Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Influence of Different Storage Media, Temperatures and Time Duration on Susceptibility of *Ornithobacterium rhinotracheale*

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ARTICLE HISTORY

Received: March 20, 2012
Revised: April 17, 2012
Accepted: April 26, 2012

Key words:
Diagnosis
ORT
Transport medium
Viability

ABSTRACT

Ornithobacterium rhinotracheale (ORT) is an important respiratory pathogen of chickens and turkeys. Isolation of the bacterium from diseased birds is necessary for serotyping, to determine the antimicrobial susceptibility for an effective therapy and to produce autogenous vaccines. A series of experiments was carried out to determine optimal conditions for storage of swabs soaked in ORT suspension. Swabs were immersed in viable ORT suspensions with different bacterial counts and then stored under different conditions. At several time points the viable ORT count in the swabs was determined. Dry cotton swabs as well as three transport media, namely Amies gel medium (AG), Amies gel medium with charcoal (AC), and Stuart gel medium (SG) were tested. ORT could be reisolated from dry swabs stored at room temperature for up to five days and from swabs stored in the media at room temperature for more than seven days. Differences among the transport media were minor. The minimal number of cfu in the ORT-suspension, in which the swabs were soaked, was 10^5 cfu/ml for successful reisolation of ORT one day post immersion from swabs stored at room temperature in AC medium, and 10^6 cfu/ml was successful for reisolation from dry swabs. Higher inoculation doses and storage at 4°C prolonged the period in which ORT could be reisolated. Storage of dry swabs at -20°C allowed reisolation of ORT at a constant level for at least 5 d.p.i. Inoculation of swabs with ORT and *E. coli* reduced the period for which ORT could be reisolated.

To Cite This Article: Numee S, R Hauck, and HM Hafez, 2012. Influence of different storage media, temperatures and time duration on susceptibility of *Ornithobacterium rhinotracheale*. Pak Vet J, 32(3): 438-442.

INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is an important respiratory pathogen of chickens and turkeys with worldwide distribution (Chansiripornchai *et al*., 2007; Murthy *et al*., 2008; Tabatabai *et al*., 2008; Ghanbarpour and Salehi, 2009). ORT is a fastidious, Gram negative, oxidase positive rod. It grows slowly but can be isolated on blood agar at 37°C under microaerophilic conditions (Van Empel and Hafez, 1999, Hafez and Vandamme, 2011). By agar gel precipitation test 18 serotypes can be distinguished (Van Empel and Hafez, 1999; Chin *et al*., 2008). Since clinical signs and post-mortem lesions of ORT infections are not sufficiently specific to allow diagnosis, laboratory methods are needed for definite diagnosis. While detection of nucleic acids by PCR is reliable and fast (Hassanzadeh *et al*., 2010), isolation of the bacterium is necessary for serotyping, to determine the antimicrobial susceptibility for an effective therapy, and to produce autogenous vaccines.

However, many factors can interfere with isolation of ORT such as the time of sampling, presence of secondary infections and shipment from farm to the diagnostic laboratory. While ORT can readily be isolated from infected birds in an early stage of the infection, the recovery of ORT in later stages may fail (Kilic *et al*., 2009). After an ORT infection, other bacteria, especially *E. coli* (Sakai *et al*., 2000; Sprenger *et al*., 2000), can induce secondary infections. Because these bacteria have a higher tenacity and grow faster, they may overgrow the fastidious and slowly growing ORT when isolation is tried.

Mostly tracheal swabs or swabs taken from lungs or air sacs at post mortem are sent instead of organs. Swabs for microbiological analysis are usually placed in various media for transport to the laboratory. Swabs could be placed into a nonnutritive transport medium, which keeps

the bacteria viable, but does not permit overgrowth of one pathogen by other bacteria present in the sample (Rosa-Fraile *et al*., 2005, Morosini *et al*., 2006). A large number of studies evaluated different swabs and transportation systems with a variety of anaerobes and fastidious aerobes (Thompson and French, 1999; Morosini *et al*., 2006). However, there is no information in the literature on the comparative performance of various transport systems in regard to ORT.

Thus the objective of this study was to determine optimal conditions for collection, and storage conditions for the samples to be transported to laboratory for successful ORT detection.

