conclusion

Shewanella putrefaciens is as yet rarely responsible for clinical syndrome in humans. However the infection with this organism is on the rise in recent years causing septicaemia, pneumonia, otitis media and wound infections. So attention should be devoted to unusual pathogens.

Table 1: Antibiotic Senstivity pattern of Shewanella putrefaciens

Drugs	MIC	Interpretation
Amikacin	<=16	
Amox/K Clav	<=8/4	S
Amox/K Clav	<=8/4	S
Aztreonam	8	I
Cefazolin	<=8	
Cefepime	<=8	S
Cefaperazone/Sulbactum	<=16/8	S
Cefotaxime	<=1	S
Cefotaxime/K Clavulanate	<=0.5	
Cefoxitin	>16	
Ceftazidime	4	S
Ceftazidime/K Clavulanate	<=0.25	
Cefuroxime	<=4	
Ciprofloxacin	<=1	S
Colistin	<=2	
Doripenem	<=1	S
Ertapenem	<=0.5	S
Gentamycin	<=4	
Imipenem	<=1	S
Levofloxacin	4	I
Meropenem	<=1	S
Pip/ Tazo	<=16	S
Tetracycline	<=4	S
Ticar/K Clav	<=16	S
Tigecycline	<=2	S
Trimeth/Sulfa	<=2/38	S

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Case Report

Candida Krusei Prosthetic Joint Infection Following Fungaemia in a Patient with Total Hip Replacement

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Abstract

Fungal prosthetic joint infections are rare; with *Candida albicans* being the most frequently reported pathogen in English literature. Immunocompromised state, as well as extensive use of the newer triazole, fluconazole to suppress fungal infections has lead to an increase in fungaemia due to albicans and non-albicans candida. Here, we report a case of disseminated *Candida krusei*, in an immunocompromised patient with total hip replacement one month prior following fracture neck of left femur. Patient was deemed infection free following prosthetic joint removal, thorough debridement and antifungal therapy.

Key words: *Candida krusei*, Prosthetic Joint Infections, fungaemia , Biofilm

Introduction

Prosthetic replacement surgery for hip, knee, shoulder and elbow joints has become routine because of the magnificent success of these procedures in restoring function. Infection is the second most common cause of prosthetic joint failure. This is a devastating complication associated with additional surgery, antimicrobial treatment, prolonged rehabilitation as well as the possibility of renewed disability or even death. Emphasis must be laid on accurate, timely diagnosis and appropriate treatment of prosthetic joint infections (PJI). The actual incidence of fungal infections is not known, but is estimated to be about 1% of all PJI. *Candida albicans* is most commonly reported causing candidial fungal prosthetic joint infections. Conventional treatment of fungal PJI usually includes removal of prosthesis followed by a long term antifungal therapy.[1] No case of *Candida krusei* infection of prosthesis has been reported till date in literature. We report a case of total hip replacement infected with this emerging pathogen following fungaemia.

Case report

An 82 year old woman, who had undergone a total hip replacement one month earlier following fracture neck of left femur was admitted in our hospital with a 1 day history of fever(100.4^0 F) with chills, severe pain and limitation of movements of the left hip. There was no recent history of injury around the hip. Associate co-morbid conditions were Insulin Dependent Diabetes Mellitus, hypertension, hypothyroidism, anaemia, electrolyte imbalance and Ischemic Heart Disease.

On clinical examination pulse rate was 80 / min, blood pressure was 130 / 80 mm of Hg, and respiratory rate 20 / min. A 4x4cm deep trophic ulcer extending up to a depth of 5 cm into the surgical

scar, with foul smelling discharge, present over the left greater trochanter and a 2x2cm superficial healing bed sore over the left gluteal region were noted. Erythematous plaques with whitish discharge and maceration present over the inner side of the thighs were diagnosed as candidial intertrigo and grew *C.krusei* on culture.

Laboratory investigations showed haemoglobin-10.4 gm%, Total Leucocyte Count of 19,700 cells / cu mm with N-86%, L-13% and E-1%. ESR was 40 / hr. X-ray showed posterior dislocation of the Austin Moore Prosthesis into the acetabular cavity. Ultrasound abdomen showed right moderate hydronephrosis with cholecystolithiasis. ECHO showed an irregular rhythm (atrial fibrillation) with sclerotic aortic valve. Repeated urine cultures yielded *C.krusei* which was treated with fluconazole. Blood culture isolated *Candida krusei*.

