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Induction to Viable but Non Culturable State and Resuscitation of *Salmonella* and *Pseudomonas*

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Abstract

Salmonella and *Pseudomonas* were induced to Viable but Non Culturable state by starvation and temperature up shift to 50°C. The change in osmolarity by addition of more than 10%NaCl also resulted in induction into VBNC state of both Salmonella and Pseudomonas. In the VBNC state these cultures lost their capacity to reduce nitrate. *Salmonella* also lost the capacity to ferment mannitol, sorbitol, xylose and citrate utilization. On the other hand, for *Pseudomonas* the capacity to ferment mannitol was lost in VBNC state. While serum and egg yolk could not help the resuscitation of these cultures but favourable temperature between 35°C - 40°C and enrichment of media would help in resuscitation of these cultures. There were distinct differences in the VBNC state with respect to many enzyme activity.

Keywords: *Salmonella* | *Pseudomonas* | Culture

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Introduction

Bacteria are considered a diverse group and are almost omnipresent in nature. At times conditions may prevail which may not be conducive to the overall well being or growth of the living system. Almost every living system experiences such odd conditions. Bacteria often face conditions that are not conducive to growth and multiplication. This may led to enter into various states which includes the VBNC (Viable but Non Culturable) state. A characteristic of these cells is that, though very much viable and metabolically active, they show a loss of Culturability on routine culture media. Various workers have studied and given evidences of bacteria entering the VBNC state. (Muela *et al.*, 2008, Signoretto *et al.*, 2000, Shleeva *et al.*, 2004, Asakura *et al*., 2008).

Moreover VBNC state is not a permanent state and the VBNC state organisms can be reverted back to a culturable state. This phenomenon is known as 'Resuscitation'. Various factors like temperature up shift increase in nutrient concentrations, and supplementation of additional substances and at times presence of host cells can cause resuscitation.

It is observed that the organisms retain their virulence potential upon resuscitation by many workers as in *Mycobacterium tuberculosis* (Laam li *et al.,* 2014) *Legionella sps* (Steinert *et al.,* 1997, Al Bama *et al.,* 2013). *Vibrio sps*. (Baffone *et al.,* 2003, Sun *et al.,* 2008) A number of virulence and toxin genes are expressed even in the VBNC state (Vora *et al.,* 2005). This can be particularly dangerous as the non detected VBNC state cells might gain entry into host body and reasocitate in favourable conditions in the host body causing diseases, the cause of which may remain unknown. Thus it is highly essential to intensely study hundreds of pathogens which may remain in our surroundings in the VBNC state and may well be the cause of recurrent infections.

Materials and Method

Induction of VBNC State of the Isolates

The isolates were subjected to nutrient deprivation and starvation by either prolonged incubation or by altering the media composition. This was found to be the most effective inducing factor for getting the VBNC state .the media composition was altered by gradually changing the contents from 100% to 10% For *e.g.*: The nutrient content of nutrient broth was reduced 13 gm / litre to up to 0.5 gm per litre (Lemke and Leff, 2006, Rahman *et al.,* 2001).

Induction into VBNC state using osmotic pressure

Salt and sugar concentration in the medium were increased from 2% to 10% for generating severe osmotic shock. The isolates were grown in high osmotic conditions by incubating at 37°C till the VBNC state is achieved. (Inglish and Sagripanth 2006)

Induction of VBNC state using elevated temp of incubation

The isolated were grown at high temp of incubation up to 50°C fir getting VBNC states (Reissbrodt *et al.,* 2002)

Resucitation of the VBNC states of the Isolates

Resuscitation of VBNC state was carried out by different methods. The media composition was altered and enriched with either egg yolk or serum. The VBNC organisms were grown in the enriched media and incubated at 37°C until resuscitation occurs. In some of the cases the resuscitation was done by temperature modulation or temp downshift as done by many workers. (Mukamolova *et al.,* 1998 and Downing *et al.,* 2005; Reissbrodt *et al.,* 2002.)

Comparative Morphological, Biochemical Characterization, Carbohydrate fermentation and Enzyme Studies were done to compare the Normal state, VBNC and Resuscitated state.

It is very essential to examine a large number of food items that are available in the market to ascertain the existence of VBNC state of different microbes. The improvement of human health necessitates a thorough and deep study in this field.