MATERIALS AND METHODS

Strains used to contaminate the swabs: Gentamycin resistant ORT strain of serotype A (B3263/91) was used as standard strain for all experiments and was kindly provided by Intervet International Boxmeer, The Netherlands. Additionally the effect of the bacterial counts in the ORT suspension, in which the swabs were soaked, and storage temperatures were tested with field isolates F56/10 (serotype A), F488/10 (serotype B), and F94/09 (serotype E), which were isolated and serotyped in our laboratory as described by Hafez and Sting (1999). ORT was grown on 5% sheep blood agar plates with 10 µg/ml gentamycin. The plates were incubated microaerobically in 5% CO_2 atmosphere at 37°C for 48 hr.

Escherichia coli strain GB 1927/10/3 was used to determine the effect of storage of ORT together with *E. coli* on ORT reisolation. It was isolated from turkeys and classified as susceptible against gentamycin by agar diffusion test. It was grown on Columbia agar (Oxoid, Wesel, Germany) at 37°C for 24 h.

For preparation of the inocula plates were flooded with PBS. An initial bacterial suspension containing 10^7 – 10^8 cfu/ml in PBS was prepared for each experiment by adjusting turbidity to McFarland standard 0.5.

Viable bacterial counts: Viable bacterial counts were determined by preparing a tenfold dilution series in PBS. Then 100 µl of each dilution were streaked on plates with Drigalski spatula and incubated as described above. ORT colonies were visually counted after 48 h.

Comparison between different transport media and dry swabs: Dry cotton swabs and three different transport media, namely i) Amies gel medium (AG), ii) Amies gel medium with charcoal (AC) and iii) Stuart gel (SG) medium, (all COPAN, Brescia, Italy) were used. Swabs were immersed for 2 min in an ORT suspension with a McFarland turbidity of 0.5 and then placed into their respective transport media. Dry swabs were stored in sterile glass tubes. The swabs were held at room temperature. After various time intervals two swabs of each medium were suspended in 1 ml sterile PBS each and the bacterial counts were determined.

Effect of ORT concentrations on the viability after storage of dry cotton swab at room temperature: In a first experiment sterile dry cotton swabs were used as bacterial carriers and immersed for 2 min in an ORT suspension with a McFarland turbidity of 0.5. Then they were removed and kept in sterile glass tubes at room temperature. After various time intervals two swabs of each storing temperature were suspended in 1 ml sterile PBS each and the bacterial counts were determined.

In a second experiment with similar design swabs were stored at 4°C or -20°C for various time intervals.

Dry swabs absorbed in different ORT concentrations: Starting with an ORT suspension with a McFarland turbidity of 0.5 a tenfold dilution series was prepared in sterile PBS to a dilution of $1:10^6$. Sterile, dry cotton swabs were immersed for 2 min in each dilution. Then they were removed and kept in sterile glass tubes at room temperature. After various time intervals two swabs of each concentration were suspended in 1 ml sterile PBS each and the bacterial counts were determined.

Effect of ORT concentrations on the reisolation after storage of swabs in AC media at different temperatures: Starting with an ORT suspension with a McFarland turbidity of 0.5 a tenfold dilution series was prepared in sterile PBS to a dilution of $1:10^4$. Swabs of the transportation system using AC medium were immersed for 2 min in the undiluted suspension as well as in the $1:10^2$, $1:10^4$ and $1:10^6$ dilutions. The swabs were placed into their plastic devices containing the medium and held at room temperature. After various time intervals two swabs of each combination of inoculation concentration and storage temperature were suspended in 1 ml sterile PBS each and the bacterial counts were determined. The experiment was repeated with field strains F56/10, F488/10, and F94/09, of which only the undiluted inocula and the $1:10^2$ dilutions were tested.

Effect of storage of ORT in mixed culture with *E. coli* on the reisolation: Eight suspensions containing different concentrations of ORT and/or E. coli were prepared by mixing equal parts of ORT and *E. coli* suspensions. Suspensions were either adjusted to McFarland standard 0.5 (about. 10^7 cfu/ml) or diluted $1:10^2$ (about 10^5 cfu/ml). Swabs of the AC transportation system were immersed for 2 min in the bacterial suspensions. The swabs were placed into their plastic devices containing the medium and held at room temperature. At various time intervals two swabs of each inoculum were streaked directly on 5% sheep blood agar plates with gentamycin and on Gassner agar (Oxoid, Wesel, Germany).