The patient was posted for closed reduction which was unsuccessful and so an open reduction was done. However the reduction was unsuccessful and there was repeated posterior dislocation. The infected AMP implant was removed with thorough debridement of the infected tissue and evacuation of pus in the subcutaneous planes. Gentamicin impregnated cement was moulded onto a Steinman pin in the shape of prosthesis and placed in the acetabular cavity.

The infected tissue, pus and prosthesis was cultured and showed growth of *Candida krusei, Klebsiella ozaenae* and *Staphylococcus epidermidis.*

The patient was treated with Gatifloxacin 400mg OD for 2 weeks and Voriconazole 400mg BD loading dose followed by 200mg BD for 2 weeks. Escharotomy and daily dressings failed to heal the ulcers and Split Thickness Skin Graft was done. Patient was advised total hip replacement. But patient refused surgery and discharged against medical advice and lost for follow up.

Discussion

Candida infection of prosthetic joints is uncommon. Only 30 cases have been reported previously, *C. parapsilosis* and *C. albicans* being the two most common organisms[2].*Candida* infections of prosthetic joints mostly involves hip and knee prostheses than smaller joints due to the longer duration of surgery, low blood flow to cortical bone, and the formation of large haematoma around the devices. These haematomas can devascularise the tissue and prevent the entry of antibiotics[3].

Candida PJI infection is usually associated with prolonged antibiotic treatment, immunocompromised state, I.V drug abuse, prolonged indwelling catheter, rheumatoid arthritis, diabetes mellitus, obesity, poor nutrition and advanced age. [2, 3]The route by which *Candida* reaches the implanted joints cannot be determined in most cases. However in our patient, *C. krusei* isolated in the blood, urine and from the whitish discharge from the thigh indicates it could be haematogenous route. Several studies have found that about 70% of patients develop colonisation by C.krusei before the onset of infection. Gastrointestinal tract was the most frequent site of colonisation followed by respiratory tract.[4,5] This was observed in our case too, as we had isolated the C. krusei from urine and discharge from the inner side of the thigh for which patient received fluconazole. Fungaemia most often develops in immunocompromised patients who undergo antifungal prophylaxis with fluconazole [5, 6]

C.krusei has great ability to colonise inert surfaces such as implants and catheters by virtue of its cell surface hydrophobicity[7].This property may have the clinical implication on the course and treatment of infections linked to medical devices. Slow growth of organisms in biofilms accompanied by changes in cell surface composition affecting the host defence mechanism, restricted penetration of drugs through the matrix and unique biofilm-associated patterns of gene expression.[3] Polymethylemethacrylate cement appears to predispose toward infection by inhibiting phagocytosis and complement function and favours biofilm formation.[8,9]

Although the standard treatment of Candida prosthetic joint infection has not been established, it should include effective antifungal therapy, thorough debridement, and removal of the implants.Intravenous amphotericin B has been the mainstay of treatment for invasive candidiasis Treatment with amphotericin B is restricted by its toxicity. The ease of administration, availability in both oral and intravenous forms and the lack of toxicity have given fluconazole an edge over amphotericin B. However, the emerging non-albicans species, such as *C. krusei and C. glabrata* that are resistant to fluconazole, are susceptible to voriconazole and caspofungin [9].

Most of the prosthetic infections are caused by bacteria. Whenever there is no improvement with antibacterial one should look for fungal causes especially when patient is immunocompromised. An increased understanding of the epidemiology and

pathogenicity of *C. krusei* will aid us in prophylaxis and treatment of infections caused this yeast.

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Short Communication

RNAs in the Acid Tolerance Response and Virulence of *Salmonella*

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*Salmonellaenterica*serovarTyphimurium is a major enteric pathogen capable of causing severe gastroenteritis as well as systemic infections. The intestinal tracts of a diverse range of domestic and wild animals serve as a reservoir for *Salmonella* and consequently infection mainly occurs through the consumption of food and water contaminated with animal waste. This species is responsible for 93.8 million cases of gastroenteritis (80.3 million estimated food-borne), with 155,000 deaths(Majowicz et al., 2010). *Salmonella* spp. are able to survive a variety of stress conditions (pH, high osmolarity, low oxygen tension and bile salts) in their environmental niche, the gastrointestinal tract and the Salmonella containing vacuole(SCV) within macrophages, reflecting the adaptability of these pathogens. Following ingestion, Salmonella traversethe intestinal epitheliumutilizing a mechanism encoded by the horizontally acquired *Salmonella* Pathogenicity Island 1 (SPI1) type 3 secretion system (T3SS). In immunocompromised patients, *Salmonella* are able to cause typhoid like fever owing to their ability to replicate within macrophages.This survival is mediated by *Salmonella* Pathogenicity Island 2 (SPI2) genes encoding another T3SS(Fàbrega & Vila, 2013).