Results and Discussion

In case of Nutrient deprivation or starvation almost all the organisms entered into VBNC state when the nutrient supplied was reduced to around 60%. It was observed that *Salmonella* entered into VBNC state far quickly than Pseudomonas. Similarly the temperature

modulation showed a very distinct variation whereas the temp up shift to about 50°C resulted in induction into VBNC state. It was noted that the difference in the total and viable counts was starkly different in case of nutrient deprivation in comparison to temperature up shift. Therefore it seems that starvation is better for induction into VBNC state. When osmotic pressure was varied by the addition of salt and sugar it is observed that addition of sugar did not induce any of the cells into VBNC state. Both *Salmonella and Pseudomonas* tolerated the additional supplementation of sucrose. However, addition of sodium chloride at 10% resulted in induction into VBNC state. Lower than 10% of sodium chloride addition did not result in induction into VBNC state.

As can be seen from table 8 that none of the organisms could be resuscitated in 72 hrs by the addition of serum in the medium. The cfu/ml did not rise above 10/ml in any of the cultures. This indicates that serum does not act as stimuli for resuscitation. Most of the colonies that grew on the cfu plates were of diminished size and did not show the typical colony characteristics of both the cultures under study. The serum contents are not able to provide the conducive environment or nutrient for resuscitation.

Egg yolk is generally considered to be rich in proteins and other growth factors. however supplementation of egg yolk at around 10 ml level could partially resuscitate as cfu rise above 10 /ml marginally as can be seen from table 9. In comparison to serum egg yolk was able to resuscitate partially but did not sufficiently resuscitate the VBNC cultures. The colony characteristics on the cfu plates were much larger in size in comparison to the colony size with serum addition.

Temperature up shift between 35 to 40°C resuscitated as the cultures with increase in cfu /ml as can be seen from table 10.Both the cultures showed typical colony characteristics upon resuscitation.

Enrichment of the media by adding additional amount of dehydrated medium reduced the starvation effect and increased the cfu/ml thereby resuscitating both the cultures. The colony characteristics of both the cultures were found to be same as the characteristics shown by normal viable and culturable cells.

In biochemical tests both *Salmonella* and *Pseudomonas* lost their capacity to reduce nitrate in their VBNC state and all these cultures regain this property upon resuscitation. In *Pseudomonas* loss of citrate utilization was observed. *Salmonella* also loses the capacity to ferment Mannitol, Sorbitol and Xylose (Table 15).Although *Pseudomonas* in their normal state hardly ferments most of the sugars but they do ferment mannitol. However in VBNC state they lose this capacity to ferment mannitol as can be seen from the table 16. Both *Salmonella* and *Pseudomonas* lost the Catalase property during entry to VBNC. The lack of Catalase in the VBNC state clearly states the necessary stress that is responsible for entry into the VBNC state. *Salmonella* apart from Catalase it loses the lysine activity. Similarly *Pseudomonas* also shows loss of Arginine dehydrolase activity

In the present study nutrient deprivation induced both *Salmonella* and *Pseudomonas* to enter into VBNC state. As can be seen from table 1 when nutrients are very low the ratio between the viable count and the total count is very high and cfu/ml is far below 10. Similar difference between the viable and total count has been reported by other workers. (Chaveerach *et al.,* 2003). Prolonged incubation resulted in successful establishment of VBNC state. The elevation of temperature of incubation overall 50°C results in induction into VBNC state but low temperature did not favour induction into VBNC state. 'Osmotic pressure due to addition of salt resulted in induction into VBNC state but additional sugar addition did not act as a factor for induction into VBNC state. Also resuscitation could not be attained by the addition of serum or egg yolk. However temperature modulation resulted in resuscitation within 72 hrs .Similarly the increase in the nutrient content resulted in resuscitation. The resuscitation should be improved further by increasing the incubation period. Such work has been reported by many workers. (Pawlowski *et al.,* 2011, Amel *et al.,* 2008, Panutdaporn 2006, Bovill and Mackey, 1997)

In the present study distinct changes were observed in the biochemical characteristics of the organisms. While Catalase activity was absent in VBNC cells the lack of Catalase must have resulted in stress of reacting oxygen species .Distinctly nitrate reduction and argine dehydrolase activity have been markedly accepted in the VBNC state.