RESULTS

Comparison between different transport media: The total ORT count in the inoculum was $10^{7.3}$ cfu/ml. The viable counts of reisolated ORT from swabs kept in Amies gel medium and AC medium as well as in Stuart gel medium were similar. Until the end of the experiment on day 7 reisolation counts showed a slow but steady decline from about $10^{5.2}$ cfu to 10^3 cfu, but ORT was reisolated from all swabs stored in media throughout the experiment. In contrast the viable bacterial counts from dry swabs decreased faster, and 6 and 7 days post inoculation (d p. i.) no ORT was reisolated from dry swabs (Table 1).

Table 1: Mean (n=2) $\log_{10}$ of cfu *Ornithobacterium rhinotracheale* (ORT) reisolated from dry swabs and swabs of transport systems with Amies gel (AG) medium, Amies gel medium with charcoal (AC) or Stuart gel (SG) medium stored at room temperature for various time intervals.

Medium	ORT count in inoculum (cfu/ml)	Storage time								
		3 h	6 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d
Dry	$10^{7.3}$	6.27	5.45	5.86	4.73	2.40	2.95	3.48	-*	-
AG	$10^{7.3}$	5.36	5.66	5.34	5.11	4.39	4.10	3.70	3.11	2.98
AC	$10^{7.3}$	5.13	5.45	5.28	4.85	4.45	4.54	3.40	3.55	3.37
SG	$10^{7.3}$	5.16	5.37	5.15	4.48	4.43	4.38	3.30	3.46	3.26

*no ORT reisolated

Dry swabs stored at different temperatures: Viable ORT counts in the inocula were $10^{7.7}$ cfu/ml in the first experiment and $10^{7.6}$ cfu/ml in the second experiment. ORT counts obtained from dry swabs stored at room temperature declined quickly within the first two days p. i. to $10^{1.6}$ cfu (Table 2). In contrast viable ORT counts from dry swabs stored at 4°C stayed almost constant at about 10^6 cfu after 2 days in the first experiment and $10^{4.5}$ cfu in the second experiment for the first three days, before decreasing sharply to about 10^2 cfu at day 5. Storage of the dry swabs at -20°C allowed recovery of ORT at a constant level of about $10^{4.7}$ cfu (Table 3), till 2nd day.

Table 2: Mean (n=2) $\log_{10}$ of cfu *Ornithobacterium rhinotracheale* (ORT) reisolated from dry swabs stored at room temperature (RT) and at 4°C for various time intervals.

Storage temperature	ORT count in inoculum (cfu/ml)	Storage time			
		3 h	6 h	1 d	2 d
RT	$10^{7.7}$	5.99	6.26	4.15	1.63
4 °C	$10^{7.7}$	6.13	6.20	5.99	5.75

Effect of ORT storage on dry cotton swab at room temperature: The ORT count in the undiluted inoculum was $10^{7.7}$ cfu/ml. The two highest inoculation doses of $10^{7.7}$ cfu/ml or $10^{6.7}$ cfu/ml allowed viability until 1 d p. i. From swabs inoculated with $10^{5.7}$ cfu/ml or $10^{4.7}$ cfu/ml ORT could be recovered 3 hours post inoculation (h.p.i.) and 6 h p. i. From swabs inoculated with $10^{3.7}$ cfu/ml only 3 h p. i. ORT was reisolated. From swabs inoculated with $10^{2.7}$ cfu/ml or $10^{1.7}$ cfu/ml no ORT was found viable (Table 4).

Table 3: Mean (n=2) $\log_{10}$ of cfu ($10^{7.6}$) *Ornithobacterium rhinotracheale* (ORT) reisolated from dry swabs stored at 4°C and at -20°C for various time intervals.

Storage time	Storage temperature (°C)	
	4	-20
3 h	4.80	4.79
6 h	4.75	4.78
1 d	4.58	4.76
2 d	4.42	4.70
3 d	4.42	4.55
4 d	2.39	4.50
5 d	2.00	4.59

Susceptibility of ORT concentrations after storage of swabs in AC media stored at different temperatures: The ORT count in the undiluted inoculum was $10^{7.8}$ cfu/ml. From swabs inoculated with undiluted suspension and stored at room temperature reisolation counts were between 10^4 cfu and 10^5 cfu until 4 d p. i.. Afterwards they declined. On day 7 p. i. reisolation was still possible from reference strain A (B3263/91) as well as from field isolates F56/10 and F488/10, but not from field isolate F94/09. 14 d p. i. no reisolation was possible from all tested swabs. In contrast from swabs inoculated with undiluted suspension and stored at 4°C up to $10^{3.7}$ cfu were reisolated 14 d p. i., only field isolate F94/10 could not be reisolated from these swabs.