The adaptive response of *Salmonella* to acid stress conditions termed the Acid Tolerance Response (ATR) is vital to their ability to survive and ultimately cause infection. The ATR received attention through pioneering work by Foster and Hall (J. W. Foster & Hall, 1990; J. Foster, 1991, 1993) and defines a system that induces resistance to normally lethal pH, termed as acid challenge, following growth under mild acid exposure, termed as acid adaptation. This response could enhance survival of the bacteria in acidic foods as well as increase survival to harsher pH in the stomach and SCV. Depending on the growth phase, the ATR could be induced either during the log phase or the stationary phase both of which are functionally distinct. The log phase ATR was first described by shifting log-phase *S.* Typhimurium cells grown at pH 7.6, to mild acid (pH5.8, adjusted with hydrochloric acid) for one doubling and subsequent challenge at lethal pH (pH3.3, adjusted with hydrochloric acid). Subsequent studies utilized the same procedure while varying the pH values and exposure times for adaptation and challenge (J. Foster, 1993, Wilmes-riesenberg, Bearson, & Foster, 1996, (Baik, Bearson, Dunbar, & Foster, 1996; Bang, Kim, Foster, & Park, 2000). The stationary phase ATR was induced by growing overnight cultures to attain a final pH of 7.4 to 4.3 followed by challenge at pH 3.0(Lin, Lee, Frey, Slonczewski, & Foster, 1995). It was observed that stationary phase cells allowed to grow at a pH<5.0, showed significantly better survival than those adapted at higher pH values.

Salmonella have long served as model organisms for studying pathogenesis, virulence, gene regulation and evolution with most of the focus on proteins and their involvement. However, it is only of recent that *Salmonella* has come to the fore as a model organism for studying non-coding RNA mediated regulation. The diverse families non-coding RNAs may fall into include small RNAs (sRNAs) that are regulatory in nature, cis-regulatory elements (riboswitches and thermometers) and RNAs that bind to and interact with proteins.It is only in the last two decades that sRNAs have started to gain momentum as important

regulators in diverse species. They range in size from 50 to 500 nucleotides, do not contain expressed open reading frames (ORFs) and are largely conserved in related species. They bring about their regulatory effects either by binding (cis or trans) to target mRNA and affecting their translation / stability, or by directly binding to proteins and modulating their activity (Altuvia, 2007). Translational repression is brought about by blocking of the ribosome binding site due to sRNA binding and activation, by unraveling of secondary structure that inhibit ribosome binding.

Both, acid tolerance and virulence are essential to the efficient survival and propagation of *Salmonella* in a host. Various protein coding genes involved in the ATR have also been shown to be essential for virulence such as the Mg^{2+} proton translocating ATPase encoded by *atp*. Consequently an *atp* mutant was found to be avirulent(Garcia-del Portillo, Foster, & Finlay, 1993). Similarly, atrC and fur mutants were found to be less virulent as compared to their wild-types (Wilmes-riesenberg et al., 1996). Similar studies in *Listeria monocytogenes*have shown that some acid tolerant mutants displayed increased virulence when compared to their wild-type(Hill, 1996).Additionally, the glutamate decarboxylase (GAD) system has also been shown to be important for both low pH survival and overall virulence. Similarly, several sRNAs have been shown to be involved in response to acid stress, with probable roles in virulence as well. For example, in *E.coli*the DsrAsRNA is a well known acid resistance regulator, with probable roles in virulence (Lease, Smith, Mcdonough, & Belfort, 2004). Other sRNAssuch as RprA, ArcZ and GcvB have also been shown to play similar roles with still more (IsrM, IsrC, IsrE) being implicated in virulence (Tracy, Gaida, & Papoutsakis, 2010). The link between the different acid tolerance systems and virulence should further be examined particularly in the case of enteric pathogens and with a focus on sRNA regulators involved. This would help provide a clear picture on the regulatory circuits that play roles in survival and pathogenesis.

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Article in supplement or special issue

[3] Fleischer W, Reimer K. Povidone iodine antisepsis. State of the art. Dermatology 1997;195 Suppl 2:3-9.

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Reference from electronic media

[9] National Statistics Online—Trends in suicide by method in England and Wales, 1979-2001. www.statistics.gov.uk/downloads/theme_health/HSQ 20.pdf (accessed Jan 24, 2005): 7-18. Only verified references against the original documents should be cited. Authors are responsible for the accuracy and completeness of their references and for correct text citation. The number of reference should be kept limited to 20 in case of major communications and 10 for short communications.

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