It is very essential to examine a large number of food items that are available in the market to ascertain the existence of VBNC state of different microbes. The improvement of human health necessitates a thorough and deep study in this field.

S. No	Organism	Number of Colonies (cfu/ml)/Total Count (a*10^3)								Induction into VBNC state
		12gms	10gms	8gms	6gms	4gms	2gms	1gms	0.5gms	
1	Salmonella	37/52	32/50	20/48	7/46	6/46	5/46	2/44	1/45	+
2	Pseudomonas	36/48	32/44	15/45	12/45	10/44	7/48	7/48	5/47	+

+ = Positive Reaction: - + Negative Reaction ; ND=Not Detected

Table 1: Induction into VBNC state of different organism by Nutrient Deprivation

S. No	Organism	Morphology	Gm Reaction
1	Salmonella	Very short rods with broad width	Gm –ve
2	Pseudomonas	Short rods, almost coccobacilli	Gm –ve

Table 2: Morphological variation in VBNC State

S.No	Organism	Incubation in days	Number of colonies/ Total Count(a*10^3)	Morphology
1	Salmonella	40	05/46	Very short rods with broad width
2	Pseudomonas	50	07/59	Short rods almost coccobacilli

Table 3: Induction into VBNC state by prolonged incubation

S. No	Organism	No. of colonies(Cfu/ml)/ Total Count ($a*10^3$)				Induction into VBNC State
		39°C	42°C	46°C	50°C	
1	Salmonella	47/49	40/48	30/48	9/49	+
2	Pseudomonas	46/52	42/52	12/54	10/51	+

+ = Positive Reaction: - + Negative Reaction ;ND=Not Detected

Table 4: Induction into VBNC state by High Temperature Incubation

S. No	Organism	No. of colonies (Cfu/ml)/Total Count ($a*10^3$)				Induction into VBNC State
		20°C	10°C	6°C	4°C	
1	Salmonella	41/51	32/49	30/52	10/49	06/47
2	Pseudomonas	32/46	30/48	18/49	07/49	02/47

+ = Positive Reaction: - + Negative Reaction ;ND=Not Detected

Table 5: Induction into VBNC state by Low Temperature Incubation

S. No	Organism	No. of Colonies (Cfu/ml)/ Total Count ($a*10^3$)					Induction into VBNC State
		2%	4%	6%	8%	10%	
1	B.cereus	40/48	37/49	37/46	15/48	07/49	+
2	E.coli	45/49	42/49	37/48	16/46	06/48	+

+ = Positive Reaction: - + Negative Reaction ;ND=Not Detected

Table 6: Induction into VBNC state by Osmotic Pressure using Salt

S. No	Organism	No. of Colonies (Cfu/ml)/Total Count($a*10^3$)					Induction into VBNC State
		2%	4%	6%	8%	10%	
1	Salmonella	30/40	30/42	35/42	34/46	37/43	ND
2	Pseudomonas	47/49	46/48	32/45	34/46	36/44	ND

+ = Positive Reaction: - + Negative Reaction ;ND=Not Detected

Table 7: Induction into VBNC state by osmotic pressure using Sugar

S. No	Organism	Concentration of Serum in ml								
		0.1	0.5	1	2	4	6	8	10	Incubation Period
		No. of Colonies(Cfu/ml)/Total Count($a*10^3$)								
1	Salmonella	0/44	0/46	1/44	1/44	4/44	7/48	10/46	10/45	72 hrs
2	Pseudomonas	1/45	1/46	2/46	2/44	2/43	6/44	7/48	8/47	72 hrs

Table 8: Resuscitation by addition of Serum

S.No	Organism	Concentration of Egg yolk								
		0.1	0.5	1	2	4	6	8	10	Incubation Period
		No of Colonies(Cfu/ml)/Total Count ($a*10^3$)								
1	Salmonella	0/45	1/46	2/44	4/44	6/48	8/46	10/48	14/46	72 hrs
2	Pseudomonas	0/45	2/48	2/46	2/44	4/48	6/46	8/46	12/48	72 hrs

Table 9: Resuscitation by addition of Egg Yolk

S. No	Organism	Temperature of Incubation				
		4+-2°C	10+-2°C	37+-2°C	40+-2°C	Incubation Period
		No of Colonies(Cfu/ml)/Total Count ($a*10^3$)				
1	Salmonella	5/44	6/48	45/48	45/49	72 hrs
2	Pseudomonas	4/44	5/47	45/48	38/48	72 hrs