Inoculation with the $1:10^2$ dilution of the inoculum allowed reisolation until 2 d p. i. from swabs stored at room temperature. ORT counts reisolated from swabs inoculated with the $1:10^2$ dilution of the inoculum and stored at 4°C decreased only slowly from about $10^{3.3}$ cfu 1 d p. i. to about 10^2 cfu 7 d p. i.. 14 d p. i. reisolation was not possible. From swabs inoculated with $10^{3.8}$ cfu/ml or $10^{1.8}$ cfu/ml of the reference strain A (B3263/91) no ORT could be reisolated 1 d p. i. (Table 5).

Effect of storage of ORT in mixed culture with *E. coli* on the reisolation: From swabs inoculated with $10^{7.0}$ cfu/ml ORT without *E. coli* ORT was reisolated until 6 d p. i., from the swabs inoculated with $10^{5.0}$ cfu/ml ORT without *E. coli*, ORT was reisolated until 3 d p. i.. Absorbing the swabs additionally with *E. coli*, regardless of the bacterial counts used, shortened the period in which ORT was reisolated to 3 days and 2 days p. i., respectively. *E. coli* was reisolated from all swabs whose inoculum had contained *E. coli* throughout the whole experiment, and it even frequently grew on the blood agar containing gentamycin (Table 6).

Table 4: Mean (n=2) $\log_{10}$ of cfu *Ornithobacterium rhinotracheale* (ORT) reisolated from dry swabs inoculated with suspensions containing different ORT counts and stored at room temperature for various time intervals.

ORT count in inoculum (cfu/ml)	Storage time at room temperature			
	3 h	6 h	1 d	2 d
$10^{7.7}$	6.83	6.08	5.15	-*
$10^{6.7}$	5.31	5.09	2.45	-
$10^{5.7}$	3.82	3.77	-	-
$10^{4.7}$	3.46	3.35	-	-
$10^{3.7}$	2.15	-	-	-
$10^{2.7}$	-	-	-	-
$10^{1.7}$	-	-	-	-

*no ORT reisolated

DISCUSSION

A series of experiments was conducted to determine optimal conditions for storage of swabs absorbed with ORT. These conditions should help to determine the possible optimal conditions for shipment of the swabs from farm to the diagnostic laboratory.

Three transport media, namely Amies gel medium, Amies gel medium with charcoal (AC) and Stuart gel medium were included in the investigation and compared to dry swabs. Amies medium is a variation of Stuart medium containing further additives (Amies, 1967). Charcoal can be added to the medium to help neutralize compounds which are toxic to the bacteria (Gästrin *et al*., 1968; Khursheed and Lang, 1996), but its addition to media is not necessarily correlated with better performance (Human and Jones, 2004). Stuart medium was originally intended as transport medium for gonococci (Stuart, 1946). All three media have been shown suitable for transport of a variety of different bacteria (Barber *et al*., 1998).

Table 5: Mean (n=2) $\log_{10}$ of cfu *Ornithobacterium rhinotracheale* (ORT) reisolated from swabs inoculated with suspensions containing different ORT counts and stored at room temperature and at 4 °C in Amies gel medium with charcoal for various time intervals.(reshuffle the following indicated data to make it in proper descending order of ORT count in the inoculums column)

ORT strain	ORT count in inoculum (cfu/ml)	Storage temperature	Storage time							
			1 d	2 d	3 d	4 d	5 d	6 d	7 d	14 d
B3263/91	$10^{7.8}$	RT	4.6	4.7	4.6	4.0	3.1	2.1	1.5	-*
		4 °C	5.4	5.3	5.5	5.4	4.8	4.3	4.3	3.7
F56/10	$10^{7.4}$	RT	4.5	4.5	4.4	4.2	3.5	2.9	2.6	-
		4 °C	5.2	5.0	5.4	5.3	4.5	4.2	4.1	3.4
F488/10	$10^{7.2}$	RT	4.8	4.7	4.6	4.5	3.5	2.6	2.5	-
		4 °C	5.5	5.3	5.4	5.2	4.7	4.3	4.2	3.8
F94/09	$10^{7.5}$	RT	4.4	4.5	4.3	3.9	3.6	2.5	-	-
		4 °C	5.3	5.2	5.2	5.0	4.5	4.1	3.7	-
B3263/91	$10^{5.8}$	RT	2.8	2.6	-	-	-	-	-	-
		4 °C	3.3	2.7	2.8	2.5	2.4	2.5	2.6	-
F56/10	$10^{5.4}$	RT	2.8	2.4	-	-	-	-	-	-
		4 °C	3.3	2.9	2.9	2.6	2.3	2.2	2.2	-
F488/10	$10^{5.2}$	RT	2.4	2.0	-	-	-	-	-	-
		4 °C	3.3	2.5	2.5	2.4	2.2	2.0	2.0	-
F94/09	$10^{5.5}$	RT	2.8	2.6	-	-	-	-	-	-
		4 °C	3.2	2.6	2.4	2.2	2.0	1.9	1.9	-
A (B3263/91)	$10^{3.8}$	RT	-	-	-	-	-	-	-	-
		4 °C	-	-	-	-	-	-	-	-

Table 6: Reisolation of *Ornithobacterium rhinotracheale* (ORT) and *E. coli* from swabs inoculated with suspensions containing different ORT and *E. coli* concentrations, stored at room temperature in Amies gel medium with charcoal for various time intervals.

ORT suspension	E. coli suspension	Agar for reisolation	Storage time (d)							
			1	2	3	4	5	6	7	14
10^7 cfu/ml	PBS[1]	Blood	ORT	ORT	ORT	ORT	ORT	ORT	-*	-
		Gassner	-	-	-	-	-	-	-	-
10^7 cfu/ml	10^5 cfu/ml	Blood	ORT	ORT	ORT,*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
10^7 cfu/ml	10^7 cfu/ml	Blood	ORT	ORT, *E. coli*	ORT, *E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
10^5 cfu/ml	PBS[1]	Blood	ORT	ORT	ORT	-	-	-	-	-
		Gassner	-	-	-	-	-	-	-	-
10^5 cfu/ml	10^5 cfu/ml	Blood	ORT, *E. coli*	ORT, *E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
10^5 cfu/ml	10^7 cfu/ml	Blood	ORT, *E. coli*	ORT, *E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
PBS[1]	10^7 cfu/ml	Blood	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
PBS[1]	10^5 cfu/ml	Blood	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*

[1]instead of the bacterial suspension sterile PBS was added; *no bacterial growth

Differences of the transport media in regard to viable ORT count were minor. Similar results were obtained by Barber *et al.* (1998), who tested ten different systems with several bacterial species. However, transport systems using the same medium from different manufacturers may produce different results (Morosini *et al*., 2006). Dry swabs kept ORT viable between 2 and 5 d p. i. at room temperature, but their performance was variable. Dry swabs in aerobic tubes previously had been shown suitable for transport of bacteria (Roelofsen *et al*., 1999). Possible advantages are the cost and that dry swabs do not allow multiplication of other bacteria that might overgrow ORT.

Higher inoculation doses and storage at 4°C prolonged the period in which ORT could be reisolated. The same influence of storage temperature on viability has been described for some bacterial species (Human and Jones, 2004), while other combinations of bacteria and media yielded similar results at 4°C and room temperature (Tvede and Hoiby, 1992; Human and Jones, 2004). Surprisingly, storage of dry swabs at -20°C allowed reisolation of ORT at a constant level for at least 5 d p. i. The recovery rates were similar, regardless of whether the reference strain or a field isolate was tested.

Inoculation of swabs with ORT and *E. coli* showed that additional immersion of swabs with secondary pathogens can compromise reisolation of ORT. This experiment also underlined the low viability of ORT compared to *E. coli*. which explained the lower isolation rate compared to high detection rate using PCR or immunohistochemistry (van Veen *et al*., 2000; Hafez and Vandamme, 2001). From second day after p. i. it was also possible to isolate *E. coli* on blood agar containing gentamycin. This indicated that *E. coli* multiplied in the transport medium to such numbers that it could overcome the adverse effect of the gentamicin used into the blood agar.

In conclusion for a successful isolation of ORT swabs may be stored in transport medium and brought to the laboratory as earlier as possible. Moreover, swabs may be refrigerated during transportation and at the laboratory, if they are not to be processed immediately. There is no data about the counts of ORT in organs of naturally infected birds, and probably they vary depending on the involved strain, intensity of infection and stage of the infection.

Therefore, several ORT counts in the inoculum were tested and the results showed that this parameter was the most influential. So the selection of a sample for swabbing that contains a high amount of ORT with as few other bacterial load is important for a successful reisolation in the laboratory.

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