Table 10: Resuscitation by Temperature modulation

S. No	Organism	Concentration of Nutrient media (gms/ltr)			Incubation Period
		13	35	40	
		No. of Colonies(Cfu/ml)/ Total Count($a*10^3$)			
1	Salmonella		4/44	20/42	22/42
2	Pseudomonas		4/44	45/46	25/42

Table 11: Resuscitation by increase in Nutrient Content

S. No	Organism	Gram Reaction	Motility	Spore Staining	Morphology
1	Salmonella	Gm -ve	Actively motile	Non sporulating	Very short rods with broad width

Table 12: Morphological Characterization of Resuscitated Cultures

S. No	Characteristics	Organism		
		Normal State	VBNC state	Resuscitated state
1	Indole	-	-	-
2	Methyl Red	+	+	+
3	Voges-Proskauer	-	-	-
4	Citrate	-	-	-
5	Nitrate reduction	+	-	+
6	H_2S	ND	ND	ND

+ = Positive Reaction: - + Negative Reaction ;
ND=Not Detected

Table 13: Comparative Biochemical characteristics of *B.cereus*

S. No	Characteristics	Organism		
		Normal State	VBNC state	Resuscitated state
1	Indole	-	-	-
2	Methyl Red	-	-	-
3	Voges-Proskauer	-	-	-
4	Citrate	+	-	+
5	Nitrate reduction	+	-	+
6	H_2S	-	-	-

+ = Positive Reaction: - + Negative Reaction;
ND=Not Detected

Table 14: Comparative Biochemical characteristics of *E.coli*

S. No	Characteristics	Organism		
		Normal State	VBNC state	Resuscitated state
1	Glucose	+	+	+
2	Galactose	ND	ND	ND
3	Maltose	+	+	+
4	Mannitol	+	-	+
5	Lactose	-	-	-
6	Inulin	ND	ND	ND
7	Fructose	ND	ND	ND
8	Arabinose	-	-	-
9	Sorbitol	+	-	+
10	Starch	ND	ND	ND
11	Sucrose	-	-	-
12	Xylose	+	-	+

+ = Positive Reaction; - + Negative Reaction;
ND=Not Detected

Table 15: Comparative Carbohydrate fermentation of *B.cereus*

S. No	Characteristics	Organism		
		Normal State	VBNC state	Resuscitated state
1	Glucose	-	-	-
2	Galactose	ND	ND	ND
3	Maltose	-	-	-
4	Mannitol	+	-	+
5	Lactose	-	-	-
6	Inulin	-	-	-
7	Fructose	-	-	-
8	Arabinose	-	-	-
9	Sorbitol	-	-	-
10	Starch	ND	ND	ND
11	Sucrose	-	-	-
12	Xylose	ND	ND	ND

+ = Positive Reaction; - + Negative Reaction;
ND=Not Detected

Table 16: Comparative Carbohydrate fermentation of *E.coli*

S. No	Characteristics	Organism		
		Normal state	VBNC state	Resuscitated state
1	Oxidise	-	-	-
2	Catalase	+	-	+
3	Urease	-	-	-
4	Arginine dehydrolase	-	-	-
5	Esculin hydrolysis	-	-	-
6	Acetate utilization	-	-	-
7	Phenylalanine deaminase	ND	ND	ND
8	Ornithine decarboxylase	-	-	-
9	Lysine	+	-	+

+ = Positive Reaction; - + Negative Reaction;
ND=Not Detected

Table 17: Comparative enzyme study of *B.cereus*

S. No	Characteristics	Organism		
		Normal state	VBNC state	Resuscitated state
1	Oxidase	-	-	-
2	Catalase	+	-	+
3	Urease	-	-	-
4	Arginine dehydrolase	+	-	+
5	Esculin hydrolysis	ND	ND	ND
6	Acetate utilization	+	-	+
7	Phenylalanine deaminase	-	-	-
8	Ornithine decarboxylase	+	-	+
9	Lysine	+	-	+

+ = Positive Reaction: - + Negative Reaction;
ND=Not Detected

Table 18: Comparative enzyme study of *E.coli*

Conclusion

Both *Salmonella* and *Pseudomonas* sps could be induced to VBNC state and were resuscitated by suitable stimulating factors.

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